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# Evaluating the toxicity of nitrogen-functionalized carbon nanotubes

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# EVALUATING THE TOXICITY OF NITROGEN-FUNCTIONALIZED CARBON NANOTUBES

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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has been approved by the Examining Committee
for the thesis requirement for the Master of Science
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To T.K.

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# CHAPTER I

## INTRODUCTION AND BACKGROUND

Over twenty years ago, widespread attention was drawn to the new age of nanomaterials when carbon nanotubes (CNTs) were first described in the scientific literature. In a 1991 letter to the Nature Publishing Group, the synthesis and structure of these "needle-like tubes" was described (Iijima 1991). Today, over two decades later, the scientific community has dramatically expanded its understanding of these intriguing molecules.

Countless applications for CNTs exist at present, and new potential uses are conceived at an exponential rate. Despite such strides, it has been noted that inadequate progress has been made regarding safety, toxicity, and environmental implications. As the use of CNTs inevitably expands, such considerations will become increasingly imperative (Dhawan and Sharma 2010). Many studies have explored CNT toxicity effects on mammalian cells. However, much understanding of this subject has yet to be acquired. Information on bacterial toxicity is even more limited, and there is ample opportunity for research to fill this knowledge gap. Through study of the antimicrobial properties of CNTs, potential environmental effects can be evaluated, as well as implications and limitations of proposed technological applications. While microbes do not serve as perfect models, research of this kind can also unearth information regarding toxicity on higher organisms, potentially including humans.

A body of research supports the conclusion that several CNT varieties possess antimicrobial properties. However, information garnered from such studies stresses the highly variable nature of these antibacterial effects. Many traditional methods for toxicity assessment have also proven incompatible with these nanoparticles, hindering progress in such research (Dhawan and Sharma 2010).

Further investigation is needed to understand the potential repercussions of carbon nanotubes' entrance into water treatment systems. Chlorine, chloramines, ozone, and other commonly used chemical disinfectants are known to react with organic compounds in water treatment streams to produce harmful disinfection byproducts (DBPs) (Krasner, Weinberg et al. 2006). Current research does not include thorough investigation of whether carbon nanotube toxicity is affected by such chemical disinfection processes.

Two primary research objectives motivated this study. First, this research aimed to investigate whether CNT washing was associated with an effect on observed cytotoxicity. We hypothesized that washing would remove such impurities, such as N-Nitrosodimethylamine (NDMA), from the CNTs, resulting in a reduced antimicrobial effect. This study intended to obtain relative toxicity information for several N-functionalized CNTs to further illuminate possible toxicity mechanisms. Secondly, we set out to examine whether CNTs that have been reacted with hypochlorous acid, a strong oxidizer, exhibit different cytotoxic effects than their unreacted counterparts. We hypothesized that reaction with hypochlorous acid would elicit a chemical reaction, likely to form NDMA. This chemical reaction would likely result in enhanced CNT toxicity. Through this research, some of the methods used in previous publications have been modified and refined in order to examine these hypotheses (Kang, Pinault et al. 2007, Pasquini, Sekol et al. 2013).

# CHAPTER II

# LITERATURE REVIEW

Various studies have been conducted to elucidate both the mechanism and degree of antimicrobial effects of CNTs. The following literature review provides a synopsis of the existing body of research, as well as the methods used in such studies. Moreover, this background will justify the relevance and importance of the study undertaken.

## A background on carbon nanotubes

At the molecular level, carbon nanotubes (CNT) resemble cross-linkages of carbon atoms in a cylindrical structure. Several varieties of CNT have been synthesized, but the simplest groups into which these are categorized are single-walled carbon nanotubes (SWNT) and multi-walled carbon nanotubes (MWNT). SWNTs consist of a single cylinder, while MWNTs are composed of two or more concentric tubes (Iijima 1991). Vast arrays of carbon nanotubes have been produced in laboratory settings, featuring a variety of chiralities and functional groups conferring specific attributes to the molecule (Terrones 2003).

Overall, the widespread appeal of carbon nanotubes across multiple scientific disciplines results from their many fascinating properties. Most notably, carbon nanotubes are known to possess superior electric and thermal conductivity, as well as remarkable strength and flexibility (Terrones 2003).

## Assessing bacterial cell viability

In order to understand the toxicity of carbon nanotubes, selection of appropriate methods to assess cell viability is needed. A variety of methods to evaluate bacterial viability are available, each considering different indications of what constitutes a "viable" cell. The following Figure 1 portrays a variety of indications that can be considered in the appraisal of cell viability. Membrane integrity, metabolic activity, cell

reproduction, and membrane potential all serve as indications of viability. Methods that produce quantifiable measures of these factors, therefore, could be ideal for the evaluation carbon nanotubes' antibacterial effects.

However, it must be stressed that not all methods are suitable for such studies. Previous research serves as a cautionary tale to those embarking on new research in this field. Many classes of CNTs have properties that are poorly characterized; as a result, it is essential to ensure that the CNTs being studied will not interfere with the method used (Wörle-Knirsch, Pulskamp et al. 2006). It is recommended that thorough characterization of CNTs, including properties such as size distribution, surface area, surface chemistry, solubility, and agglomeration state, are determined before conducting toxicity experiments (Dhawan and Sharma 2010).

Although researchers have successfully implemented various methods, comparison of results between separate studies remains a challenge. Research in this area is still in its infancy, and standardized procedures for evaluating carbon nanotube toxicity have yet to be established (Dhawan and Sharma 2010). The unique physical and chemical properties of different CNT varieties pose a challenge, however; a method that works for one CNT type may be unsuitable for another.

Success has been found with methods that evaluate cellular integrity and metabolic activity as indications of viability. Research available on this subject has been pioneered only recently, and often involves a variety of methods in order to provide for validation of results. Membrane integrity has provided a common measure of such studies; fluorescent microscopy and nucleic acid quantification methods are frequently utilized in such experiments (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008). In addition, changes in metabolic activity and genetic expression have been investigated through fluorescent microscopy and microarray methods, respectively (Kang, Herzberg et al. 2008). Traditional plating methods have been useful for quantitative toxicity assessment, and provide for a more comprehensive indication of cell viability (Liu, Tang

et al. 2007, Pasquini, Sekol et al. 2013). However, plating offers little insight into the toxicity mechanisms. Other studies have investigated deviations from a standard bacterial growth curve as an indication of viability reduction (Arias and Yang 2009, Yang, Mamouni et al. 2010).

Each of the aforementioned techniques features unique benefits to understanding the mechanism of CNT toxicity, but when used piecemeal, these render an incomplete picture of the microbe-CNT interaction. By employing multiple approaches within a single study, researchers are also able to verify consistency of results between methods.

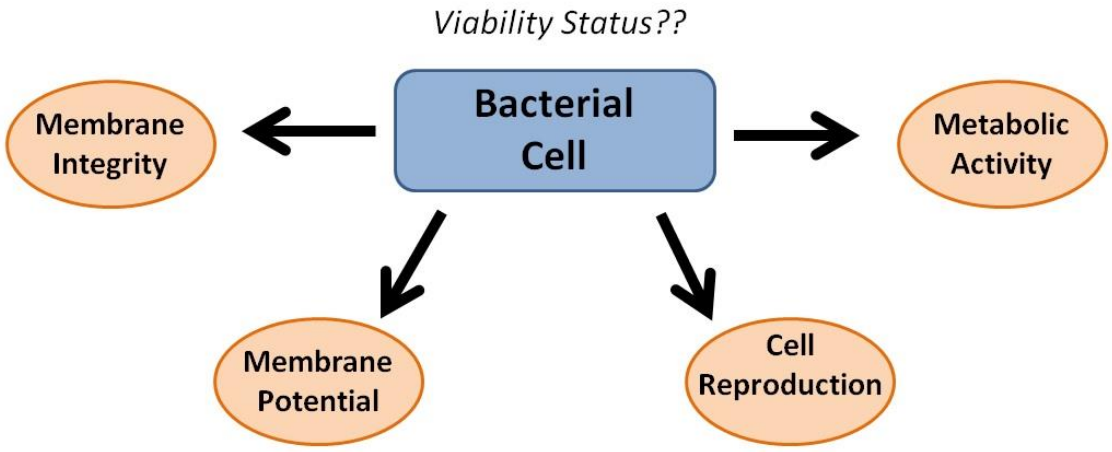



Figure 1. Indications for the assessment of bacterial cell viability.

## Selection of a model organism

Selection of a model organism is an important consideration to be made before commencing any biological experiment. There are many merits to the use of mammalian cells in toxicity studies, but microbes provide a generally simpler, cost-effective alternative. Bacteria may be an especially good choice for studies investigating environmental toxicity implications.

*Escherichia coli* is widely available and generally simple to use as a model in biological experiments. As a result, *Escherichia coli* has been used repeatedly in carbon nanotube toxicity studies, particularly as strain K12 (Kang, Pinault et al. 2007, Pasquini, Hashmi et al. 2012).

Other investigations have incorporated multiple bacterial models, including *Escherichia coli, Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus epidermidis* to assess differences between Gram Negative and Gram Positive bacteria. Inherent differences in the cell wall structure of these microbes have been helpful in understanding the toxicity effects of CNTs (Kang, Mauter et al. 2009). It has been proposed that subtle mechanical differences in microbial cell structure influence the degree of CNT toxicity (Liu, Wei et al. 2009). Yet other microbes evaluated in such studies include *Staphylococcus aureus* and *Salmonella typhimurium* (Arias and Yang 2009, Liu, Wei et al. 2009). Such previously mentioned research demonstrates that there are obvious advantages to the inclusion of multiple microbes in CNT toxicity studies.

## Antimicrobial properties and mechanism of toxicity

The toxic effects of carbon nanotubes on bacteria have been previously demonstrated in laboratory settings. Through implementation of multiple approaches, researchers have begun to elucidate possible mechanisms of toxicity. However, the available literature suggests that these are complicated interactions that cannot be explained by a single mechanism.

In 2007, it was demonstrated that after incubation in a suspension with SWNT, *Escherichia coli* bacteria succumbed to substantial decrease in viability compared to a control. Similar results were observed after passing the bacterial suspension through a SWNT-coated filter. Interestingly, it was noted that cells in suspension not in direct contact with SWNT aggregates did not suffer a significant decrease in viability. This observation insinuated that physical contact was necessary for exertion of a toxic effect. Moreover, scanning electron microscopy revealed striking changes in cell morphologies had occurred to cells in contact with the SWNT-coated filter. The flattened appearances of these cells suggested that membrane damage had led to considerable cytoplasm loss. Additional experiments confirmed that SWNT-exposed cells experienced significant metabolic inactivation as well as a loss in cytoplasmic material. These findings further solidified the conclusion that a physical disruption to the cell membrane had occurred (Kang, Pinault et al. 2007).

In a follow-up investigation, the cellular toxicity of multi-walled carbon nanotubes (MWNT) was compared to that of SWNTs (Kang, Herzberg et al. 2008). Through similar methods used in the 2007 study, it was concluded that MWNT were antimicrobial, although less toxic in comparison to SWNTs (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008). In addition, this study employed microarray analysis to explore the expression of genes commonly associated with cellular stress responses. Elevated transcription of these genes was observed in both MWNT and SWNT-exposed cells, although effects were considerably heightened in SWNT-exposed cells. Through these results, it was hypothesized that the smaller diameter of SWNTs may be a key to understanding their effects on cell toxicity. Results of this study caused speculation that carbon nanotubes exert their cytotoxic effects by physical piercing of the cell membrane. Moreover, microarray results also suggested CNTs may also exert toxicity through oxidative stress (Kang, Herzberg et al. 2008).

Later research endeavors were in accordance with these previously described findings on SWNT toxicity (Kang, Pinault et al. 2007, Liu, Wei et al. 2009). In a 2009 study, the term "nano darts" was coined to describe the toxicity mechanism observed for SWNTs. Better dispersed CNTs were found to be much more toxic than CNT aggregates. Results of this study prompted speculation that the movement of SWNTs in solution induced progressive cell degradation through piercing of the cell wall. The study generated the conclusion that dispersion of SWNTs appeared to enhance their antimicrobial effects. This trend was observed for multiple microbe types, including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis.* In addition, it was found that toxicity was enhanced by increasing SWNT concentration and by raising the shaking speed during incubation (Liu, Wei et al. 2009). These protocol adjustments effectively increase the likelihood of physical interaction between cells and CNTs. As a result, this study was supportive of the original hypothesis that CNT toxicity is contact-dependent (Kang, Pinault et al. 2007, Liu, Wei et al. 2009).

In 2012, additional evidence was presented to support the hypothesis that carbon nanotubes induce mechanical damage on bacteria. When *E. coli* producing β-galactosidase enzyme were incubated in suspension with CNTs, significant increases in extracellular enzyme activity were measured in solution. Increased presence of this enzyme suggests that the cell membrane had been substantially compromised, allowing for quantifiable leakage of cytoplasmic materials (Amarnath, Hussain et al. 2012). Akin to previous findings, this trend was observed to a greater degree for SWNT in comparison with MWNT (Kang, Herzberg et al. 2008, Amarnath, Hussain et al. 2012). In addition, MWNT with a larger surface area to volume ratio was associated with greater extracellular enzyme activity than larger MWNT (Amarnath, Hussain et al. 2012).

An investigation in 2010 sought to understand the effects of carbon nanotube length on the observed bactericidal effects. In this experiment, cultures of *Salmonella typhimurium* were incubated with SWNTs of three different lengths (<1 μm, 1-5 μm, and

~5 μm). As carbon nanotube length was increased, greater time was required to reach an exponential growth phase, as determined by the culture turbidity. Through these results, it was suggested that with increasing length, more carbon nanotube aggregates tend to involve bacterial cells. This increased incidence of direct microbe to nanotube contact thereby enhances antimicrobial effect (Yang, Mamouni et al. 2010). Consequently, this research joined several preceding studies to support the emerging hypothesis of contact-dependent CNT toxicity (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009, Yang, Mamouni et al. 2010).

As exhibited in these previously mentioned studies, it is believed that the physical characteristics of unmodified carbon nanotubes contribute to their toxic effects. Results of the multiple aforementioned studies agree that cell inactivation appears to be caused by physical contact with carbon nanotubes (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009, Yang, Mamouni et al. 2010). The literature strongly suggests that CNT aggregation kinetics are extremely significant to the understanding these CNT-microbe interactions (Yang, Mamouni et al. 2010).

## Modified carbon nanotubes

Modifications to the structure of carbon nanotubes have been noted to induce effects on their observed antimicrobial activity. Such alterations can be achieved by covalently bonding components to a carbon nanotube (functionalization), or alternatively, by noncovalent modification such as wrapping. Such processes have the potential to expand the range of possible CNT applications, making them of great interest to researchers.

Not surprisingly, CNT functionalization has been shown to illicit varying effects on toxicity. Literature review suggests CNT modification complicates the contact-dependent toxicity mechanism suggested to occur with pristine (unfunctionalized) CNTs

(Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009, Yang, Mamouni et al. 2010).

It has been reported that greater antimicrobial effects were achieved by functionalizing multi-walled carbon nanotubes with arginine and lysine. This enhanced toxicity was demonstrated against several gram-negative species including *Escherichia coli* and *Salmonella typhimurium*, as well as the resistant *Staphylococcus aureus*. It has been suggested that the positively charged amino acid functional groups may adsorb the negatively charged bacterial membrane. When the cell membrane is disrupted in this manner, sudden cell lysis may occur (Zardini, Amiri et al. 2012).

Research conducted in 2012 studied the effects of SWNT surface functionalization on the observed antimicrobial effects. After several studies, it was concluded that the functionalized CNT varieties actually induced milder cytotoxic effects than their pristine counterparts. A hypothesis arose that it was not the chemical properties of the functional groups, but rather the effects on CNT aggregation that elicited these findings (Pasquini, Hashmi et al. 2012). This conclusion is supportive of a hypothesis proposed by previous research on pristine CNTs (Yang, Mamouni et al. 2010, Pasquini, Hashmi et al. 2012).

In 2009, a study found that surface functional groups appear to greatly affect antimicrobial efficiency of CNTs, although not all functional groups behave alike. SWNTs functionalized with –OH and –COOH groups exerted strong toxic effects on both gram-positive and gram-negative microbes at a range of CNT concentrations. However, these bactericidal effects appeared to be highly buffer-dependent. It was suggested that with increasing ionic strength of solution, the intensity of CNT-microbe interactions is decreased, thereby reducing the antibacterial effect (Arias and Yang 2009). This finding is in agreement with several previous studies stressing the importance of CNT to microbe contact (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Arias and Yang 2009, Liu, Wei et al. 2009). On the contrary, SWNTs with -$NH_2$ functional groups

exhibited antimicrobial activity only at higher concentrations. MWNTs with –OH, -COOH, and $–NH_2$ functional groups were also investigated, but did not illicit any significant antimicrobial activity (Arias and Yang 2009). These results also challenged the hypothesis of contact-dependent toxicity (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Arias and Yang 2009, Liu, Wei et al. 2009). While contact may play an important role, this data provided evidence that other mechanisms also play a significant role (Arias and Yang 2009).

Various forms of noncovalent modification of carbon nanotubes have also been explored. The antimicrobial properties of carbon nanotubes are reported to be significantly enhanced when coupled with a silver coating. Silver ions are believed to enter into bacterial cells to ultimately disrupt the normal process of cell reproduction. In this study, *Escherichia coli* and *Staphylococcus aureus* cultures were incubated with suspensions of silver-coated multi-walled carbon nanotubes. Serial dilutions and subsequent platings were performed, revealing a significant reduction in colony formation compared to a control. It was concluded that the bactericidal rate of these modified carbon nanotubes increases with the thickness of the silver coating. It was proposed that the large surface area to volume ratio of carbon nanotubes helps to enhance the inherent antimicrobial properties of silver (Liu, Tang et al. 2007). In addition, the proposed piercing mechanism of carbon nanotubes may allow for increased transport of toxic silver into bacterial cells.

Through the literature presented, it is evident that modification of carbon nanotubes appears to substantially complicate the dynamics of the CNT-microbe interaction. Not only do the chemical properties of functional groups play a role, but solution chemistry and resulting aggregation kinetics may also be extremely important.

## Environmental fate and implications of carbon nanotubes

As use of this nanotechnology expands into more industrial and commercial applications, it is essential to consider certain environmental implications. Nanomaterial release to the environment could potentially occur both accidentally or intentionally via point and nonpoint sources. Such release events can ultimately affect surface waters, groundwater, and air. Consideration of the safety of carbon nanotubes should include not only an assessment of toxicity, but also an understanding of the exposure hazard of these materials (Wiesner, Lowry et al. 2006).

While the environmental fate of carbon nanotubes remains elusive, there is evidence to support the vulnerability of natural aqueous environments. Assessment of such risks poses challenges, due to the difficulty in mimicking the various environmental conditions in a controlled laboratory setting. However, various studies have provided insight into how CNTs might behave in a natural water body.

In a 2007 study, it was concluded that multi-walled carbon nanotubes remained stabilized in solutions containing natural organic matter. This observation could indicate the potential for extensive dispersion of this nanomaterial in natural environments (Hyung, Fortner et al. 2006). Separate research agreed that aggregation kinetics of MWNTs was substantially reduced in solutions with conditions characteristic of natural aquatic environments (Saleh, Pfefferle et al. 2008). In light of such findings, it is evident that environmental CNT release should be of concern to proponents of this technology (Hyung, Fortner et al. 2006, Saleh, Pfefferle et al. 2008).

Comprising the foundation of essentially any natural environment, bacteria may be especially susceptible to any environmental release events. Mentioned previously, the discovery of the buffer dependence of functionalized CNT toxicity provides implications for natural aquatic environments (Arias and Yang 2009). Not all natural aquatic environments feature the same chemistry and composition; as a result, CNT behavior may vary significantly depending on the receiving water body.

Others have conducted studies to better understand the impacts of carbon nanotubes in conditions specific to many aquatic environments where environmental release could be an issue. To assess such ramifications, the effects of SWNT and MWNT in river water and wastewater effluent have been evaluated. It was determined that SWNTs resulted in a significant increase in cell inactivation in a sample from Mill River in New Haven, CT. SWNT also exhibited substantial toxic effects on a wastewater effluent sample obtained from a rotating biological contactor treatment system in Wallingford, CT. MWNT exposure resulted in a significant increase in cell inactivation, though the toxic effects were milder in comparison to SWNT (Kang, Mauter et al. 2009). This comparison of SWNT and MWNT toxicity suggested that previous research was in accordance with studies of actual environmental samples (Kang, Herzberg et al. 2008, Amarnath, Hussain et al. 2012).

As a part of this same study, experiments were conducted using both gram-positive (*Staphylococcus epidermis* and *Bacillus subtilis*) and gram-negative (*Escherichia coli* and *Pseudomonas aeurginosa*) monocultures. In each of these cultures, both SWNTs and MWNTs resulted in increased cell inactivation. While SWNT toxicity was consistently high for all cultures, the degree of MWNT toxicity was quite variable, and will require further research to fully understand. Interestingly, *B. subtilis* appeared to be more resistant than other strains to the toxic effects of either of these nanotube types. It was proposed that the thick peptidoglycan layer present in the cell wall of this gram-positive microbe provided added rigidity to avoid penetration by the nanotubes. Ultimately, it was acknowledged that such monoculture experiments provided unreliable predictions of the effects of carbon nanotubes in the wastewater and water treatment plant samples (Kang, Mauter et al. 2009). This conclusion is especially significant as many researchers are attempting to use laboratory-generated data to extrapolate potential environmental effects.

Review of the aforementioned studies provides valuable considerations for investigators. Natural environments contain highly dynamic communities of microbes, as well as several variable abiotic factors that are challenging to mimic in a laboratory setting. It is impossible to forgo laboratory-controlled experiments for such investigations, but researchers should be aware of the possible shortcomings of their research when interpreting results.

Stems from from The entrance of CNTs into engineered water treatment systems is also of interest. Traditional water and wastewater facilities often apply strong chemicals, especially disinfectants, for treatment of water and wastewater streams. Existing studies have not yet evaluated the effects of such disinfecting agents on carbon nanotubes. It is known that certain organic nitrogen-containing compounds serve as precursors for the formation of N-Nitrosodimethylamine (NDMA) (Choi and Valentine 2002). As a result, it is of interest to assess the possibility that interaction with disinfectants can alter the toxic effects of CNTs. Specifically, it is unknown whether or not CNTs functionalized with nitrogen surface groups can serve as precursors for DPBs such as N-Nitrosodimethylamine (NDMA).

## Utilizing antimicrobial properties of carbon nanotubes

Much of the aforementioned literature on microbial toxicity was presented such to illuminate the potential risks of carbon nanotube use. However, the antimicrobial properties of CNTs also provide opportunity for new classes of applications.

Numerous researchers have proposed plans for technologies to benefit the biomedical industry. Among such developments is the SWNT-containing antimicrobial biomaterial described in 2010. Substantial reduction in viability of *Escherichia coli* and *Staphylococcus epidermis*, common biomedical pathogens, was observed after contact with this substance. Researchers have envisioned that when applied as a film on

biomedical devices, CNTs could significantly reduce the incidence of hospital acquired infection (Aslan, Loebick et al. 2010).

Water treatment technologies may also stand to benefit from the antimicrobial properties of carbon nanotubes. A 2011 study discussed an innovative method for water purification using an anodic multi-walled carbon nanotube microfiltration system. By applying a small voltage to the filter, the exceptional conductivity of CNTs was utilized to increase pathogen removal from an *Escherichia coli* suspension. This method was shown to successfully remove all bacteria from the suspension, as well as inactivating 74% of cells collected on the filter (Vecitis, Schnoor et al. 2011). Proposed technologies such as this indicate that there may be great potential for application of carbon nanotubes in water purification.

# CHAPTER III

# MATERIALS AND METHODS

## Chemicals, media, bacterial strains, and growth conditions

The E. coli K-12 strain MG1655 was donated by Dr. David Weiss from the Department of Microbiology at the University of Iowa. This strain was grown on Lennox L Broth Base (Invitrogen) as a liquid culture or on 1.5% Bacto Agar (BD) plates. A growth curve was developed for this bacterial strain in LB media growing at $37^0$C with 200 rpm shaking. Sodium Chloride used in isotonic saline solutions was of ACS grade from Research Products International Corporation. All other chemicals used were of reagent grade or better.

The LIVE/DEAD BacLight viability kit (Life Technologies Corporation) was used to distinguish live and dead cells under a fluorescent microscope. This kit contains the nucleic acid-binding dyes propidium iodide (PI) and SYTO® 9. SYTO® 9 is membrane permeable, and can therefore stain all cells regardless of viability. Propidium iodide can only enter into a cell through a compromised cell membrane, and is therefore an ideal indicator for dead cells. Prior to fluorescent microscopy experiments, a 1:1 mixture (by volume) of each dye was prepared for application to cell suspensions, as recommended by the manufacturer.

## Statistical analyses

Where applicable, treatments were evaluated for statistical significance using a Student's paired t-Test with a two-tailed distribution with a 95% confidence interval ($p < 0.05$).

In DNA efflux and cell plating viability assays, it was necessary to account for slight variation in cell suspension density between replicates. This consideration was especially relevant in order to conduct sound statistical analyses of the data. In these experiments, treatment data was normalized according to a practice advised by existing

literature (Valcu and Valcu 2011). This approach entailed dividing all control and treatment samples by the mean of the control data (Valcu and Valcu 2011).

## Carbon nanotubes

Various nitrogen-functionalized CNTs were utilized in these experiments. All CNTs were manufactured by Nano Labs (NL) or Carbon Solutions (CS). Nano Labs CNTs included in this research are amine functionalized single-walled CNTs (NL $SWNH_2$) and amine functionalized multi-walled CNTs (NL $MWNH_2$). Carbon Solutions CNTs include amide functionalized single-walled CNTs (CS $SWNH_2$) and PABS functionalized single-walled CNTs (CS PABS). All carbon nanotubes were stored in a 1 g/L aqueous suspension, and were sonicated for at least 30 minutes prior to use. These CNT types and their associated surface moieties are included in Table 1.

## Laboratory instruments, equipment and software

Cultures were incubated in a New Brunswick Scientific I2400 Incubator Shaker, which was monitored using a mercury thermometer to ensure accurate temperature control.

A Beckman Model J2-21M centrifuge was used for all cell pelleting and washing steps described.

Cell culture density was analyzed using a Cary 50 Bio UV-Visible Spectrophotometer and Cary WinUV Concentration Application software by Varian.

An Eppendorf Electroporator 2510 and 0.1 cm-gap electroporation cuvettes from Molecular BioProducts were used in the electroporation process.

The concentration of plasmid DNA in the filtrate was measured using the Qubit Fluorometric Quantitation platform.

An Olympus BX-61 microscope was used in fluorescent microscopy assays. Olympus cellSens® Dimension digital imaging software was used for image capture and visualization.

ImageJ image analysis and processing software was used for quantification of dead cells in fluorescent microscopy assay images.

Fluorescent microscopy viability assay

A protocol found in the existing literature was used as the basis for the cell viability assay (Kang, Pinault et al. 2007). MG1655 from an LB culture plate was used to inoculate 5 ml of LB in a 15 ml culture tube. The culture was left to propagate overnight at $37^0$ C and 200 rpm. An aliquot (20 µl) of the overnight culture was used to inoculate 125 ml fresh LB in a sterile 250 ml Erlenmeyer flask. The culture was grown to early exponential growth phase, to a target cell density of $OD_{600}$ =0.3-0.4. When the cell density had achieved the appropriate value, cells were collected by centrifugation for 5 minutes at 3000 RPM (1381 x g) in a 50 mL polypropylene tube. The supernatant was discarded, and the cells were washed twice with 10 ml of a filter sterilized 0.85% NaCl solution to remove traces of growth media.

The cells were resuspended in 150 ml of 0.85% NaCl to an $OD_{600}$ of roughly 0.12 to 0.2, which was found to provide an adequate number of cells per frame in the microscope window. The resuspended cells were distributed equally across 3-250 mL pre-autoclaved Erlenmeyer flasks. Carbon nanotubes were added to the flasks appropriately to achieve a 10 µg/ml final concentration. These suspensions were placed in a $37^0$C incubator-shaker with gentle shaking (75 rpm) for 1 hour. After 1 hour, a 500 µl aliquot was removed from each of the suspensions and transferred to a sterile 1.8 ml microcentrifuge tube.

Cell suspensions and a prepared mixture of fluorescent dyes were transported on ice to the University of Iowa Central Microscopy Research Facility. Upon arrival at the Microscopy Facility, 1.5 µl of the fluorescent dye mixture was added to the 500 µl cell suspensions. After a 15 minute incubation period, 10 µl of suspension was placed on a

glass slide and covered with a glass cover slip. The slide was placed on the stage of the Olympus BX-61 fluorescent microscope, and the image was focused at the 40x objective.

Frames were selected in the viewfinder to contain a target of 100 cells per frame for suspended (unattached to a CNT aggregate) cells. An image was captured with the "Green Fluorescent Protein" setting to view the "live" (SYTO® 9-stained) cells. Immediately, the microscope was changed to the "Texas Red" setting to capture an image of only the "dead" (propidium iodide-stained) cells. Images of 15-20 frames were captured for each suspension viewed. Frames for aggregates were selected by the presence of cells attached to a CNT aggregate, which appeared as a large clump of fluorescently-stained cells. Image capture for aggregates followed the same protocol as described for suspended cells.

The percentage of dead cells was quantified using ImageJ image analysis software. For freely suspended cells, a direct counting method was applied to determine the percentage of dead cells. A composite image was created by merging color channels from both the "live" and "dead" images for a single frame. Total cells in the resulting composite images were manually counted using the ImageJ Cell Counter Plugin tool. Cells in the "dead" image were manually counted to quantify the number of dead cells appearing with the frame. For each frame, the quantity of "dead" cells was divided by the quantity of total cells to obtain a percentage of dead cells. This process was repeated for each captured frame to obtain average percent dead and standard deviation values for the treatment.

For cells attached to CNT aggregates, an area estimation method was employed to evaluate the percentage of dead cells. A composite image was created as described previously. The resulting image was converted into a binary (black/white) image, and the total cell area was then obtained using the software. The "dead" cell image was converted into a binary image, and the dead cell area (in pixels) was obtained. For each frame, the dead cell area was divided by the total cell area to obtain a percentage of dead

cells in the image. This process was repeated for each captured frame to obtain average percent dead and standard deviation values for each treatment. Figure 2 illustrates the procedure used for this area-based estimation method.

DNA efflux viability assay

Electrocompetent MG1655 cells were prepared according to a published protocol (Russell 2001). The plasmid pUC19 from New England Biolabs (50 pg/µl) was used in the transformations, which had been included as a control plasmid in a New England Biolabs NEB5α competent cells kit.

Several combinations of transformation conditions were used, including variations of the voltage settings used on the electroporator, as well as the mass of plasmid DNA. The operation manual for the Eppendorf Electroporator 2510 recommended electroporator conditions of 12-19 kV per cm; this corresponded with 1.2-1.9 kV for the 0.1 cm-gap cuvettes used. Three different voltages were used to cover this range; 1.2 kV, 1.5 kV, and 1.7 kV. The Eppendorf Electroporator 2510 operation manual recommended that 10 pg-25 ng of plasmid DNA be applied for each transformation. Two different pDNA masses were used in the transformations performed, including 50 pg and 100 pg of pDNA, which corresponded with a 1 and 2 µl aliquot of the 50 pg/µl pUC19 plasmid. In total, 6 separate transformations were performed, each using a combination of the voltage and pDNA concentrations specified above.

Prepared electrocompetent cells (50 µl) and the appropriate volume (either 1 or 2 µl) of pUC19 pDNA was added to a 500 µl microfuge tube, and flicked gently to mix. The tube was incubated on ice for approximately 1 minute before being transferred to a prechilled electroporation cuvette. The cuvette was inspected briefly to ensure that there were no visible bubbles inside. A kimwipe was used to gently wipe condensation from the cuvette, and the cuvette was inserted into the electroporator instrument and the appropriate voltage applied. Immediately after electroporation, the cells were transferred

to a 1.8 ml microfuge tube, and 1 ml of sterile SOC medium was added to the cells. Cells were placed in the incubator-shaker at 200 rpm at $37^0$ C for 1 hour outgrowth. After the outgrowth, 100 µl of 1:2 and 1:4 dilutions of the cells were plated on prewarmed SOB plates supplemented with 20 mM $MgSO_4$ and 100 µg/ml ampicillin. Plates were incubated overnight at $37^0$ C.

Inspection of plates showed that all transformation conditions produced viable colony forming units (CFUs). Four individual well-isolated CFUs were selected from plates and used to inoculate 5 ml of LB supplemented with ampicillin (100 µg/ml). Cultures were grown overnight at $37^0$ C with shaking at 200 rpm. The same four transformant colonies, along with control MG1655 cells without pUC19 plasmid, were streaked onto a new LB plate supplemented with ampicillin. These plates were incubated overnight at $37^0$ C.

Inspection of streak plates showed that all four transformant colonies were able to propagate in the presence of ampicillin, while the control culture of MG1655 did not grow. This confirmed that ampicillin resistance had been acquired by the transformant colonies, confirming the presence of pUC19 plasmid. Four overnight cultures of the MG1655/pUC19 transformant colonies were processed with the Qiagen QIAprep Spin Miniprep Kit to extract plasmid DNA. All four samples were eluted with 50 µl elution buffer, and 4 µl of sample was applied to a 1% agarose gel and visualized under UV light to verify presence of pUC19 plasmid. Two of the colonies confirmed to have plasmid by gel electrophoresis were used to prepare frozen stock cultures to be stored in the $-80^0$ C freezer in glycerol for future need.

The protocol used for this assay was adapted from a published method (Kang, Pinault et al. 2007). Sterile LB (125 ml) supplemented with ampicillin antibiotic (100 µg/ml) was inoculated with an overnight culture of MG1655 containing pUC19 plasmid. The culture was grown until exponential phase was reached ($OD_{600}$=0.3-0.4), and then collected by centrifugation at 3000 rpm (1381 x g) for 5 minutes. Cells were washed

once with filter sterilized 0.85% NaCl solution (10 ml). Cells were resuspended in 0.85% NaCl solution to an $OD_{600}$=0.12-0.2). The resuspended cell suspension was divided into 50 ml aliquots split amongst sterile 250 ml Erlenmeyer flask. One of these flasks was reserved as the control; sonicated carbon nanotubes were added to the remaining flasks to achieve desired final CNT.

The flasks were incubated at $37^0$C for 1 hour with shaking (200 rpm). After the incubation period, a 3 ml aliquot of each sample was passed through a 0.22 µm Millex low-binding PVDF (Millipore) membrane. The Qubit High Sensitivity dsDNA fluorometry kit was used to measure the concentrations of each sample. A new calibration was performed with each new pDNA measurement. DNA calibrations were performed using the dsDNA standards for the Qubit High Sensitivity DNA concentration assay.

Adsorption experiments were conducted in order to account for DNA not measured during fluorometric concentration determination due to sorption onto CNT mass. The protocol used for CNT adsorption determination was adapted from a published method (Kang, Pinault et al. 2007).

Overnight 100 mL MG1655 containing pUC19 plasmid was prepped using the Qiagen HiSpeed Maxiprep kit to isolate pUC19 plasmid DNA. Two 1-ml aliquots were obtained. pDNA concentrations were measured using the Qubit Broad Range dsDNA fluorometry kit.

Separate experiments were performed for each carbon nanotube variety. Sterile 0.85% NaCl solution (50 ml) was added to a sterile 250 ml Erlenmeyer flask. Pre-sonicated carbon nanotubes were added to the flask to achieve a 10 µg/ml final concentration. Previously isolated pUC19 pDNA was added to the flask to achieve a final concentration of ~150 ng/ml. Flasks were incubated for 60 minutes at $37^0$C with 200 rpm shaking.

After 60 minutes, a 3 ml aliquot was removed from each flask and filtered through a 0.22 µm Millex low-binding PVDF (Millipore) membrane. Plasmid DNA concentrations in the filtrate were measured and adjusted for dilution and normalized to the control flask (no carbon nanotubes).

Cell plating viability assay

The plating viability assay protocol was adapted from a method detail in the existing literature (Pasquini, Sekol et al. 2013). MG1655 from an LB culture plate was used to inoculate 5 ml of LB in a 15 ml culture tube. The culture was grown overnight at $37^0$ C with 200 rpm shaking. An aliquot (20 µl) of the overnight culture was used to inoculate 125 ml fresh LB in a sterile 250 ml Erlenmeyer flask. The culture was grown to early exponential growth phase, to a target cell density of $OD_{600}$ =0.3-0.4. When the cell density had achieved the appropriate value, cells were collected by centrifugation for 5 minutes at 3000 RPM (1381 x g) in a 50 mL polypropylene tube. The supernatant was discarded, and the cells were washed once with 10 ml of a filter sterilized 0.85% NaCl solution to remove traces of growth media.

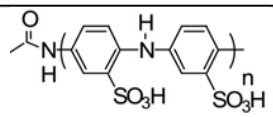
The cells were resuspended in roughly 15 ml of 0.85% NaCl to achieve $OD_{600}$ of roughly 0.05. An aliquot (2.5 ml) of the resulting cell suspension was added to each treatment flask plus a control to achieve a final volume of 25 ml and a theoretical $OD_{600}$ of approximately 0.005. This dilution was found to provide an appropriate number of colony forming units (CFUs) per plate with a $10^{-3}$ dilution. The appropriate volume of CNT suspension was added to the reaction flasks to achieve the desired final concentrations.

Treatment and control flasks were placed in a $37^0$C incubator-shaker with shaking (200 rpm) for 1 hour. After 1 hour, a 50 µl aliquot was removed from the suspension and placed into a sterile 1.8 ml microcentrifuge tube with 450 µl 0.85% NaCl solution to yield a $10^{-1}$ dilution. This sample was diluted further to produce a final $10^{-3}$ dilution. A

100 µl aliquot from the $10^{-3}$ dilution was applied to an LB 1.5% agar plate and distributed uniformly using a sterile plate spreader. This was done three times to generate three spread plates for each treatment. This procedure was repeated at 3 hour incubation time to acquire data over a longer CNT-exposure timespan.

Plates were wrapped with parafilm and were incubated overnight in a $37^{0}$C incubator. The following day, colony forming units (CFUs) were counted on each plate. Triplicate experiments were performed for each CNT type.

Table 1. Carbon nanotube varieties used in experiments and their associated abbreviated names and surface moieties.

| **CNT formulation** | **Abbreviated name** | **CNT surface moiety** |
|---|---|---|
| Nano Labs amine functionalized single-walled | NL SW($NH_2$) | -C=ONH-$CH_2CH_2$-$NH_2$ |
| Nano Labs amine functionalized multi-walled | NL MW($NH_2$) | -C=ONH-$CH_2CH_2$-$NH_2$ |
| Carbon Solutions amide functionalized single-walled | CS SW($NH_2$) | -C=O$NH_2$ |
| Carbon Solutions PABS functionalized single-walled | CS PABS |  |

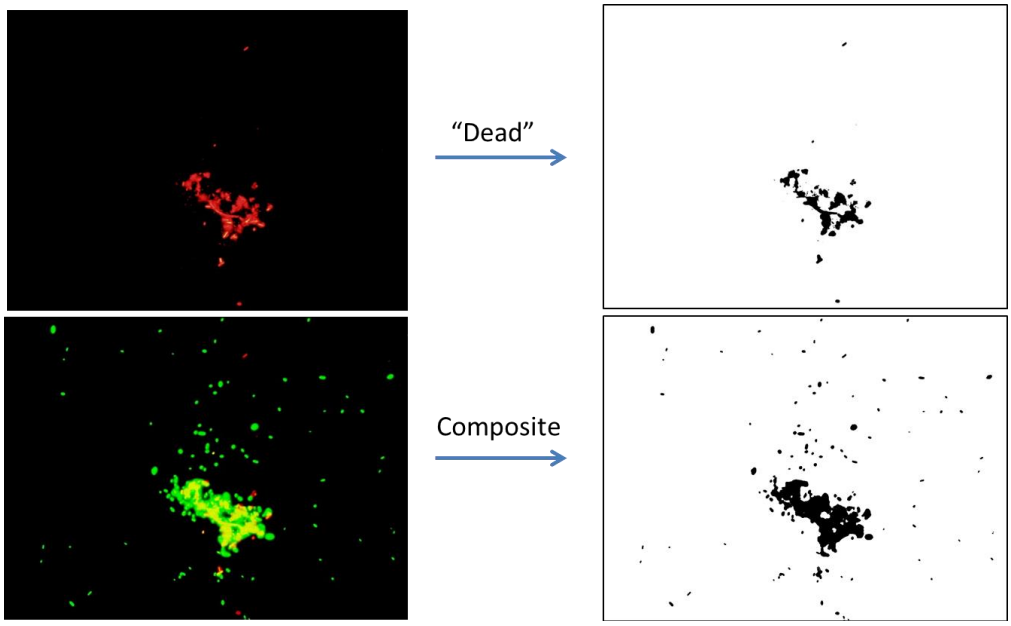



Figure 2. Illustration of the area-based viability estimation method for fluorescent microscopy assays.

# CHAPTER IV

# RESULTS AND DISCUSSION

This chapter presents and discusses results of this research (August 2012 through January 2013). As revealed by the current literature, modifications to CNTs have been known to result in marked variations in their chemical and toxicological properties. Research described henceforth aimed to evaluate such toxicity effects through the study of various N-functionalized CNTs.

Existing research has not yet established a standardized method for the evaluation of CNT toxicity on microbes; however, precedent has been set for the application of multiple approaches in such assessments. Several of such methods were employed in our study, but modification of protocols was often required in order to carry out our investigations. Because method development and modification comprised a substantial part of this research endeavor, much of this chapter consists of discussion of such procedural considerations. Several studies were undertaken to better understand the effects of carbon nanotube exposure on the viability of our model organism, *Escherichia coli*.

## Fluorescent microscopy viability assay

Initial efforts undertaken in this study considered the bacterial cell membrane integrity as an indication of viability. As described earlier, the existing literature contains repeated suggestions that a membrane-piercing mechanism is an underlying cause of CNT toxicity (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009). Consequently, investigation of such an effect appeared to be a logical starting point for this study. The experiments undertaken were modified from protocols that were implemented successfully in previous studies (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009). As described previously, the LIVE/DEAD BacLight viability kit for fluorescent microscopy was used in these investigations.

Use of this technique revealed many important procedural details omitted from the referenced published articles (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008). Such considerations included microscope camera exposure time, camera sensitivity settings, appropriate magnification, method for selecting frames, selecting the appropriate stain concentration, and cell quantification protocols. Leanne Pasquini, an investigator from Yale who conducted similar assays, was contacted for expertise about some of these questions (Pasquini, Hashmi et al. 2012). Preparations for these experiments included estimation of appropriate cell suspension concentration, stain incubation time, and microscope exposure time.

In consideration of trends observed in previous studies, we evaluated attached and unattached (suspended) bacterial cells separately through methods described previously (Kang, Pinault et al. 2007). Figure 3 depicts an example composite image of freely suspended cells captured by this method. In the image, "live" cells are stained green (SYTO® 9) and membrane-compromised "dead" cells are stained red (propidium iodide). There are a few instances of yellow cells, which resulted from dual-stained cells. Such cells were considered "dead," per recommendation of the manufacturer. A separate image (Fig. 4) depicts a sample image of attached bacterial cells in a CNT suspension.

After a standard methods protocol was developed, a live/dead control experiment was conducted to confirm the reliability of the fluorescent microscopy assay. This experiment (Fig. 5) resulted in an average percent dead of 99.1% for the dead (isopropyl alcohol-treated) control, and 9.4% for the live control. These results appeared to be reasonable, and CNT experiments were commenced based on the success of this experiment.

Viability differences between washed and unwashed CS $SWNH_2$ were first investigated. The first experiment (Fig. 6) on this CNT type included image capture of freely suspended cells only. The aforementioned figure also features results of a follow-up experiment performed to also include capture of bacteria associated with CNT

aggregates. As seen in the figure, results were inconsistent between the two replicates. Differences between each of the replicates were statistically significant ($p < 0.05$), indicating poor reproducibility of the method. However, it was observed that there was a higher percent dead for cells associated with CNT aggregates than for freely suspended cells. This trend was consistent with findings of previous literature (Kang, Pinault et al. 2007).

Despite the poor reproducibility of the previous experiment, additional CNTs were investigated using this method. Washed and unwashed varieties of NL MW($NH_2$) were evaluated (Fig. 7) in comparison to a control. In this study, the control featured a greater percent of dead cells (23.5%) compared to those obtained in previous studies (Kang, Pinault et al. 2007). In addition, the unwashed treatment was associated with a significantly lower percentage of dead cells (3.0%) than the control. These results did not seem to be reasonable, and suggested methodical errors.

Two experiments were performed using the fluorescent microscopy assay to investigate the effects of chlorine reaction on NL SW($NH_2$) toxicity. These experiments (Fig. 8) used different free chlorine concentrations (25 mg/L and 50 mg/L) to investigate the possibility of a chlorine-concentration effect. In the 25 mg/L chlorine reaction, no significant difference was observed between the control treatment and the unreacted CNT or the chlorine-reacted CNT treatments. A significant difference ($p < 0.05$) was found between the control and reacted (50 mg/L) NL SW($NH_2$) treatments. However, no significant difference was observed between any other treatments. Since no significant difference was found between the reacted and unreacted CNT treatments, there was no support of our hypothesis that chlorine reaction induces enhanced toxicity for this CNT type.

As evidenced by the lack of experimental data, CNT aggregates became increasingly difficult to locate under the microscope as additional experiments were performed. In order to alleviate this problem, an experiment was attempted using NL

SW($NH_2$) with increased mass loading (20 µg/ml from 10 µg/ml). However, this modification did not seem to increase the appearance of CNT aggregates under the microscope.

On the rare occasion that aggregates were located under the microscope, they were often difficult to bring into focus. Such poorly focused aggregates tended to take up more area in the image; this raised concerns about the reliability of the area-based viability estimation method. In addition, viability results were often inconsistent between trials. As mentioned previously, consistently low viability of control cells compared to previous studies raised suspicion of a procedural error (Kang, Pinault et al. 2007). For these reasons, alternative techniques were explored to assess loss in membrane integrity.

## DNA efflux viability assay

DNA efflux viability assay experiments were conducted as an alternative method to evaluate CNT toxicity on *E. coli* bacteria. Preparations for these experiments began in May 2013. In congruence with the fluorescent microscopy viability assessments, the DNA efflux assay served as a method to quantify apparent cell membrane damage. The fluorescent microscopy technique provided a measurement of a material entering into the cell (stain). In contrast, the DNA efflux assay provides quantification of DNA passage out of the cell. Also akin to the fluorescent microscopy viability assay, the DNA efflux method provides an assessment of toxicity effects associated with acute CNT exposure. This approach was adopted and modified from previous studies, where it was employed to successfully study various CNT types (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008).

As described previously, *E. coli* cells were electroporated with a plasmid to provide an "indicator" of cytoplasmic material efflux before these experiments were started. The high-copy number plasmid pUC19 was selected for this purpose to provide for greater sensitivity of the assay.

The DNA efflux data reveals trends associated with membrane disruption resulting from acute exposure. Figure 9 provides a compilation of triplicate DNA efflux data for washed and unwashed batches of three CNT types. Washed and unwashed Carbon Solutions SWNTs ($NH_2$) and Carbon Solutions PABS CNTs were associated with statistically significant DNA efflux increase compared to a control. Nano Labs SWNTs ($NH_2$) exhibited no such effect. Previous research has made similar revelations, finding that that antimicrobial activity was not exhibited for amine-functionalized ($NH_2$) SWNTs at concentrations up to 100 µg/ml (Arias and Yang 2009). The unwashed CS PABS CNTs were associated with a significant increase in DNA efflux in comparison with the washed CNTs. No such effect of washing was observed with any other CNT type in this figure.

Data resulting from triplicate DNA efflux experiments with washed and unwashed NL MW($NH_2$) are provided in Figure 10. DNA efflux resulting from incubation with washed NL MW($NH_2$) is not significantly different from a control. Interestingly, the unwashed CNTs produced less DNA efflux than the control. This observation has not yet been explained, but may relate to DNA sorption by the CNTs that was not adequately captured in the sorption experiments conducted.

A single DNA efflux experiment was conducted to investigate the effects of chlorine reaction on NL SW($NH_2$) CNTs. The resulting data (Fig. 11) revealed that neither the unreacted nor the chlorine-reacted (50 mg/L HOCl) induced a significant change in DNA efflux compared to the control. However, the reacted CNTs produced a significantly lower amount of DNA efflux compared to the washed (unreacted) CNTs. This is a perplexing result, and may also be explained by the DNA sorption activity of these CNTs.

Trends were observed relating CNT suspension stability to DNA efflux effects. Differences between the various CNT types were readily observed during experimentation. CS PABS (Fig. 12) remained extremely stable in aqueous solution,

resembling a homogenous solution even after incubation. Conversely, aggregates of CS SW($NH_2$) formed readily (Fig. 13), appearing as prominent clumps by the end of an hour-long incubation period. NL SW($NH_2$) (Fig. 14) also remained poorly-suspended in aqueous solution, tending towards aggregation and settling.

Table 2 provides numerical information describing observed CNT settling constants (collected by Edgard Verdugo) and reiterates measured DNA efflux values for washed CNTs. Carbon Solutions PABS CNTs were found to be one of the most stable CNTs in aqueous suspension, and also induced the greatest increase in DNA efflux. Conversely, Nano Labs SW($NH_2$), which induced an insignificant increase in DNA efflux, settles much more readily in aqueous solution. Previous literature has described a relationship between increased CNT dispersivity and cell viability loss; this trend also appears to occur in these results (Liu, Wei et al. 2009). This table of data also provides a useful comparison of the NL SW($NH_2$) and NL MW($NH_2$) CNTs. Although these two CNTs are both amine-functionalized, the single-walled variety was associated with greater average DNA efflux (1.29, normalized to control) than the multi-walled version (1.10, normalized control). This finding is consistent with previous literature suggesting that the smaller diameter of SWNTs makes them more prone to induce cellular membrane damage than MWNTs (Kang, Herzberg et al. 2008).

In order to provide context for the DNA efflux results, "dead" control experiments were performed in triplicate. As a part of these experiments, cells were boiled for 10 minutes before quantifying dsDNA concentration in the solution. These experiments induced DNA efflux in great excess (average 23.7, normalized to control; S.D. 4.7) compared to any of the CNTs evaluated. However, an extended period of such a high temperature is quite an extreme condition. It is certainly possible for significant cell damage to occur under less harsh circumstances.

As illustrated in the previous figures, washing the CNTs was not associated with any change in the observed DNA efflux. To our knowledge, the washing procedure

primarily removed residual NDMA from the CNTs. NDMA is not known to induce an acute toxic effect on bacteria, nor is it associated with a membrane perforation effect. As a result, this lack of a washing effect can be explained rationally.

While the DNA efflux viability assay should provide for adequate quantification of membrane damage, it may not properly assess other reductions in cell viability. This limitation was acknowledged, and lead to the inclusion of another technique in this study.

### Cell plating viability assay

Although cell membrane perturbation has been repeatedly proposed as a CNT toxicity mechanism, various other mechanisms could also be important to consider (Kang, Pinault et al. 2007, Kang, Herzberg et al. 2008, Liu, Wei et al. 2009). Although the fluorescent microscopy and DNA efflux techniques provide for an interesting assessment of CNT effects, they are primarily indicative of acute toxicity resulting in loss of cell membrane integrity. As a result, these methods may be inadequate to capture other mechanisms of toxicity.

In order to provide a more comprehensive toxicity assessment, a plating viability assay was incorporated in this research. This technique should theoretically illuminate other toxicity effects, including but not limited to, metabolic disruption. Preparations for these cell plating viability experiments began in August 2013. As discussed earlier, previous CNT toxicity studies have successfully applied plating experiments, and provided guidance in the development of methods for these experiments (Pasquini, Sekol et al. 2013).

Plating experiments featured an increased CNT mass loading and decreased bacterial suspension concentration in comparison to previous DNA efflux experiments. It is believed that this procedural change enhanced the incidence of CNT to microbe contact. These changes were adopted from a previous study, for the purpose of obtaining more pronounced reductions in CFUs (Pasquini, Sekol et al. 2013). In order to illuminate

potential time-dependent effects, plating assays were also extended to include 3-hour incubation data.

Using the plating viability technique, exposure effects on bacterial viability were evaluated for several varieties of washed and unwashed CNTs. Results of these experiments (Fig. 15) provide worthwhile insight into the toxicity mechanisms at hand. Using this plating viability method, Nano Labs SW($NH_2$) induced the greatest CFU reduction of all CNTs evaluated. This effect was not observed in previous DNA efflux experiments, providing possible indication that this type of CNT exerts toxicity by non-mechanical means.

In contrast, Carbon Solutions PABS CNTs, which elicited the highest DNA efflux, were marked by milder CFU reduction compared to Nano Labs SW($NH_2$). A possible explanation for this may be that the degree of DNA efflux observed for CS PABS was not significant enough to cause extensive cell viability reduction. Although an increase in DNA efflux was produced, Carbon Solutions SW($NH_2$) were not associated with significant CFU reduction. Once again, the perceived degree of cell membrane damage may not have been substantial enough to reduce CFU formation. No washing effect was observed for the Carbon Solutions SW($NH_2$) or PABS-functionalized CNTs. Greater CFU reduction was found for unwashed Nano Labs SW($NH_2$) compared to the washed variety only at 3 hours.

The plating technique was employed to assess chlorine-reaction effects on Nano Labs SW($NH_2$) toxicity. Results of these experiments are illustrated in Figure 16. Experiments were conducted to confirm that reduced CFU formation did not result from the presence of residual free chlorine in the reaction. Free chlorine detection assays available were not sensitive enough for these purposes, so an alternate approach was taken to ensure a negligible free chlorine effect. Two separate CNT-chlorine reactions were prepared; these CNT suspensions were reacted for 3 and 22 days, respectively. No significant difference in CFU formation was found after exposure to CNTs reacted for 3

days compared to CNTs from a 22-day reaction. Since free chlorine would be expected to dissipate with time, this result suggested a negligible effect of any free chlorine residual.

Contrary to the initial hypothesis, reaction with chlorine did not produce any observable reduction in CFU formation compared to unreacted CNT. A separate batch of CNTs was also processed for these experiments, having undergone a chlorine reaction followed by a dechlorination step with sodium sulfite to remove residual chlorine. Surprisingly, these CNTs resulted in significantly reduced toxicity. CFU formation resulting from these CNTs was not statistically different from a CNT-free control. These findings suggested that the chlorination-dechlorination process induces a chemical change to the CNT, virtually eliminating toxicity. Although further research is needed to fully understand this effect, the possibility of a surface functional group transformation should be considered. This result has implications for the use of CNTs in engineered treatment systems. It is possible that, pending additional research, this coupled chlorination-dechlorination process could serve as a means to reduce the toxicity of CNTs as required. Results of this study also provide insight to researchers wishing to use CNTs the presence of strong oxidizing agents. The possibility of a chemical change occurring on the CNT itself must not be ruled out. Moreover, this finding warrants additional research on the effects of chemical disinfection on other CNTs, both functionalized and pristine.

Experiments were performed using SWNTs featuring a –COOH functional group to provide for comparison. In accordance with previous research, incubation with carboxylated CNTs completely inhibited CFU formation (Arias and Yang 2009). Although the aminated Nano Labs SWNTs resulted in significant CFU reduction, these results suggested that the carboxylated SWNTs had a much more potent effect. The substantial toxicity differences observed between these CNT types has been described previously, but warrants further investigation (Arias and Yang 2009).

A separate set of triplicate experiments was conducted to assess the effect of chlorine reaction on CS SW($NH_2$) CNTs. These results (Fig. 17) also indicated that the chlorine-reacted CNTs were not associated with a significant change in CFU reduction. Both unreacted and chlorine reacted varieties of CS SW($NH_2$) CNTs did not produce significantly different CFU formation compared to the control. These results were also in line with the experiments to assess the effect of chlorine reaction on NL SW($NH_2$) CNTs. For both of these CNT types, chlorine reaction alone did not induce any changes as observed by the plating viability assay.

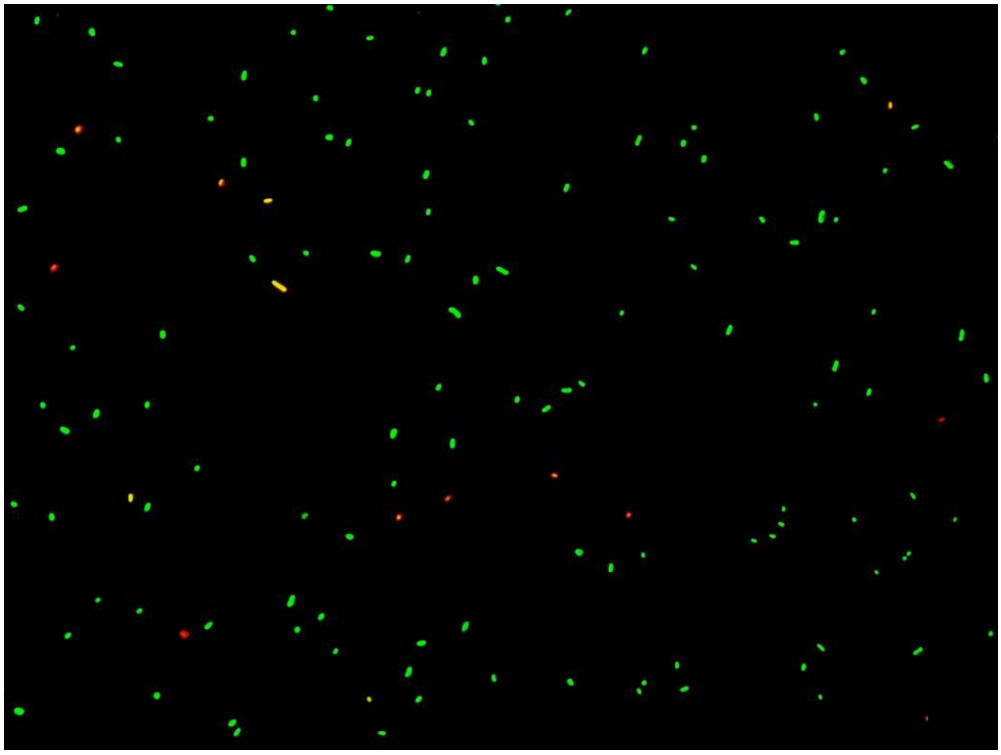


Figure 3. Sample frame of freely suspended cells stained in fluorescent microscopy viability assay.

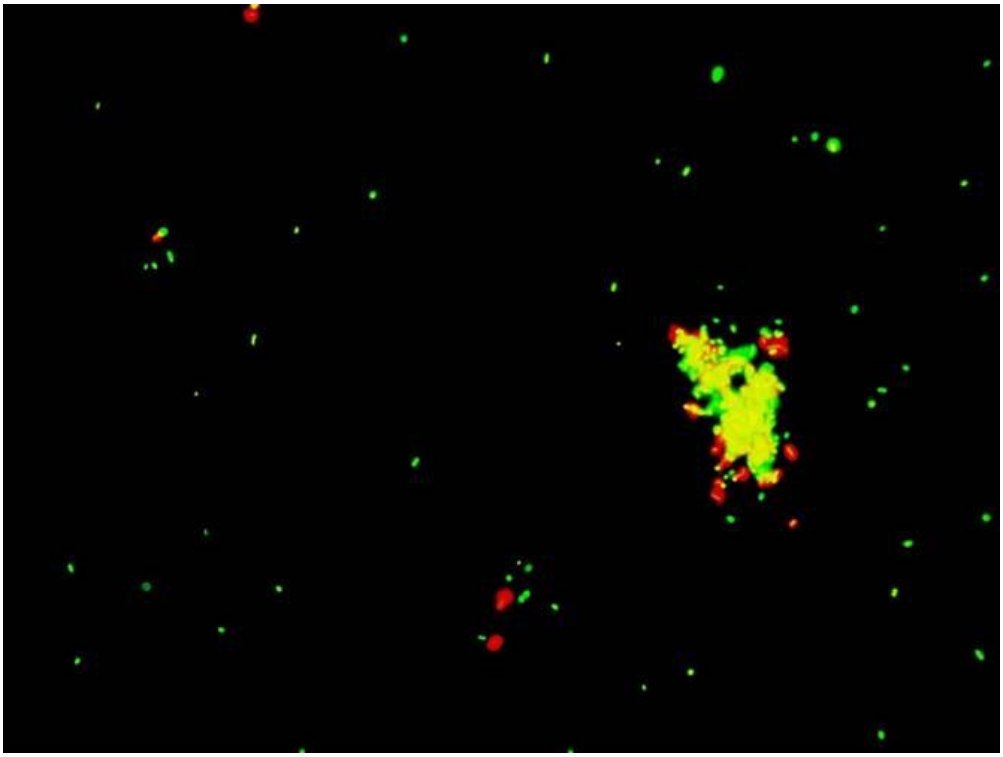


Figure 4. Sample frame of attached cells stained in fluorescent microscopy viability assay.

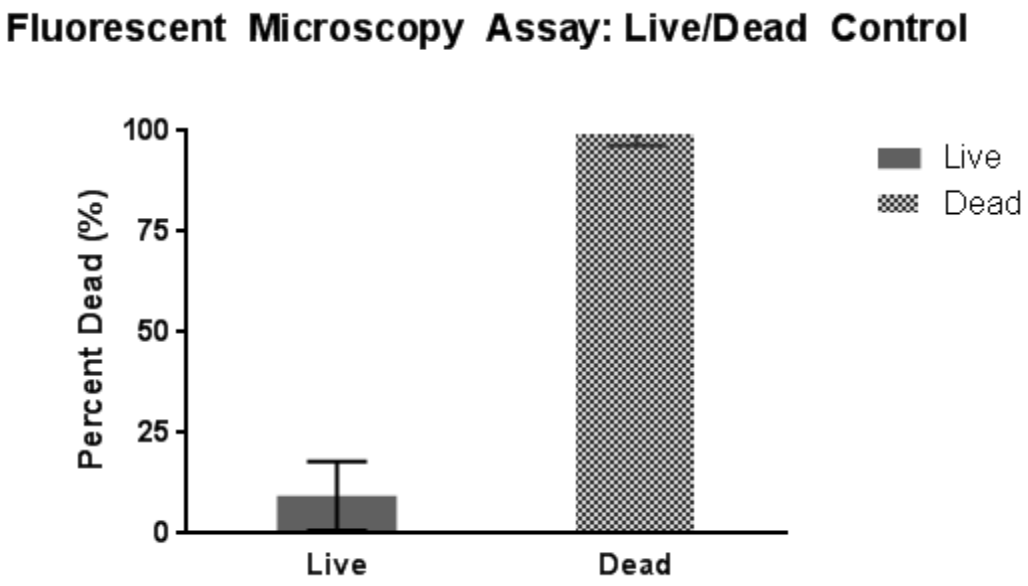



Figure 5. Average percent dead cells from a live/dead control fluorescent microscopy assay. Error bars indicate standard deviation.

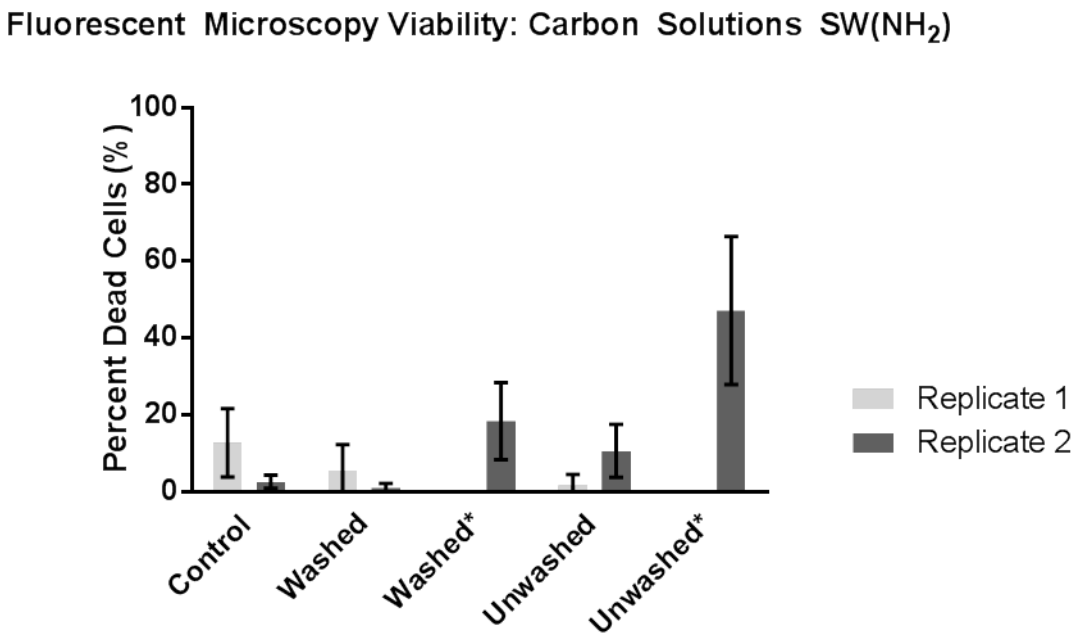



Figure 6. Average percentage of dead cells in fluorescent microscopy assay for washed and unwashed CS $SW(NH_2)$ (10 µg/ml) treatments for replicate experiments. Treatments denoted by an asterisk (*) indicate cells attached to CNT aggregates. Error bars indicate standard deviation.

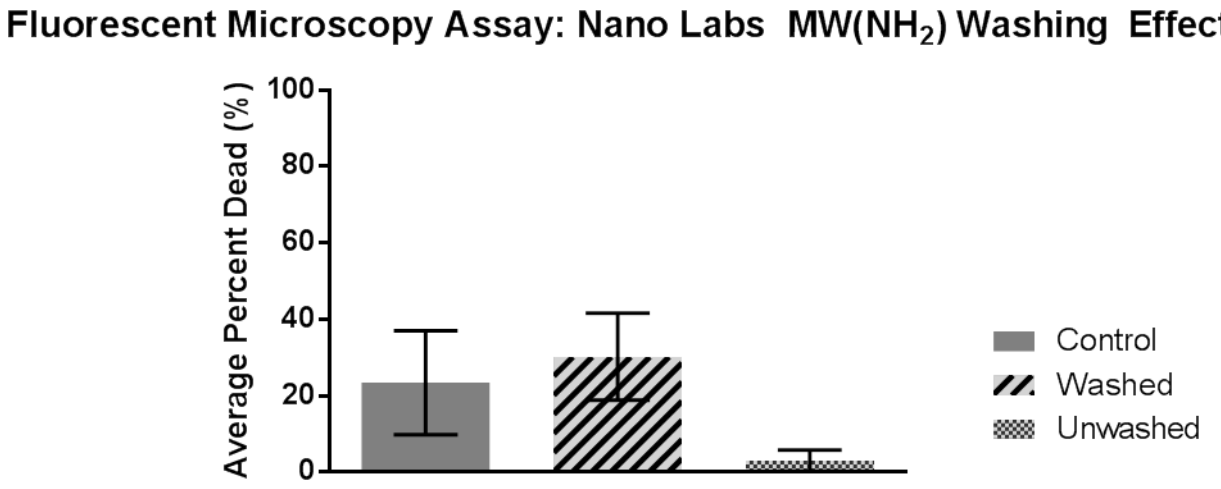



Figure 7. Average percentage of dead cells in fluorescent microscopy assay for washed and unwashed NL MW($NH_2$) (10 µg/ml) treatments. Error bars indicate standard deviation.

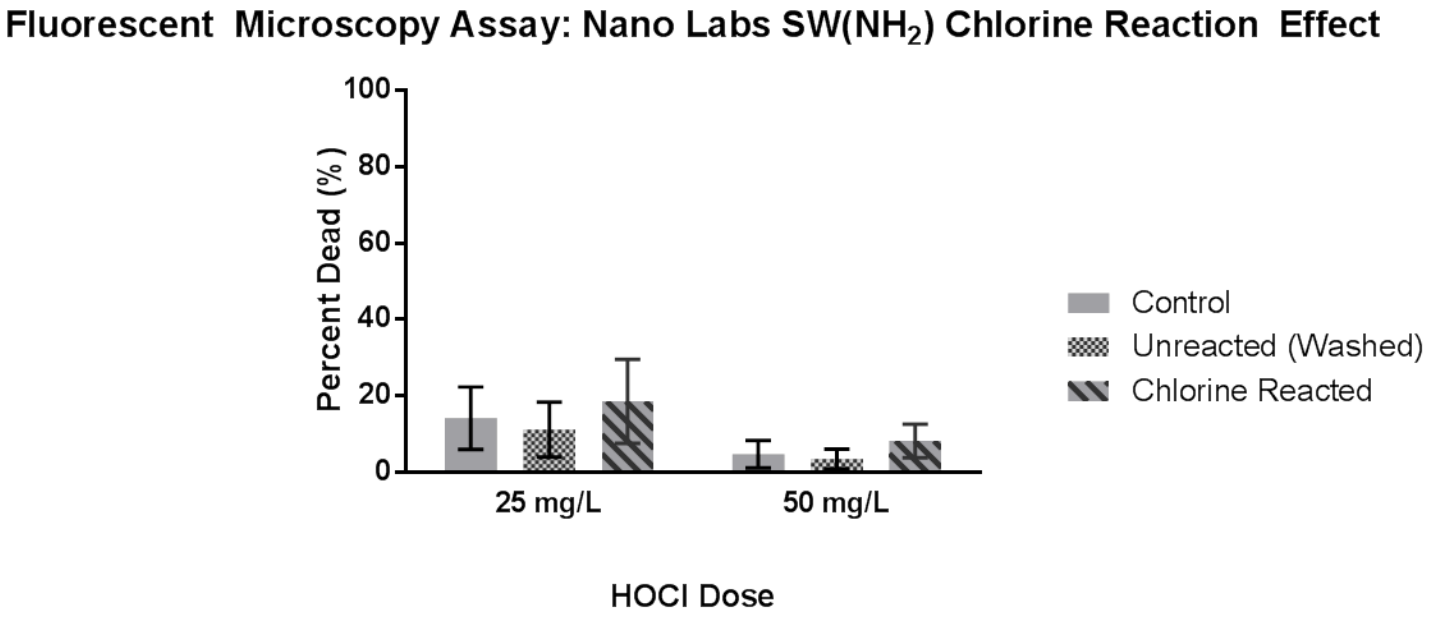



Figure 8. Average percentage of dead cells in fluorescent microscopy assay for washed and chlorine-reacted (25 mg/L and 50 mg/L HOCl) NL SW($NH_2$) (10 µg/ml) treatments. Error bars indicate standard deviation.

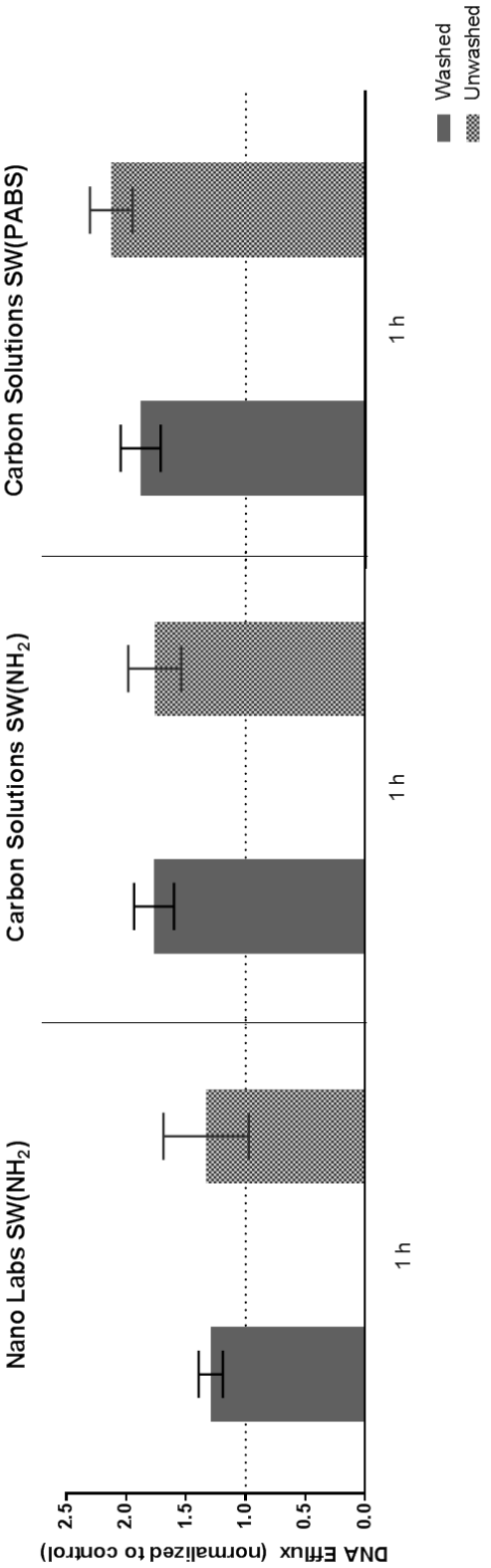



Figure 9. Comparison of DNA efflux (normalized to control) after 1 hour incubation with washed and unwashed CNT varieties (10 µg/ml). Standard deviation error bars obtained from triplicate experiments.

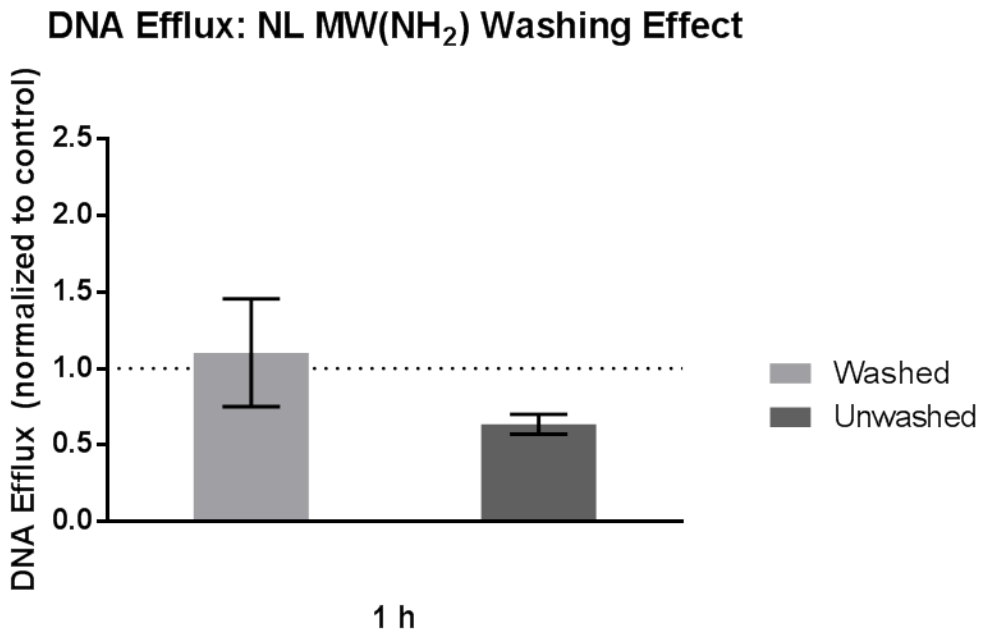



Figure 10. DNA efflux (normalized to control) after 1 hour incubation with washed and unwashed NL MW($NH_2$) (10 µg/ml). Standard deviation error bars obtained from triplicate experiments.

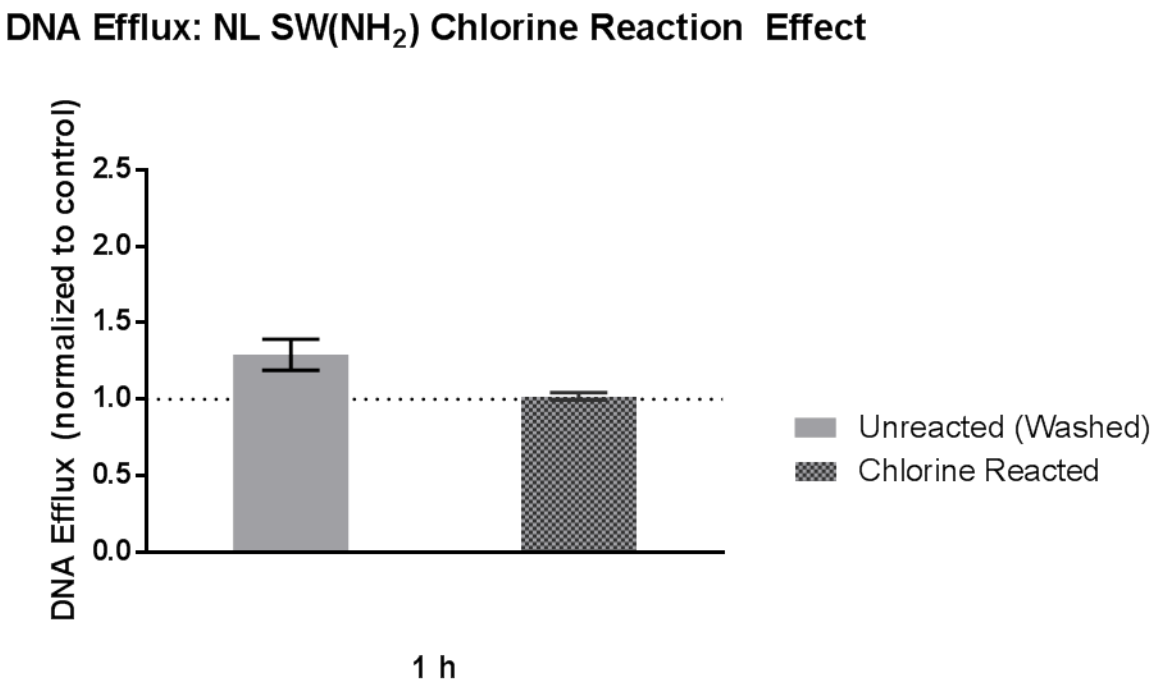



Figure 11. DNA efflux (normalized to control) after 1 hour incubation with unreacted and chlorine-reacted (50 mg/L HOCl) NL SW($NH_2$) (10 µg/ml). Standard deviation error bars obtained from triplicate experiments.

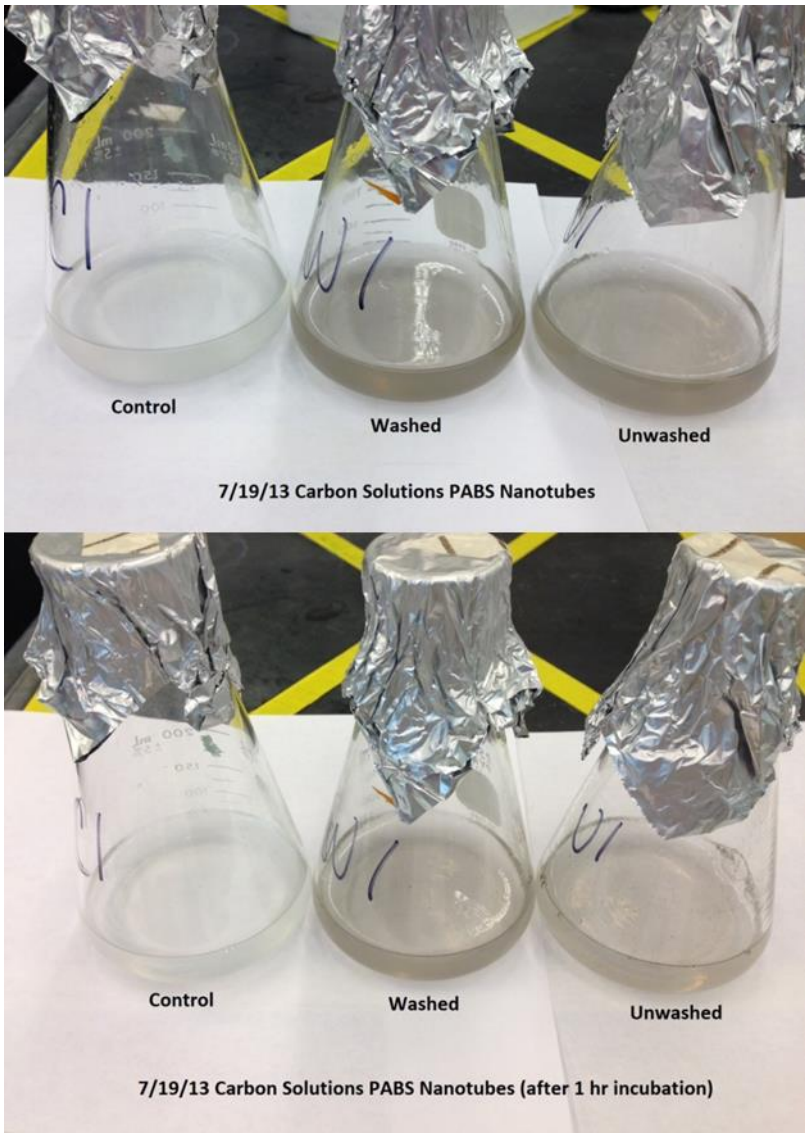



Figure 12. Suspension stability of washed and unwashed CS PABS (10 µg/ml) before and after 1 hour incubation (200 rpm).

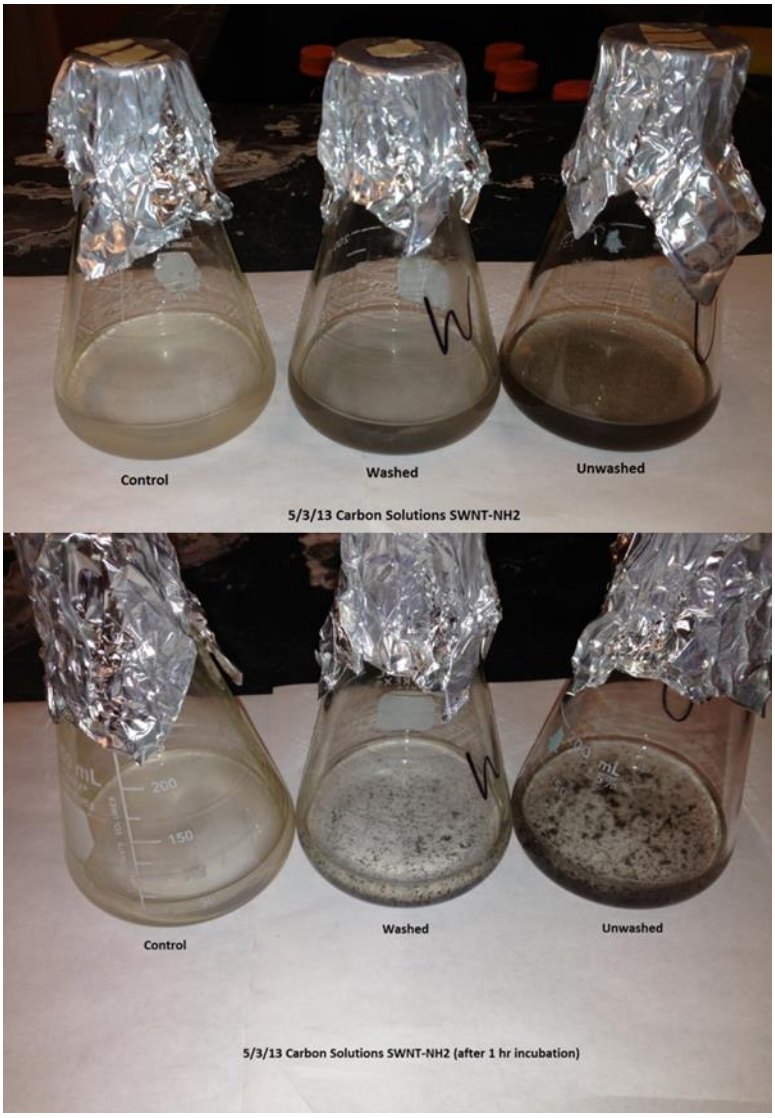



Figure 13. Aggregative tendency of washed and unwashed CS SWNTs (NH2) (10 µg/ml) before and after 1 hour incubation (200 rpm).

Figure 14. Aggregative tendency of washed NL SW($NH_2$) CNTs (10 µg/ml) after sonication.

Table 2. Comparison of observed CNT settling velocities ($K_{obs}$) and associated DNA efflux data (normalized to control).

| **CNT name** | **$k_{obs}$ (exponential fit)** | **DNA efflux (normalized to control)** |
|---|---|---|
| NL SW($NH_2$) | 0.011 | 1.29 |
| NL MW($NH_2$) | 0.022 | 1.10 |
| CS SW($NH_2$) | 0.0001 | 1.77 |
| CS SW PABS | 0.0001 | 1.88 |

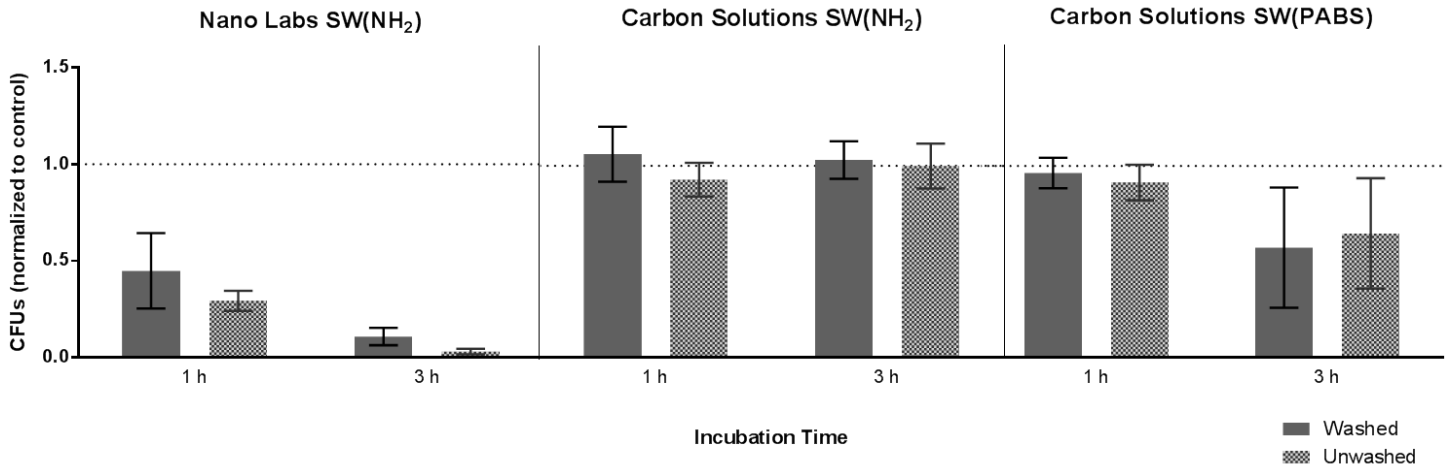



Figure 15. CFU formation (normalized to control) after 1 and 3 hours incubation with various washed and unwashed CNTs (200 µg/ml). Standard deviation error bars obtained from triplicate experiments.

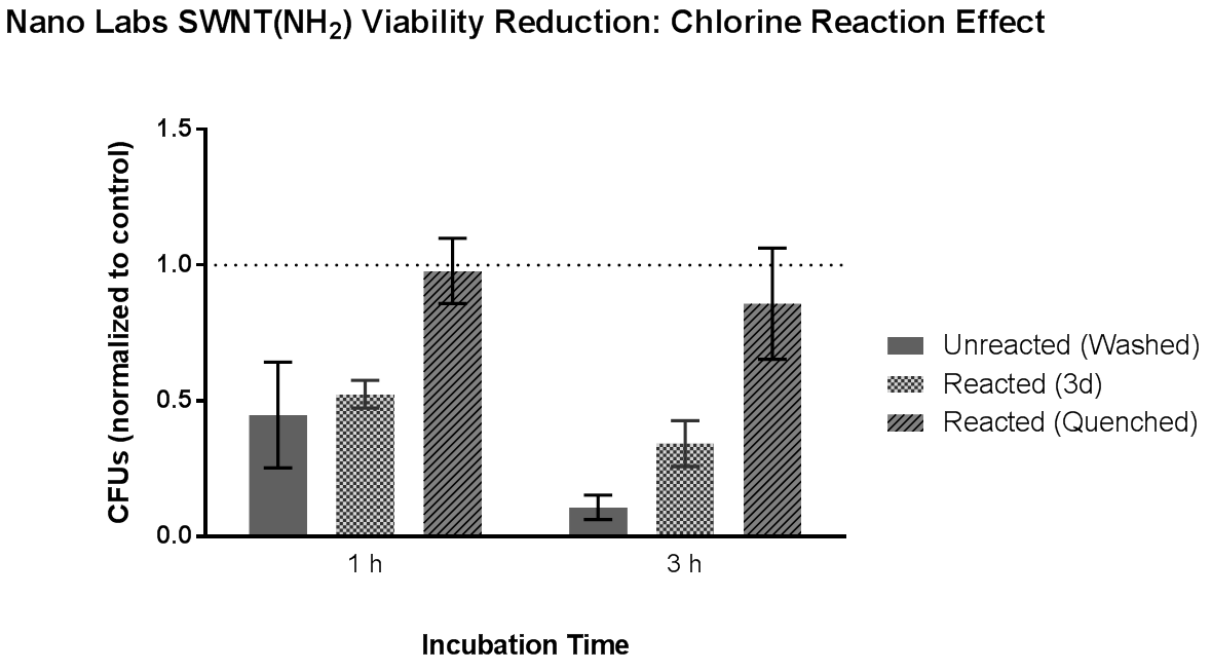



Figure 16. CFU formation (normalized to control) after 1 and 3 hours incubation with NL SWNTs ($NH_2$) (200 µg/ml) unreacted and reacted with chlorine (50 mg/L HOCl). Standard deviation error bars obtained from triplicate experiments.

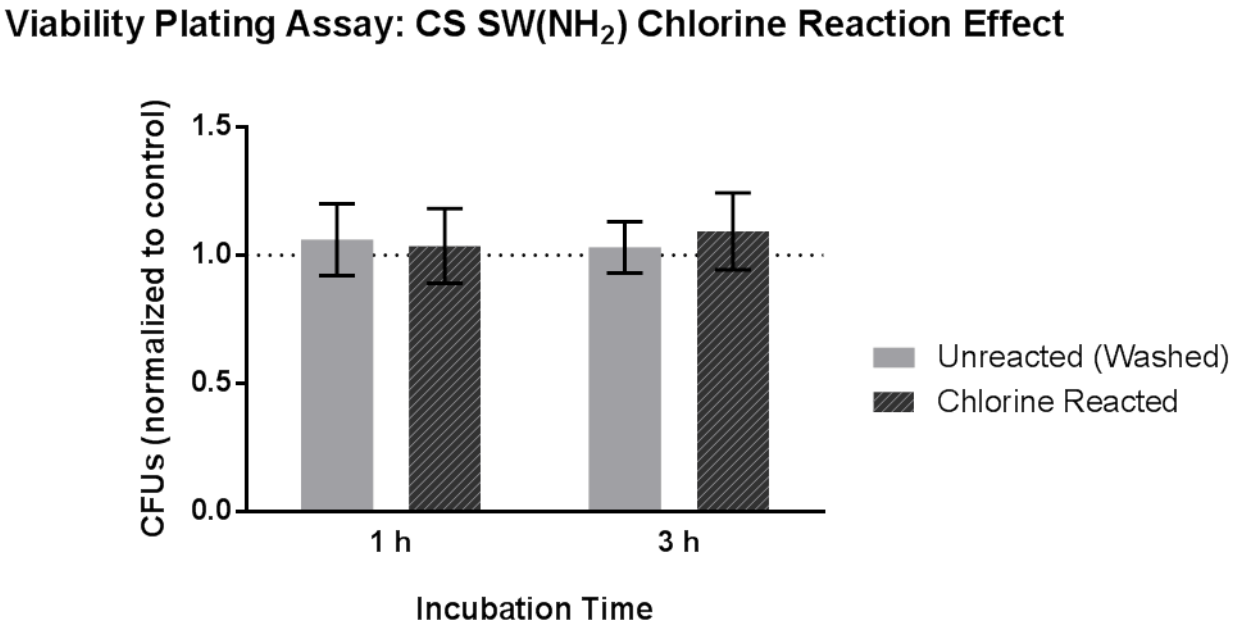



Figure 17. CFU formation (normalized to control) after 1 and 3 hours incubation with CS SWNTs ($NH_2$) (200 µg/ml) unreacted and reacted with chlorine (50 mg/L HOCl). Standard deviation error bars obtained from triplicate experiments.

# CHAPTER V

# ENGINEERING SIGNIFICANCE AND FUTURE RESEARCH

## Engineering significance

For over twenty years, carbon nanotubes have incited interest because of their remarkable physical properties. Our understanding of CNTs has developed rapidly since their characterization in the early 1990's, and grand visions have emerged regarding how to harness the power of this exciting class of nanomaterials. However, the safety and environmental implications of this technology remains poorly characterized. Continued research in the area of nanomaterial safety is necessary for the responsible development of new technology and applications utilizing CNTs.

It is essential to consider microbes, which are vital to the health and homeostasis of essentially any natural environment, in these safety evaluations. Moreover, by understanding the effects on bacteria, we are provided with further insight into potential consequences for eukaryotic cells and multicellular organisms. The intent of this research was to assess the potential cytotoxic effects of several classes of nitrogen-functionalized CNTs, as well as their disinfection byproducts, on *Escherichia coli* bacteria. In addition, this research served to understand if washing CNTs caused a reduction in toxicity. Through this study, we also aimed to develop a better understanding of which methods are most effective for cytotoxicity evaluation.

Through this work, it is evident that when possible, it is desirable to utilize multiple methods to assess cell viability changes. Several previous studies have also employed the use of multiple techniques in similar studies. This practice is of great utility for the confirmation of data consistency. In addition, deviations in data between methods can be useful for understanding the mechanisms behind CNT toxicity.

## Future research

A plethora of experiments have demonstrated that certain carbon nanotube types are capable of causing cellular damage to bacteria, most likely due to membrane disruption. However, as demonstrated here and in previous literature, interactions between CNTs and bacteria appear to be remarkably dynamic and may depend on a variety of factors. For this reason, it is clear that more research is needed to elucidate the exact mechanism of interaction, as well as the role played by other factors.

It has been stressed that the lack of standardized methods to assess nanomaterial toxicity greatly impedes progress in the field. Without appropriate guidelines in place, meaningful comparison of data between research groups is fruitless and unreliable (Dhawan and Sharma 2010). Identifying methods suitable for the expansive assortment of CNTs and their unique chemical properties is undoubtedly, an arduous task. Nevertheless, establishment of such standards should be a priority for future research endeavors, as it would pave the way for considerable strides in discovery.

In the future, useful information could be obtained by extending this research to include different types of bacteria beyond *Escherichia coli*. As discussed previously, other microbes that have been studied include *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis,* and *Salmonella typhimurium*. Utilizing microbes of both gram positive and gram negative varieties could provide further clarity regarding the mechanisms by which these particular classes of CNTs exert their antimicrobial effects.

Many of the methods employed in this study were refined as needed throughout the data acquisition phases of this experiment. As a result, the viability plating assay utilized a different CNT concentration and cell suspension density compared to the DNA efflux assay. It may be valuable to repeat the DNA efflux experiments using a CNT concentration and cell density consistent with the plating viability experiments. Completing such experiments could allow for better data comparison between the two assays.

Inclusion of additional techniques in the evaluation of CNT toxicity may be worth consideration in future studies. The viability plating experiments suggested that another mechanism may account for the toxicity of NL SW($NH_2$) CNTs beyond membrane puncture. It may be possible to understand this effect further through the incorporation of other laboratory techniques. Previous research has implemented DNA microarray techniques, which are indispensable for quantifying subtle changes in genetic expression (Kang, Herzberg et al. 2008). Incorporation of a microarray technique could potentially illuminate subtle cellular changes that would be difficult or impossible to detect through other laboratory techniques.

It can be argued that the current study only served to investigate acute toxic effects of these CNT varieties. Undoubtedly, it would also be interesting to investigate potential effects of chronic exposure to the CNTs included in this study. However, substantial protocol modification, possibly including a different model organism, would be required to conduct such experiments.

Finally, this research provided a brief investigation into the effect of chlorine reaction on CNT toxicity. Although chlorination alone did not appear to produce a change in CNT toxicity, results of this study suggested that a coupled chlorination-dechlorination reaction significantly reduced toxicity of NL SW($NH_2$) CNTs. Further analysis of this effect, and a possible surface chemical transformation, are of great interest for future research. In addition, it would be worthwhile to observe potential effects of disinfection on CNT toxicity using an extended pool oxidizing agents, such as ozone, as well as ultraviolet disinfection.

During experimentation, this study uncovered a substantial difference in the apparent toxicity of carboxylated SWNTs (-COOH) in comparison with the other CNTs investigated. In light of the chlorine reaction data, we intend to investigate the effects of free chlorine reaction, as well as the use of other disinfection processes, on these carboxylated CNTs.

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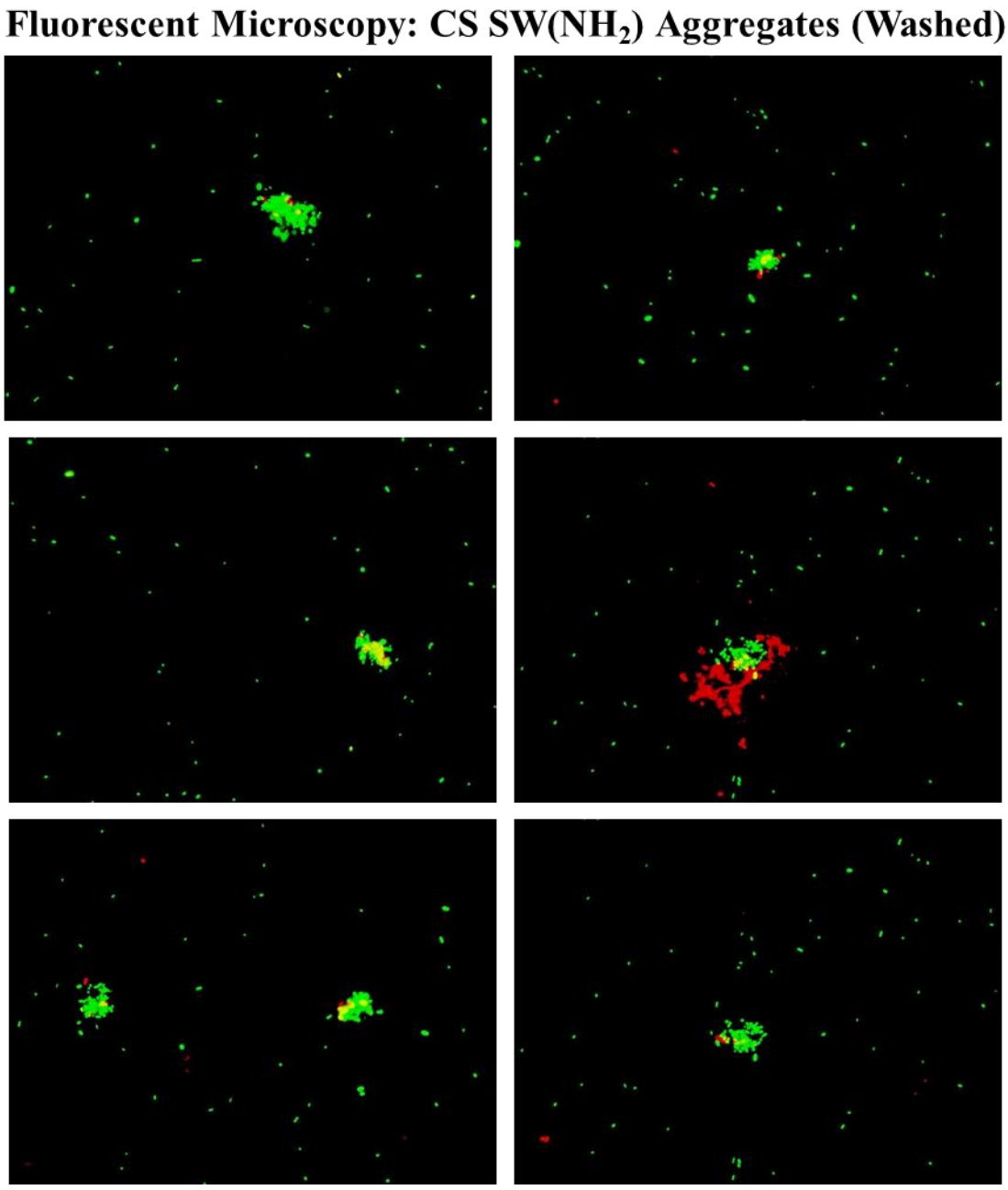
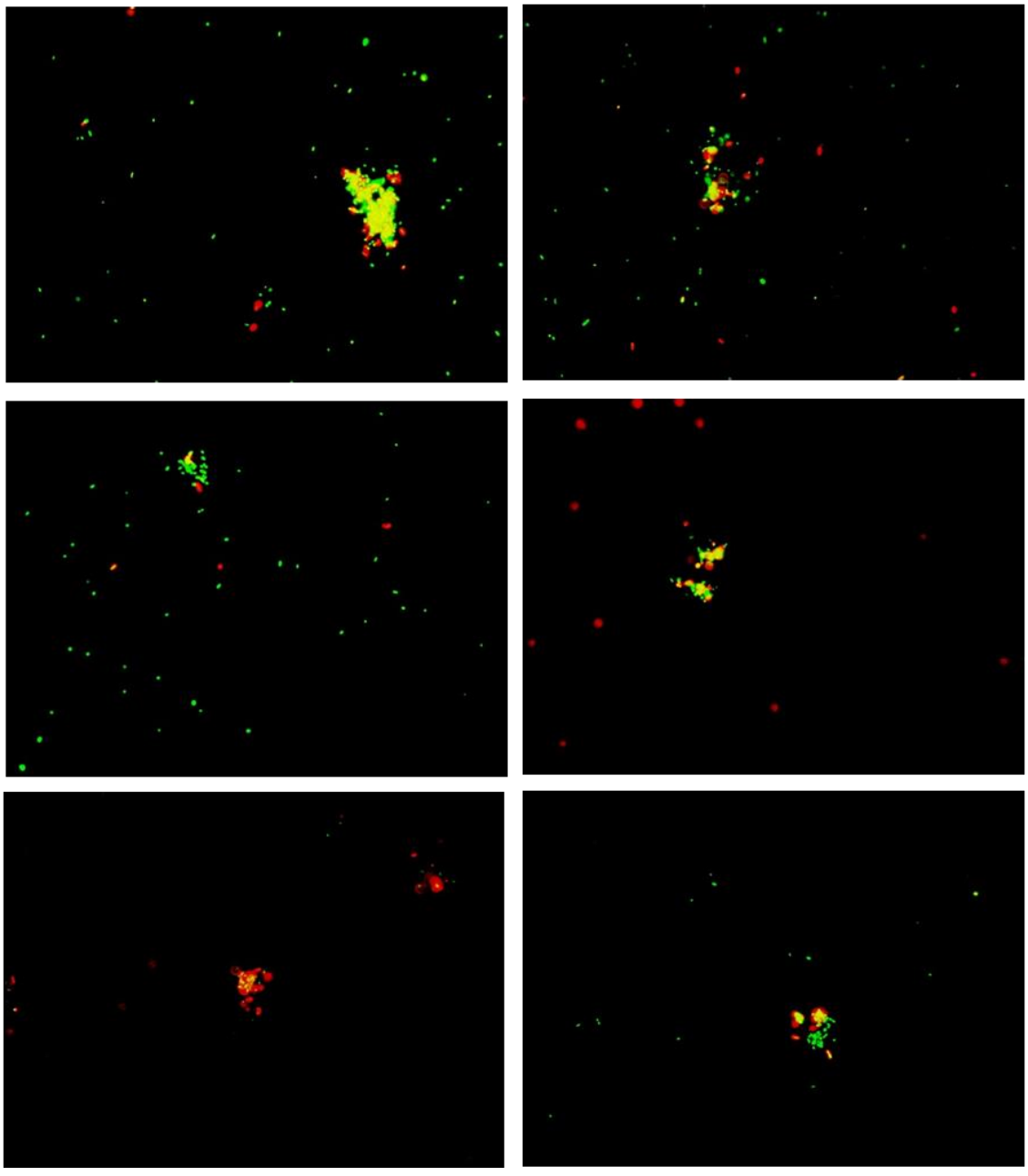
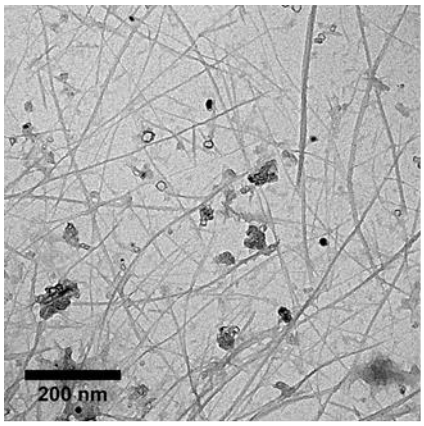
# APPENDIX A

## SELECTED IMAGES

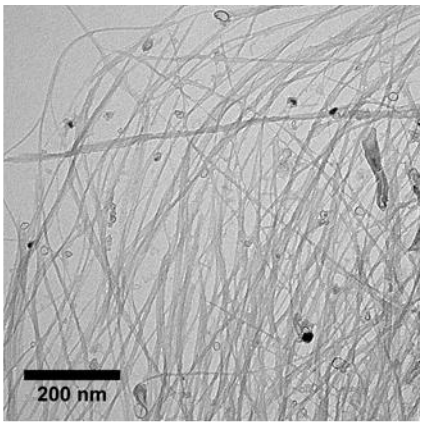


**Fluorescent Microscopy: CS SW($NH_2$) Aggregates (Unwashed)**

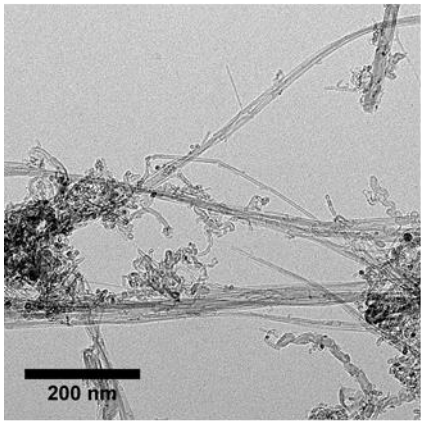




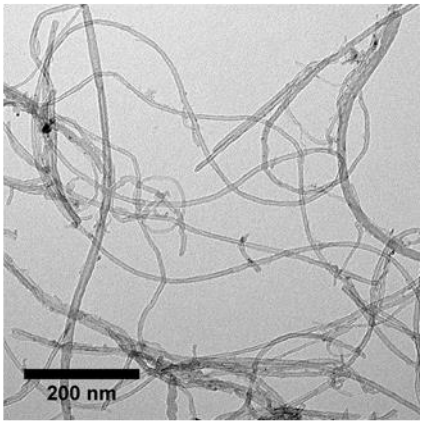

TEM Image: Carbon Solutions PABS SWNT




TEM Image: Carbon Solutions SW($NH_2$)




TEM Image: Nano Labs MW($NH_2$)




TEM Image: Nano Labs SW($NH_2$)

# APPENDIX B

## SELECTED RAW DATA

### Live/Dead Fluorescent Microscopy Data

| Dead | | | Live | | |
|---|---|---|---|---|---|
| Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead |
| 0 | 24 | 100.0 | 43 | 2 | 4.4 |
| 0 | 21 | 100.0 | 40 | 5 | 11.1 |
| 0 | 6 | 100.0 | 33 | 20 | 37.7 |
| 0 | 17 | 100.0 | 57 | 3 | 5.0 |
| 0 | 14 | 100.0 | 38 | 1 | 2.6 |
| 0 | 10 | 100.0 | 46 | 2 | 4.2 |
| 0 | 13 | 100.0 | 60 | 12 | 16.7 |
| 2 | 17 | 89.5 | 68 | 5 | 6.8 |
| 0 | 13 | 100.0 | 42 | 3 | 6.7 |
| 0 | 9 | 100.0 | 50 | 5 | 9.1 |
| 0 | 15 | 100.0 | 51 | 5 | 8.9 |
| 0 | 21 | 100.0 | 40 | 6 | 13.0 |
| 1 | 30 | 96.8 | 50 | 2 | 3.8 |
| 0 | 60 | 100.0 | 36 | 3 | 7.7 |
| 0 | 41 | 100.0 | 59 | 1 | 1.7 |
| 0 | 18 | 100.0 | 58 | 7 | 10.8 |

### FM Washing Effect: Carbon Solutions SW($NH_2$) (4/28/13)

| Control | | | Washed (Freely Suspended) | | | Unwashed (Freely Suspended) | | |
|---|---|---|---|---|---|---|---|---|
| Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead |
| 19 | 1 | 5.0 | 33 | 0 | 0.0 | 26 | 0 | 0.0 |
| 21 | 1 | 4.5 | 32 | 9 | 22.0 | 14 | 1 | 6.7 |
| 24 | 4 | 14.3 | 23 | 4 | 14.8 | 25 | 0 | 0.0 |
| 19 | 3 | 13.6 | 34 | 2 | 5.6 | 21 | 0 | 0.0 |
| 35 | 0 | 0.0 | 22 | 0 | 0.0 | 35 | 0 | 0.0 |
| 33 | 1 | 2.9 | 38 | 0 | 0.0 | 39 | 0 | 0.0 |
| 26 | 3 | 10.3 | 29 | 0 | 0.0 | 32 | 2 | 5.9 |
| 21 | 2 | 8.7 | 30 | 1 | 3.2 | 24 | 1 | 4.0 |
| 26 | 3 | 10.3 | 29 | 1 | 3.3 | 23 | 0 | 0.0 |
| 19 | 8 | 29.6 | 36 | 0 | 0.0 | 52 | 4 | 7.1 |
| 13 | 5 | 27.8 | 42 | 3 | 6.7 | 34 | 0 | 0.0 |
| 31 | 3 | 8.8 | 36 | 2 | 5.3 | 42 | 0 | 0.0 |
| 20 | 5 | 20.0 | 38 | 7 | 15.6 | 20 | 0 | 0.0 |
| 26 | 4 | 13.3 | 29 | 2 | 6.5 | 18 | 0 | 0.0 |
| 23 | 7 | 23.3 | 34 | 1 | 2.9 | 29 | 1 | 3.3 |

**FM Washing Effect: Carbon Solutions SW($NH_2$) (5/03/13)**

| | Control | | | Washed (Freely Suspended) | | | Washed (Attached) | | | Unwashed (Freely Suspended) | | | Unwashed (Attached) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Frame | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead | Live Area | Dead Area | % Dead Area | Live Count | Dead Count | % Dead | Live Area | Dead Area | % Dead Area |
| 1 | 33 | 2 | 5.7 | 39 | 1 | 2.5 | 14300 | 1264 | 8.1 | 38 | 4 | 9.5 | 5439 | 1140 | 17.3 |
| 2 | 52 | 0 | 0.0 | 34 | 1 | 2.9 | 5782 | 774 | 11.8 | 29 | 3 | 9.4 | 3114 | 4466 | 58.9 |
| 3 | 44 | 1 | 2.2 | 59 | 0 | 0.0 | 5948 | 213 | 3.5 | 26 | 11 | 29.7 | 3279 | 1282 | 28.1 |
| 4 | 74 | 2 | 2.6 | 37 | 0 | 0.0 | 8220 | 1629 | 16.5 | 26 | 3 | 10.3 | 5240 | 4140 | 44.1 |
| 5 | 51 | 1 | 1.9 | 51 | 0 | 0.0 | 9636 | 3474 | 26.5 | 31 | 1 | 3.1 | 2870 | 2738 | 48.8 |
| 6 | 33 | 2 | 5.7 | 50 | 0 | 0.0 | 6494 | 4472 | 40.8 | 30 | 6 | 16.7 | 1339 | 8001 | 85.7 |
| 7 | 47 | 1 | 2.1 | 43 | 0 | 0.0 | 5655 | 1241 | 18.0 | 36 | 3 | 7.7 | 7546 | 9104 | 54.7 |
| 8 | 61 | 3 | 4.7 | 43 | 0 | 0.0 | 4232 | 1074 | 20.2 | 31 | 5 | 13.9 | 3824 | 7354 | 65.8 |
| 9 | 80 | 2 | 2.4 | 38 | 1 | 2.6 | 4458 | 418 | 8.6 | 34 | 3 | 8.1 | 7633 | 4755 | 38.4 |
| 10 | 64 | 1 | 1.5 | 44 | 0 | 0.0 | 9627 | 1644 | 14.6 | 31 | 3 | 8.8 | 3320 | 9392 | 73.9 |
| 11 | 66 | 0 | 0.0 | 40 | 1 | 2.4 | 43942 | 21390 | 32.7 | 43 | 4 | 8.5 | 5885 | 1581 | 21.2 |
| 12 | 102 | 2 | 1.9 | 64 | 1 | 1.5 | 5125 | 1570 | 23.5 | 28 | 0 | 0.0 | 4564 | 3269 | 41.7 |
| 13 | 50 | 2 | 3.8 | 47 | 0 | 0.0 | 9409 | 1027 | 9.8 | 20 | 3 | 13.0 | 5699 | 2183 | 27.7 |
| 14 | 34 | 1 | 2.9 | 43 | 1 | 2.3 | 14656 | 3024 | 17.1 | 16 | 3 | 15.8 | 3237 | 3276 | 50.3 |
| 15 | 28 | 1 | 3.4 | 44 | 1 | 2.2 | 4499 | 1482 | 24.8 | 29 | 2 | 6.5 | 21000 | 22553 | 51.8 |

**FM Chlorine Reaction Effect: NL SW($NH_2$)**

| | Control | | | Washed (Unreacted) | | | HOCl-reacted (25 mg/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| Frame | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead |
| 1 | 19 | 2 | 9.5 | 38 | 1 | 2.6 | 21 | 1 | 4.5 |
| 2 | 23 | 5 | 17.9 | 38 | 4 | 9.5 | 31 | 4 | 11.4 |
| 3 | 24 | 1 | 4.0 | 44 | 6 | 12.0 | 30 | 1 | 3.2 |
| 4 | 36 | 3 | 7.7 | 34 | 13 | 27.7 | 15 | 3 | 16.7 |
| 5 | 43 | 3 | 6.5 | 21 | 3 | 12.5 | 22 | 7 | 24.1 |
| 6 | 41 | 14 | 25.5 | 25 | 4 | 13.8 | 29 | 7 | 19.4 |
| 7 | 42 | 5 | 10.6 | 25 | 4 | 13.8 | 20 | 10 | 33.3 |
| 8 | 29 | 5 | 14.7 | 54 | 6 | 10.0 | 28 | 10 | 26.3 |
| 9 | 24 | 5 | 17.2 | 47 | 1 | 2.1 | 19 | 10 | 34.5 |
| 10 | 20 | 8 | 28.6 | 56 | 5 | 8.2 | 28 | 4 | 12.5 |

**FM Chlorine Reaction Effect: NL SW($NH_2$)**

| | Control | | | Washed (Unreacted) | | | HOCl-reacted (50 mg/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| Frame | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead |
| **1** | 176 | 3 | 1.7 | 69 | 1 | 1.4 | 56 | 6 | 9.7 |
| **2** | 192 | 7 | 3.5 | 80 | 4 | 4.8 | 44 | 6 | 12.0 |
| **3** | 170 | 2 | 1.2 | 107 | 5 | 4.5 | 51 | 3 | 5.6 |
| **4** | 177 | 4 | 2.2 | 122 | 4 | 3.2 | 54 | 3 | 5.3 |
| **5** | 143 | 3 | 2.1 | 157 | 0 | 0.0 | 46 | 10 | 17.9 |
| **6** | 151 | 10 | 6.2 | 135 | 2 | 1.5 | 52 | 2 | 3.7 |
| **7** | 107 | 6 | 5.3 | 81 | 7 | 8.0 | 62 | 3 | 4.6 |
| **8** | 115 | 1 | 0.9 | 94 | 5 | 5.1 | 54 | 2 | 3.6 |
| **9** | 94 | 5 | 5.1 | | | | 123 | 14 | 10.2 |
| **10** | 89 | 12 | 11.9 | | | | 93 | 3 | 3.1 |
| **11** | 74 | 7 | 8.6 | | | | 166 | 20 | 10.8 |
| **12** | 80 | 8 | 9.1 | | | | 144 | 19 | 11.7 |
| **13** | | | | | | | 104 | 11 | 9.6 |

**FM NL MW($NH_2$) Washing Effect**

| | Control | | | Washed | | | Unwashed | | |
|---|---|---|---|---|---|---|---|---|---|
| Frame | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead | Live Count | Dead Count | % Dead |
| 1 | 37 | 9 | 19.6 | 59 | 7 | 10.6 | 33 | 1 | 2.9 |
| 2 | 44 | 12 | 21.4 | 65 | 26 | 28.6 | 57 | 1 | 1.7 |
| 3 | 52 | 8 | 13.3 | 32 | 24 | 42.9 | 58 | 1 | 1.7 |
| 4 | 52 | 11 | 17.5 | 44 | 19 | 30.2 | 70 | 0 | 0.0 |
| 5 | 48 | 6 | 11.1 | 22 | 19 | 46.3 | 52 | 2 | 3.7 |
| 6 | 46 | 7 | 13.2 | 57 | 14 | 19.7 | 61 | 1 | 1.6 |
| 7 | 30 | 18 | 37.5 | 69 | 15 | 17.9 | 41 | 0 | 0.0 |
| 8 | 45 | 10 | 18.2 | 38 | 24 | 38.7 | 36 | 3 | 7.7 |
| 9 | 80 | 17 | 17.5 | 61 | 9 | 12.9 | 43 | 0 | 0.0 |
| 10 | 37 | 26 | 41.3 | 65 | 19 | 22.6 | 30 | 1 | 3.2 |
| 11 | 60 | 31 | 34.1 | 55 | 43 | 43.9 | 36 | 3 | 7.7 |
| 12 | 40 | 11 | 21.6 | 75 | 33 | 30.6 | 86 | 1 | 1.1 |
| 13 | 47 | 11 | 19.0 | 41 | 22 | 34.9 | 45 | 1 | 2.2 |
| 14 | 44 | 4 | 8.3 | 44 | 23 | 34.3 | 44 | 2 | 4.3 |
| 15 | 22 | 31 | 58.5 | 40 | 26 | 39.4 | 49 | 4 | 7.5 |

**pDNA Adsorption Experiments (10 µg/ml)**

200 rpm, 1 hr, 37 C

| NL SW($NH_2$) | | NL MW($NH_2$) | | CS SW($NH_2$) (2) | | CS PABS | | HOCl-Reacted NL SW($NH_2$) | |
|---|---|---|---|---|---|---|---|---|---|
| Sample | pDNA conc (ng/ml) | Sample | pDNA conc (ng/ml) | Sample | pDNA conc (ng/ml) | Sample | pDNA conc (ng/ml) | Sample | pDNA conc (ng/ml) |
| Control 1 | 144 | Control 1 | 157 | Control 1 | 103 | Control 1 | 122 | Control 1 | 181 |
| Washed 1 | 137 | Washed 1 | 127 | Washed 1 | 33.2 | Washed 1 | 126 | HOCl 1 | 173 |
| Unwashed 1 | 141 | Unwashed 1 | 132 | Unwashed 1 | 25.1 | Unwashed 1 | 133 | | |
| Control 2 | 110 | Control 2 | 120 | Control 2 | 120 | Control 2 | 134 | Control 2 | 182 |
| Washed 2 | 94.9 | Washed 2 | 111 | Washed 2 | 32.4 | Washed 2 | 123 | HOCl 2 | 176 |
| Unwashed 2 | 92.6 | Unwashed 2 | 92.4 | Unwashed 2 | 26.7 | Unwashed 2 | 136 | | |

**pDNA Efflux Experiments**

| **NL MW($NH_2$) (10 ug/ml)** | | | | | |
|---|---|---|---|---|---|
| Date: 6/18/2013 | | Date: 6/21/2013 | | Date: 6/28/2013 | |
| Cell Concentration ($OD_{600}$) | 0.3402 | Cell Concentration ($OD_{600}$) | 0.3382 | Cell Concentration ($OD_{600}$) | 0.3859 |
| Resuspension Conc | 0.18 | Resuspension Conc | 0.2118 | Resuspension Conc | 0.1688 |
| **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** |
| Control | 87.8 | Control | 88.6 | Control | 60.1 |
| Washed | 80.3 | Washed | 86.5 | Washed | 35.6 |
| Unwashed | 24.6 | Unwashed | 28.5 | Unwashed | 18.5 |
| **CS SW($NH_2$) (2) (10 ug/ml)** | | | | | |
| Date: 7/2/2013 | | Date: 6/21/2013 | | Date: 6/28/2013 | |
| Cell Concentration ($OD_{600}$) | 0.3519 | Cell Concentration ($OD_{600}$) | 0.3395 | Cell Concentration ($OD_{600}$) | 0.3706 |
| Resuspension Conc | 0.1906 | Resuspension Conc | 0.2156 | Resuspension Conc | 0.2236 |
| **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** |
| Control | 91.8 | Control | 83.9 | Control | 105 |
| Washed | 91.8 | Washed | 82.2 | Washed | 113 |
| Unwashed | 86.6 | Unwashed | 66.3 | Unwashed | 108 |
| **NL SW($NH_2$) (10 ug/ml)** | | | | | |
| Date: 7/17/2013 | | | | | |
| Cell Concentration ($OD_{600}$) | 0.3546 | Cell Concentration ($OD_{600}$) | 0.3432 | Cell Concentration ($OD_{600}$) | 0.3338 |
| Resuspension Conc | 0.1718 | Resuspension Conc | 0.175 | Resuspension Conc | 0.1836 |
| **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** |
| Control | 72.3 | Control | 64.3 | Control | 56.3 |
| Washed | 65 | Washed | 73.2 | Washed | 77.9 |
| Unwashed | 62.3 | Unwashed | 61.9 | Unwashed | 102 |

## pDNA Efflux Experiments

| CS PABS (10 ug/ml) | | | | | |
|---|---|---|---|---|---|
| Date: 7/17/2013 | | Date: 7/18/2013 | | Date: 7/18/2013 | |
| Cell Concentration ($OD_{600}$) | 0.3434 | Cell Concentration ($OD_{600}$) | 0.3241 | Cell Concentration ($OD_{600}$) | 0.3975 |
| Resuspension Conc | 0.1988 | Resuspension Conc | 0.1972 | Resuspension Conc | 0.1976 |
| **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** |
| Control | 70.4 | Control | 75.3 | Control | 72.8 |
| Washed | 124 | Washed | 140 | Washed | 148 |
| Unwashed | 143 | Unwashed | 154 | Unwashed | 169 |
| **HOCl-Reacted NL SW($NH_2$) (10 ug/ml)** | | | | | |
| Date: 7/23/2013 | | Date: 7/24/2013 | | Date: 7/25/2013 | |
| Cell Concentration ($OD_{600}$) | 0.3468 | Cell Concentration ($OD_{600}$) | 0.3232 | Cell Concentration ($OD_{600}$) | 0.3831 |
| Resuspension Conc | 0.1937 | Resuspension Conc | 0.1841 | Resuspension Conc | 0.2089 |
| **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** | **Sample** | **pDNA conc (ng/ml)** |
| Control | 82.2 | Control | 91.3 | Control | 74.4 |
| HOCl-Reacted | 75.9 | HOCl-Reacted | 76.5 | HOCl-Reacted | 79.6 |

## Plating Viability NL SW($NH_2$) (200 µg/ml)

| | **Rep** | **Control** | | | **Washed** | | | **Unwashed** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1 hour** | 1 | 79 | 80 | 90 | 60 | 59 | 49 | 31 | 19 | 20 |
| | 2 | 110 | 125 | 113 | 33 | 43 | 45 | 31 | 42 | 31 |
| | 3 | 88 | 95 | 82 | 17 | 45 | 23 | 28 | 28 | 23 |
| | **Rep** | **Control** | | | **Washed** | | | **Unwashed** | | |
| **3 hour** | 1 | 63 | 78 | 67 | 6 | 10 | 7 | 1 | 4 | 3 |
| | 2 | 97 | 113 | 81 | 18 | 13 | 12 | 1 | 2 | 3 |
| | 3 | 88 | 69 | 61 | 7 | 3 | 4 | 3 | 2 | 2 |

## Plating Viability CS PABS (200 µg/ml)

| | **Rep** | **Control** | | | **Washed** | | | **Unwashed** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **1 hour** | 1 | 90 | 93 | 102 | 96 | 87 | 89 | 92 | 74 | 77 |
| | 2 | 149 | 155 | 162 | 157 | 145 | 139 | 150 | 150 | 155 |
| | 3 | 97 | 80 | 85 | 100 | 81 | 79 | 78 | 90 | 71 |
| | **Rep** | **Control** | | | **Washed** | | | **Unwashed** | | |
| **3 hour** | 1 | 89 | 97 | 78 | 15 | 22 | 17 | 27 | 34 | 28 |
| | 2 | 123 | 126 | 119 | 103 | 108 | 118 | 126 | 123 | 117 |
| | 3 | 85 | 77 | 89 | 40 | 67 | 49 | 46 | 54 | 54 |

**Plating Viability CS SW($NH_2$) (3) (200 µg/ml)**

| | Rep | Control | | | Washed | | | HOCl-Reacted | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 hour | 1 | 82 | 81 | 86 | 96 | 114 | 94 | 98 | 74 | 98 |
| | 2 | 97 | 100 | 97 | 97 | 87 | 98 | 93 | 79 | 105 |
| | 3 | 72 | 88 | 97 | 82 | 90 | 86 | 83 | 106 | 89 |
| | Rep | Control | | | Washed | | | HOCl-Reacted | | |
| 3 hour | 1 | 86 | 75 | 80 | 90 | 75 | 76 | 94 | 78 | 83 |
| | 2 | 91 | 73 | 75 | 79 | 93 | 83 | 110 | 81 | 83 |
| | 3 | 81 | 68 | 76 | 71 | 72 | 88 | 92 | 66 | 84 |

**Plating Viability HOCl Reacted/Quenched CNTs (200 µg/ml)**

| | Rep | Control | | | Control/Sodium Sulfate | | | PABS Reacted | | | NL SW($NH_2$) Reacted | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 hour | 1 | 176 | 177 | 153 | 187 | 186 | 172 | 153 | 146 | 129 | 169 | 174 | 154 |
| | 2 | 114 | 89 | 112 | 129 | 127 | 130 | 78 | 73 | 89 | 120 | 108 | 119 |
| | 3 | 106 | 105 | 114 | 110 | 114 | 108 | 84 | 95 | 84 | 97 | 85 | 95 |
| | Rep | Control | | | Control/Sodium Sulfate | | | PABS Reacted | | | NL SW($NH_2$) Reacted | | |
| 3 hour | 1 | 169 | 186 | 156 | 159 | 154 | 155 | 89 | 83 | 79 | 116 | 143 | 106 |
| | 2 | 102 | 104 | 102 | 93 | 95 | 111 | 29 | 26 | 25 | 59 | 104 | 92 |
| | 3 | 92 | 76 | 81 | 73 | 91 | 97 | 82 | 68 | 71 | 101 | 78 | 78 |

**Plating Viability NL SW($NH_2$) Reacted (200 µg/ml)**

| | Rep | Control (22 d) | | | Reacted (22 d) | | | Control (3 d) | | | Reacted (3 d) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 hour | 1 | 103 | 106 | 94 | 57 | 66 | 47 | 101 | 110 | 105 | 61 | 58 | 57 |
| | 2 | 95 | 96 | 100 | 53 | 41 | 45 | 86 | 105 | 85 | 40 | 44 | 54 |
| | 3 | 109 | 127 | 118 | 36 | 20 | 24 | 110 | 101 | 111 | 51 | 58 | 58 |
| | Rep | Control (22 d) | | | Reacted (22 d) | | | Control (3 d) | | | Reacted (3 d) | | |
| 3 hour | 1 | 89 | 101 | 112 | 23 | 16 | 23 | 80 | 88 | 104 | 33 | 48 | 27 |
| | 2 | 110 | 78 | 73 | 24 | 31 | 21 | 81 | 92 | 77 | 25 | 26 | 33 |
| | 3 | 103 | 113 | 104 | 12 | 7 | 13 | 92 | 100 | 108 | 29 | 24 | 36 |

**Plating Viability Carboxylated SWNTs (200 µg/ml)**

| | Rep | Control | | | Carboxylated | | |
|---|---|---|---|---|---|---|---|
| 1 hour | 1 | 103 | 106 | 94 | 0 | 0 | 0 |
| | 2 | 95 | 96 | 100 | 0 | 0 | 0 |
| | 3 | 109 | 127 | 118 | 0 | 0 | 0 |
| | Rep | Control | | | Carboxylated | | |
| 3 hour | 1 | 89 | 101 | 112 | 0 | 0 | 0 |
| | 2 | 110 | 78 | 73 | 0 | 0 | 0 |
| | 3 | 103 | 113 | 104 | 0 | 0 | 0 |