Antibacterial activity of carbon nanomaterials

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2012

Liu, S. B. (2012). Antibacterial activity of carbon nanomaterials. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/50713>

<https://doi.org/10.32657/10356/50713>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**ANTIBACTERIAL ACTIVITY OF CARBON
NANOMATERIALS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING
2012**

ANTIBACTERIAL ACTIVITY OF CARBON NANOMATERIALS

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A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

2012

Acknowledgements

My four years of study and research experience in Nanyang Technological University was enjoyable and rewarding. I would like to take this opportunity to express my gratitude to those who assisted me in either research or private life during my PhD study.

First of all, I would like to express my sincere gratitude to my supervisor, Prof Chen Yuan, for his outstanding guidance and support to my 4-year PhD study. His patience, enthusiasm, and serious attitude to the research deeply influence me. I was always impressed by his creative ideas about the projects.

Besides my supervisor, I would like to thank Dr. Wei Jun (SIMTech, A*star) for his valuable and inspiring advices for my research. Prof. Robert Hurt and Prof Agnes Kane at Brown University (USA) also helped me a lot. They provided me the opportunity to exchange and study in Brown University. It was a wonderful experience to work with their group members in Brown.

Also, I would like to take this chance to thank my colleagues and collaborators, Wang Bo, Wei Li, Wang Qiang, Shen Jianmin, Chen Fuming, Wang Hong, Si Rongmei, Jiang Wenchao, Hao Lin, Wu Ran and so many others. They are so generous to offer me helps on research.

I highly appreciate our professional officer Dr Fang Ning, Dr Wang Xiujuan, and Dr Yu Shucong and technical executives for their help in analytical instruments.

I also would like to acknowledge the financial support from Nanyang Technological University.

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List of Abbreviations

AFM	Atomic Force Microscopy
CNM	Carbon Nanomaterials
CNT	Carbon Nanotube
CVD	Chemical Vapor Deposition
DTG	Differential Thermogravimetric
DWCNT	Double-Walled Carbon Nanotube
Gt	Graphite
GO	Graphene Oxide
GtO	Graphite Oxide
LB	Luria-Bertani
MCM-41	Mobil Composition of Matter 41
MWCNT	Multi-Walled Carbon Nanotube
OD	Optical Density
PLE	Photoluminescence
rGO	Reduced Graphene Oxide
SC	Sodium Cholate
SEM	Scanning Electron Microscope
SWCNT	Single-Walled Carbon Nanotube
TEM	Transmission Electron Microscope

TGA	Thermogravimetric Analysis
UV-vis-NIR	Ultraviolet-visible-Near infrared

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Abstract

Carbon nanomaterials (CNMs), including graphene based materials, carbon nanotubes and fullerene, have been intensively studied in recent years because of their unique properties and various potential applications. This thesis focuses on the antibacterial activity of CNMs, more specifically, one-dimensional single walled carbon nanotubes (SWCNTs) and two dimensional graphene based materials, including graphite (Gt), graphite oxide (GtO), graphene oxide (GO), and reduced graphene oxide (rGO).

First, the antibacterial activity of high purity SWCNTs was investigated. The results demonstrate that individually dispersed SWCNTs exhibited much higher antibacterial activity than SWCNT aggregates toward several types of bacteria. Ultraviolet–visible spectroscopy absorption spectroscopy study at 260 nm and scanning electron microscope images reveal that the bacterial death is related to the destruction of bacterial membrane.

Next, antibacterial mechanism of SWCNTs was explored. My results suggest that inhibition of cell and oxidative stress induced by superoxide anion ($O_2^{\cdot-}$) are not the major causes. Furthermore, the effects of Cobalt on SWCNT samples can be ruled out. The physical interactions between SWCNTs and bacteria were investigated by atomic force microscopy. Results demonstrate that individually dispersed SWCNTs in solution develop nanotube networks on bacterial surface, which destroy the bacterial envelopes with leakage of intracellular contents. Further analysis indicates that a single collision between one nanotube and bacterial cell is unlikely to introduce direct physical damage. Hence, antibacterial activity of SWCNTs is the accumulation effect of large amount of nanotubes through interactions between SWCNT networks and bacterial cells.

Then, the antibacterial activity and mechanism of graphene based nanomaterials were investigated. GO dispersion shows the highest antibacterial activity, sequentially followed by rGO, Gt, and GtO. Scanning electron microscope images display that graphene nanosheets

disrupt cell membrane. No superoxide anion ($O_2^{\cdot-}$) induced reactive oxygen species production is detected. However, the four types of materials can oxidize glutathione, which serves as a redox state mediator in bacteria. Effects of lateral dimension of GO nanosheets on their antibacterial activity were also investigated. GO samples with larger lateral dimensions show stronger antibacterial activity than smaller ones. The different antibacterial activity observed among GO sheets with different lateral dimensions can be attributed to their different wrapping efficiency. Once cells are wrapped, they were biologically isolated from growth medium. Thus, cells can neither consume the nutrients nor proliferate. My results suggested that antibacterial actions of graphene based nanomaterials are contributed by membrane stress, oxidation stress and lack of nutrients. A three-step antimicrobial mechanism is applicable to graphene-based materials. It includes initial cell deposition on graphene based nanomaterials, membrane stress caused by direct contact with sharp nanosheets, and the followed superoxide anion-independent oxidation.

This study elucidated antibacterial mechanism of CNMs and several factors governing their antibacterial activity , and it provided an insight in developing strategies that can maximize the CNM antibacterial application potentials.

Chapter 1

Introduction

Carbon nanomaterials (CNMs) were intensively investigated due to their unique properties and potential applications, especially for the graphitic carbon family-graphene, carbon nanotube (CNT) and fullerene. These CNMs share the same substance: hexagonally bonded carbon. The discovery of these nanomaterials (graphene, CNT and fullerene) changes the views of scientists toward CNMs. All of these discoveries have been awarded some of the highest honors in the scientific world. Fullerene was first discovered by Richard Smalley *et al.*¹ in 1985, and he was awarded Nobel Prize in Chemistry in 1996; CNT was discovered by Sumio Iijima in 1991², and he was awarded Kavli Prize for Nanoscience in 2008; and graphene was obtained by Geim *et al.*³ in 2004, and he was awarded Nobel Prize in Physics in 2011.

Graphene is a single atomic plane of graphite (Gt),⁴ which was first obtained through micro-mechanical exfoliation of Gt.³ Graphene is the parent of graphitic forms (Figure 1-1)⁴. The carbon-carbon bond length in graphene is about 0.142 nanometers and interplanar spacing of graphene sheets in graphite is about 0.335 nm.

CNTs are hollow cylinders rolled up by a single or several layers of one-atom-thick hexagonal carbon sheets. Single-walled carbon nanotubes (SWCNTs) were first synthesized in 1993^{5,6}, while the finding of multi-walled carbon nanotubes (MWCNTs) has been traced back much earlier^{2,7}. Common CNT synthesis methods⁶ share the same principle. Atomic carbon species derived from either solid carbon sources or carbon-bearing gases organize into thermodynamically stable nanoscale CNTs on catalyst nanoparticles at high temperatures (normally 700 to 1000 °C). CNTs have diameters ranging from 0.4 nm to a few hundred nanometers (typically 1 nm for SWCNTs and tens of nanometers for MWCNTs) with high length to diameter ratios, and their lengths range from hundreds of nanometers to centimeters⁸.

Figure 1-1 Schematic structure of carbon based nanomaterials⁴

Most earlier studies of graphene and CNTs focus on their extraordinary electronic, thermal and mechanical properties, resulting from their unique two-dimensional⁹ or one-dimensional nanostructures¹⁰. Soon, their potential biomedical applications have been extensively explored because they can be easily internalized by cells^{11, 12}, and therefore can act as delivery vehicles of various molecules^{13, 14}. Furthermore, their electronic and spectroscopic properties offer opportunities in detection and treatment of diseases^{15, 16}. However, most of biomedical investigations focused on the impact on mammalian cells.

A few early studies showed that functionalized CNTs have antimicrobial activities^{17, 18}. The first direct evidence that pristine CNTs show strong antimicrobial activities appears in 2007¹⁹. The results showed that direct contact between CNTs with *E. coli* causes severe

membrane damage and subsequent cell inactivation. A few other studies on the antimicrobial activity of CNTs appear in the next several years.^{20, 21} The earliest reports on the antibacterial activities of graphene based materials, graphene oxide (GO) and reduced graphene oxide (rGO), were published in 2010^{22, 23}. These results showed that GO and rGO exhibit strong antibacterial activities no matter they are suspended in saline solution or filtered as films.^{22, 23} Most of those studies have been conducted from the prospect of understanding the environmental impacts of CNTs and graphene, using these CNMs for removal of contaminants in drinking water, and developing antimicrobial surface coatings containing CNMs.

1.1 Graphene

Graphene is a two-dimensional sheet of sp^2 -hybridized carbon,⁴ which has attracted great interests because of its wide range of unusual properties, such as its unusual structural characteristics, large specific surface area, superlative mechanical strength²⁴, high intrinsic mobility²⁵, high optical transmittance ($\sim 97.7\%$ for a single layer)²⁶, and remarkable electronic properties.²⁷ Potential applications of graphene require the production of pure graphene with high quality and high yield.

Graphene can be prepared by various methods, including micromechanical exfoliation, chemical vapor deposition (CVD) method, arc discharge, and so on.

1) Micromechanical exfoliation. Micromechanical exfoliation is the earliest method used to prepare the graphene sheet.³ The basic process for the exfoliation is repeated peeling. The bond between the layers of graphene sheet of graphite was break by the mechanical energy during the exfoliation.

2) CVD method. Although the high quality graphene with few layers can be produced by peeling using scotch-tape, the graphene yield is too low by micromechanical exfoliation method. CVD is an alternative method to produce graphene. For the CVD process, transition metals were usually used as catalysts and hydrocarbon gases were used as precursor. Direct

synthesis of graphene by CVD have produced films with 200 Ω/S sheet resistance at 85% optical transmittance,²⁸ and the size of graphene film can reach 30 inches.²⁹ Li *et al.* synthesized high quality graphene film on Cu foils by CVD method.³⁰ This film can be transferred to silicon and glass. Microwave plasma-enhanced CVD, low-temperature CVD, and radio frequency catalytic CVD *et al.* CVD process with modification also explored to synthesize graphene.³¹

Beside the micromechanical exfoliation method and CVD method, other methods, such as arc-discharge, electrochemical synthesis are also employed to produce the graphene.³¹

Graphene oxide (GO) is a graphene sheet with carboxylic groups at its edges and phenol hydroxyl and epoxide groups on its basal plane.³² GO can be chemically exfoliated from graphite oxide (GtO), which is produced by controlled oxidation of graphite.³³⁻³⁵ Thermal annealing or chemical treatment can eliminate functional groups on GO to produce reduced graphene oxide (rGO).³⁶

Figure 1-2 Schematic model of a GO sheet (A) and rGO sheet (B)³⁶

Graphite oxide (GtO) can be obtained by oxidation of graphite. Three main protocols have been developed by Brodie³³, Staudenmeier³⁴, and Hummers³⁵ to synthesize GtO. Among these methods, Hummers method was widely used due to its shorter reaction time and absence

of hazardous gas compared to the Brodie and Staudenmeier methods, which can generate high toxicity ClO_2 .

rGO was prepared by reducing GO through various methods, including using reducing agent, such as hydrazine, vitamin C, reducing sugar, carbon monoxide, and Fe, thermal treatment, electrochemical, bacterial respiration, and so on.

1.2 Single-Walled Carbon Nanotubes

CNTs are pseudo-one-dimensional carbon allotropes of high aspect ratio, large surface area, and excellent material properties such as low density, a high elastic modulus (up to 1TPa), excellent thermal conductivities and mechanical strength (range from 2.5 to 3.5GPa).³⁷ This carbon hollow graphitic nanomaterial may conceptually be viewed as “rolled-up” structures of one or more layers of graphene sheets for SWCNTs or MWCNTs, respectively. These nanocomposites belong to the family of fullerenes, the third allotropic form of carbon (others are graphite and diamond). SWCNTs, the simplest form of CNTs, are defined fully by their diameter, chiral angle and band gap. SWCNTs can be divided into three different types: metallic, semimetallic, and semiconductive, depending on the rolling action of graphene sheet.

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CVD is a widely used SWCNT synthesis method³⁹. This method provides more controllable routes to produce structure controlled SWCNTs compared to arc discharge⁴⁰ and laser ablation which are also typical CNT synthesis methods.

In CVD methods, carbon precursors, such as carbon monoxide or ethanol³⁹, decompose at high temperature. The decomposed carbon radicals then form SWCNTs on metallic particle catalysts which are placed in heated quartz tube's center. The CVD method uses transition metals, such as Fe, Co or Ni, as catalysts. The catalysts induce the catalytic dehydrogenation of carbon precursors and consequently the formation of CNTs. The growth mechanisms are essentially categorized into root growth or tip growth⁴¹, depending on the interaction strength

between the metallic nanoparticles and the substrate. The size of the metallic clusters is related to the diameter of CNTs, moreover metallic clusters can also determine whether SWCNTs, MWCNTs or carbon filaments are formed. Previous studies have shown that a smaller metal cluster would favor the formation of SWCNT and a larger one favors the formation of MWCNT or even carbon filament.⁴¹

1.3 Heterogeneous Nature of CNM samples

Unlike regular chemical agents which have well-defined size, molecular structures, properties and purity, commonly available CNM samples are a mixture of many chemical species. They include noncarbon impurities, such as transition metal residues and nanosized catalyst supports; carbon impurities, such as amorphous carbon, carbon particles, graphite and carbon fibers. CNTs are different in diameter, length, chirality (the direction in which a carbon layer rolls up to form a nanotube)⁴², morphology, surface functional groups, and defects. Graphenes are also various in layers, sizes, surface functional groups, and defects⁴³. Besides, they often aggregate together to form CNT bundles⁴⁴ or graphene aggregates³⁶ with different sizes. Thus, it is essential to find out reliable correlations between specific CNM structural or chemical characteristics and the antimicrobial activity of CNM samples, so we can increase CNMs' potentials in antimicrobial applications, while lessen their risks. To get such reliable correlations, the properties of CNMs, such as concentration, dispersion, surface functional group, and size, should be characterized in antimicrobial assays.

The exact composition and properties of CNM samples depend on how they are synthesized, purified, and functionalized.

Various purification strategies have been developed to improve the purity of CNT samples. Haddon *et al.* have summarized their common strategies⁴⁵. Most of purification methods rely on one or more of these steps: oxidation, centrifugation, or filtration. Pristine CNTs are not soluble in water because of their hydrophobic graphitic surfaces.

Functionalization is needed to obtain a homogeneous CNT aqueous dispersion. Both covalent (*e.g.* introducing carboxyl groups, amino groups or other functional groups) and noncovalent (surfactant or polymer wrapping) functionalization methods are available for achieving CNT solubilization⁴⁶. Previously, significant efforts have also been devoted to get monodispersed CNT samples through both selective synthesis and post-synthetic approaches. For instance, SWCNTs with controlled chirality, such as (9,8) nanotubes⁴⁷, (6,5) nanotubes⁴⁸, and (7,5) nanotubes⁴⁹ can be produced by chirality selective synthesis. SWCNTs with different length can also be selective grown⁵⁰. Post-synthetic approaches include selective chemistry, electrical breakdown, dielectrophoresis, chromatography and ultracentrifugation⁵¹. CNT samples with controlled diameter, length and/or chirality to some extent are already commercially available. These efforts have already been, and are likely to further speed up our understanding and eventually realizing the applications of the antimicrobial activity of CNTs.

Purification methods are different for graphene based materials according to their synthesis method. Graphene sheet from micromechanical exfoliation is relatively “clean”, because there are no chemicals used during the whole process.³ GO and rGO synthesized by oxidation and reduction process may be contaminated by various ions and chemical residues. These potential contaminations could be removed by thorough washing and dialysis. Graphene can be functionalized with the similar strategies with CNTs by both covalent and noncovalent methods.⁵² Graphene can be functionalized by various functional groups, such as carboxyl groups, amino groups, and alkyl chains. Noncovalent modification by wrapping with surfactants (such as polyethylene glycol, sodium dodecylsulfate, polyvinyl pyrrolidone, and so on) or through π - π interaction with a pyrene derivative also employed to functionalize graphene.⁴³

Also, it is worth mentioning that evaluation of CNM properties depends on many analytical techniques, such as, optical absorbance, Raman, SEM, TEM, AFM, and ICP

analysis. However, until now, widely accepted evaluation standards for establishing CNM purity levels are still not available. Thus, please note the property data provided by some CNM producers may not be reliable. Careful evaluation of CNM sample properties is necessary for their antimicrobial applications.

1.4 Toxicity of Carbon Nanomaterials

The rapid expansion of CNMs in research and industries call for the consideration of appropriate toxicological investigation in detail.⁵³ “Particle toxicity is the study of the adverse effects of tissue exposure (typically through the lungs, digestive tract or skin) to particulate matter.”⁵⁴ CNMs could enter into human body by various ways, such as inhalation, ingestion, and injection. Skin, lungs and eyes are the main portals through which carbon nanotubes may come into the body.⁵⁵ Toxicity mechanism has been hypothesized, such as oxidation stress, disruption of membrane damage.

The specific physicochemical properties of CNMs, metal impurities, size, surface area, dispersibility, surface functionalization and surface coating can also greatly influence the toxicity.⁵⁶

Metal contaminant is a major issue for the CNT toxicity study because of the catalyst metal residues in CNTs and hence the potential cytotoxicity of the heavy metal. Some reports focus on the toxicity induced by metal residues; however, the results are still inconsistent. Toxicity of SWCNTs with varying metal content has been assessed. Kagan *et al.*⁵⁷ compare the different effects of iron-rich SWCNTs and iron-stripped SWCNTs in RAW 264.7 macrophages. SWCNTs with different iron contents are both can produce superoxide radicals or NO.⁵⁷ A higher iron-content SWCNTs (30 wt%) also was reported to cause an increase in radicals, peroxide generation and antioxidant depletion indicating the oxidative stress and cellular toxicity.⁵⁸ In contrast, Tian *et al.*⁵⁹ reported that refined SWCNTs are more toxic than their unrefined counterparts (iron-rich).

The size distribution and surface area of CNTs are also found to be key factors that can influence the CNT toxicity. Length effect on CNT toxicity was investigated by Sato *et al.*⁶⁰. The results showed that the degree of inflammation around CNTs with length of 825 nm was stronger than that around CNTs with length of 220 nm in subcutaneous tissue in rats. Tian *et al.*⁵⁹ found that CNT toxicity was surface area dependent in human fibroblasts cell. Casey *et al.*⁶¹ investigated the toxicity of HiPCO SWCNTs and Arc-discharge SWCNTs, and found that HiPCO SWCNT with greater surface area display stronger cytotoxicity than arc-discharge SWCNT.

Hu *et al.* found that the high concentration of fetal bovine serum (10%) in culture medium can greatly mitigate the toxicity of GO on A549 cells, due to mitigation of interaction of cell membrane and GO by the high adsorption of fetal bovine serum on GO.⁶² Similar result was also obtained by Chang *et al.* They found that no significant toxicity was observed toward A549 cells when GO exposed to 10% fetal bovine serum.⁶³

Because of strong π - π interaction, CNMs usually bundle together in aqueous or organic solutions. The uncontrollable aggregation behavior of CNMs can influence the toxicity studies. One *in vitro* study has previously shown that the agglomeration affected MWCNT toxicity toward human cells.⁶⁴ Covalent and non-covalent (surfactant, polymer and protein *et al.*) functionalization can effectively enhance the dispersibility, and then change the toxicity of CNT. Magrez *et al.*⁶⁵ found that cytotoxicity is enhanced when the surface is functionalized after an acid treatment. Toxicity studies on graphene also showed that accumulation of hydrophobic graphene on the vero cell membrane caused high oxidative stress leading to apoptosis, whereas internalization of carboxyl functionalized hydrophilic graphene by the vero cells didn't cause any toxicity.¹²

1.5 Antimicrobial Activities of Carbon Nanomaterials

Physiochemical properties affect the antimicrobial activities of nanomaterials⁶⁶. In the past several years, many physiochemical properties have been studied, such as size, electronic properties, concentration, impurities, functionalization, solution chemistry, and incubation time. Various antimicrobial activity assays have been adopted, including Live/Dead bacterial viability assays^{67, 68}, metabolic activity assays^{22, 68}, the plate count method¹⁸, and the turbidity measurement^{69, 70}.

1.5.1 Size

Physiochemical properties of CNMs vary as a function of their sizes. This size dependence has been clearly observed in their antimicrobial activities. Lyon *et al.* compared the antibacterial activities of four stable fullerene water suspensions with various aggregates sizes, they found that smaller aggregates had stronger antibacterial activity,⁷¹ Among two different types of CNTs (SWCNTs and MWCNTs), smaller diameter SWCNTs have stronger antimicrobial activities than larger diameter MWCNTs⁶⁸. Yang *et al.* compared three SWCNT samples with different lengths (<1 μm , 1–5 μm , >5 μm), and found that longer SWCNTs displayed stronger antimicrobial activities toward *S. typhimurium* than shorter SWCNTs at the same concentration.⁷⁰ However, the length effect is not universal. Alsan *et al.* reported that shorter SWCNTs showed stronger antimicrobial activities toward both *E. coli* and *S. epidermidis*⁷².

1.5.2 Metallicity

Depending on the chirality of SWCNTs (the rolling direction of carbon sheet), an unsorted SWCNT sample usually contains 67% of semiconducting nanotubes and 33% of metallic nanotubes.⁷³ This electronic structure difference also has a great influence on the

antimicrobial effect of SWCNTs. Metallic SWCNTs showed much stronger antimicrobial activities than semiconducting SWCNTs under the same evaluation condition ⁷⁴.

1.5.3 Concentration

CNMs also showed clear concentration dependent antimicrobial activity.⁶⁹ Higher concentration of CNMs usually results in higher death rate of bacteria. Arias *et al.* reported the antimicrobial activity of SWCNT–COOH and SWCNT–OH both increase with the raise of SWCNT concentration ⁶⁹.

1.5.4 Functionalization

CNMs can be functionalized with different surface groups, which may change the antimicrobial activity of CNMs. For instance, the antimicrobial activity of three different functionalized SWCNTs (SWCNT–OH, SWCNT–COOH, and SWCNT–NH₂) on *S. typhimurium* were compared ⁶⁹. The results showed that SWCNT–OH and SWCNT–COOH have antimicrobial activities at ~50 µg/mL, while SWCNT–NH₂ only showed antimicrobial activities at much higher concentration. Also, MWCNTs functionalized with –OH, –COOH, and –NH₂ didn't show antimicrobial activities to all tested bacterial cells up to 500–875 µg/mL ⁶⁹. However, on the contrary, another study reported that functionalized MWCNTs (by sonication in a mixture of H₂SO₄ and HNO₃, thus should contain –OH and –COOH) present stronger antimicrobial activities than pristine MWCNTs ⁷⁵. Further, SWCNTs have also been functionalized with sugars, such as mannose or galactose ^{17, 18, 76}, and these materials are efficient in capturing *E. coli* ¹⁷ and *B.anthraxis* spores ⁷⁶, resulting in significant decline in bacterial spores.

1.5.5 Impurities in CNM samples

Metal residues resulting from catalysts used for CNT synthesis, are one of the major concerns for the CNT toxicity on mammalian cells.⁷⁷ However, studies on MWCNTs (with Fe

residues)⁷⁵ suggested that small amount of metal residues have little influence on the antimicrobial activity of CNTs. Kang *et al.* compared MWCNT samples containing different amount of Fe residues. MWCNTs with catalytic metal contents of nearly zero even showed slightly higher antibacterial activity than MWCNTs containing moderate levels of Fe, suggesting no correlation between residual metal content and antibacterial activity.⁷⁵ Other than metal residues, Kang *et al.* demonstrated that amorphous carbon has no noticeable effects on the antimicrobial activity of CNTs⁷⁵.

1.5.6 Solution chemistry

In CNM antimicrobial activity studies, the solution chemistry refers to physiochemical properties of culture solutions used. It includes the type of culture media, ionic strength, pH, and its dissolved natural organic matters. Arias *et al.* investigated the behavior of functionalized SWCNTs in various media, including deionized water, 0.9 % NaCl, PBS buffer, and brain heart infusion broth. They found that SWCNTs have strong antimicrobial activities in deionized water and 0.9 % NaCl. However, SWCNTs display no antimicrobial activities in PBS buffer and brain heart infusion broth. Ionic strength is a key factor affecting performance of CNT based water filters.⁷⁸⁻⁸⁰ CNT filters can remove more viruses at higher ionic strengths. Different salts have diverse effects, for example, adding CaCl₂ improves the virus removal by CNT filters, whereas, adding MgCl₂ reduced the virus removal.^{78, 79} pH of solution also influences the virus removal using CNT filters. Using the solution with pH of 3.9, CNT filters showed a higher virus removal rate than using the solution with pH of 9.0.^{78, 79} Researchers predicted that natural organic matters may alter the surface charge, aggregation behavior, and mobility of CNTs in solution, thus modifies interactions between CNTs and bacteria. Conversely, a recent antimicrobial assay showed no significant differences when river natural organic matters are present.⁸¹ This may be because of the low concentration of river natural organic matters present in the study.

1.5.7 Incubation time

Studies from different research groups all showed the antimicrobial activity of CNTs is time dependent. Longer incubation time can increase the antimicrobial effect of CNTs. Vecitis *et al.* reported that most of the SWCNT antimicrobial action toward *E. coli* occurs shortly after the contact between bacteria and SWCNTs⁷⁴. Kang *et al.* found the inactivation rate of *B. subtilis* increases with extend of incubation time from 60 to 240 mins⁸¹. SWCNT-COOH and SWCNT-OH also showed the time-dependent antimicrobial effect toward *S. typhimurium* in deionized water⁶⁹.

1.6 Antimicrobial Mechanisms of CNMs

It is important to elucidate the antimicrobial mechanism of CNMs, thus their potential applications can be realized without causing problems to human beings and environment. Unfortunately, it is still not clear exactly how CNMs kill microbes. Some possible mechanisms have been proposed.

Although production of ROS was widely detected in eukaryotic cells^{82,83} for C₆₀ toxicity, Lyon *et al.* didn't find ROS production or ROS-mediated damage in bacteria⁸⁴. Their further studies showed that C₆₀ can damage cell membrane; on the other hand C₆₀ can behave as oxidant and exert ROS-independent oxidative stress.⁸⁴

Previously, researchers hypothesized antimicrobial activity of SWCNTs was induced by the direct physical contact between bacterial cell membranes and SWCNT aggregates¹⁹. According to their observations, most of *E. coli* cells (79.9 ± 9.8 %) adhered to SWCNT aggregates was inactivated, while only few free-swimming cells (7.6 ± 2.1 %) were inactivated. It has also been reported that bacteria in contact with SWCNT networks have much less biofilm growth⁸⁵, suggesting that multiple contacts between CNTs and bacteria are necessary for the bacterial death. Besides physical damages induced by CNTs, “chemical” effects of CNTs on bacteria may be more important. Oxidative stress induced CNTs could be a major

cause of CNTs' antimicrobial activities. DNA microarray analysis was applied to study the gene expression in *E. coli* after exposure to SWCNTs and MWCNTs⁶⁸. *E. coli* can express genes which are related to oxidative stress response⁶⁸.

Compared to other synthetic CNMs, such as fullerenes and CNTs, few toxicity studies on graphene based materials are currently available.^{22, 23, 86} Only recently, it was reported that GO and rGO exhibit strong antibacterial activity.^{22, 23} The antibacterial activity of GO and rGO has been attributed to membrane stress induced by sharp edges of graphene nanosheets, which may result in physical damages on cell membranes and leakage of RNA.²³ On the other hand, it was proposed that graphene may induce oxidative stress on neural pheochromocytoma-derived PC12 cells.⁸⁷

The details of CNM antimicrobial mechanisms still require further studies.

1.7 Potential Applications

The strong antimicrobial activity of CNMs has motivated researchers to study their various potential applications. The general strategy is to use CNMs as active building blocks to form nanocomposites with various desired morphologies and functions, which may be suitable for specific purposes.

1.7.1 Antifouling surfaces and carbon nanomaterial filters

Many studies have focused on applying CNMs for water disinfection. When CNMs enter an aquatic environment, they may inactivate bacteria⁸¹. Surfaces deposited with SWCNT layers was found to be able to inhibit the formation of bacterial biofilms⁸⁵. Bacteria and viruses can be effectively removed by membrane filters containing SWCNTs or MWCNTs^{67, 78-80}. SWCNT filters were fabricated by depositing a thin layer of SWCNTs on a poly PVDF or PTFE-based microporous membrane (5 μm pore size)^{67, 78}, while MWCNT filters were made by depositing MWCNTs on a 5- μm pore size PTFE membrane⁷⁹. MWCNT filters remove

more MS2 bacteriophage viruses than SWCNT filters under similar CNT loadings. The better performance of MWCNT filters was credited to the better uniformity of MWCNT layers ⁷⁹. A dual-layer SWCNT-MWCNT hybrid filter was then produced to further improve its performance ⁸⁰. This filter showed significantly higher virus removal efficiency compared with either SWCNT or MWCNT filters alone. Further, Schoen *et al.* fabricated a water filter composed of silver nanowires, CNTs, and cotton. This composite filter is able to kill concentrated bacteria in a high throughput gravity fed device under a moderate voltage bias at 20 V ⁸⁸.

1.7.2 Antimicrobial CNM–polymer nanocomposites

Various CNM–polymer composites have been studied for their potential antimicrobial biomedical applications. A microporous film composed of SWCNTs and PVPI (polyvinylpyrrolidone-iodine) was prepared as antiseptic bands ⁸⁹. The antiseptic iodine is available on the surface of SWCNTs wrapped together in polymer. This film shows high antimicrobial activities toward *E. coli*. Aslan *et al.* dispersed SWCNTs in PLGA to form thin films. The SWCNT-PLGA film can be coated on cover glasses with the weight ratio of SWCNTs to PLGA ranging from 1/7000 to 1/70 ⁷². It can effectively decrease the viability of *E. coli* and *S. epidermidis*. Schiffman *et al.* incorporated SWCNTs into electrospun polysulfone mats, and applied them as a conformal coating ⁹⁰. The polymer mats with low SWCNTs show strong antimicrobial activities to *E. coli*. The death rate of bacteria increases from 18 to 76% when SWCNT content increases from 0.1 to 1.0 wt %. CNT/agar composite⁹¹ also has been investigated for potential photothermal antimicrobial therapy ⁹². Nepal *et al.* fabricated a multifunctional biomimetic film composed of SWCNT, DNA, and lysozyme, using a layer-by-layer assembly method. This composite film with a high Young's modulus and controlled morphology has showed excellent long-term antimicrobial activities ⁹³. CNTs coated with silver particles have attracted interests from several research groups ⁹⁴⁻⁹⁶. Silver

nanoparticles were grafted on MWCNTs with the assistance of dendritic poly (amidoamine) (PAMAM) (d-MWCNTs/Ag), and the composite showed strong antimicrobial activities against various bacteria⁹⁶. Similarly, silver coated SWCNTs were prepared through attaching Ag nanoparticles on the surface of amino functionalized SWCNTs, and they exhibited strong antimicrobial activities against *E. coli* and *S. aureus*⁹⁵. Silver coated CNTs with high antimicrobial activities can also be prepared by ion beam assisted deposition.⁹⁴ GO–silver nanoparticle (GO–Ag) composites were synthesized by various methods such as facile two-phase (toluene–water) process⁹⁷ and in situ reduction of adsorbed Ag⁺ by hydroquinone⁹⁸ or glucose⁹⁹ to enhanced antibacterial activity of original GO, which can be used as bactericidal agent for water disinfection.⁹⁸ rGO-Ag nanocomposites with excellent antibacterial activity are also be prepared by a facile synthesis approach for the potential biomedical applications, where silver nitrate was reduced by rGO in water without additional reluctant.⁹⁹ Nanocomposites containing CNTs and titanium dioxide are another class of promising antimicrobial materials, especially under light irradiation. MWCNT/TiO₂ composites were synthesized using oxidized MWCNTs with different titanium alkoxide precursors in benzene solvent. They can effectively inhibit the growth of bacteria under sunlight¹⁰⁰.

1.7.3 Targeted antimicrobial activity

In potential antimicrobial drug applications, it is desirable that CNMs can recognize harmful microbes and don't show toxic effect to other cells or organisms.¹⁵ For instance, CNMs have been intensively studied in tumor target drug delivery.¹³ However, so far few studies have investigated the targeted and specified antimicrobial activity of CNTs or functionalized CNMs in the presence of both microbial and other kind of cells. A recent study showed that the conjugate of functionalized CNT and Amphotericin B exhibits strong antifungal activity without significant toxic effects on Jurkat cells¹⁰¹. In this study, functionalized CNTs were used as a carrier of Amphotericin B other than the active

antimicrobial agent. We expect that the targeted and specified antimicrobial activity of CNMs will be studied more in the near future. Various functionalization methods would be needed to produce and control the targeted antimicrobial activity on CNMs.

1.8 Motivation and Scope

The antimicrobial activities of CNTs and graphene have been found. And potential applications of these antibacterial materials also were explored, such as antibacterial SWCNT filter and membrane. However, reliable correlations between antimicrobial activities with specific CNM characteristics and the exact antibacterial mechanism are still unclear. From a broad prospect, the antimicrobial activity of CNMs can be combined with their unique properties for developing innovative environmental and biomedical applications. Based on these considerations, attempts toward antibacterial mechanism of SWCNT and graphene with specific structure were made.

This thesis will be presented in the following structure. Synthesis of SWCNT and graphene based materials (graphite oxide, graphene oxide, and reduced graphene oxide) was described in Chapter 2. Chapter 3 presents the antibacterial activity of SWCNTs. The effects of metal residue, oxidative stress on the antibacterial activity are investigated in Chapter 4. Physical interactions between SWCNTs and bacterial cells were illustrated in Chapter 5. Chapter 6 discusses the antibacterial activity and mechanism of Gt, GtO, GO and rGO. Chapter 7 explores the effect of lateral dimension of GO on its antibacterial activity. Chapter 8 summarizes the conclusions in this thesis.

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Chapter 2

Synthesis of Single-Walled Carbon Nanotubes and Graphene Based Materials

Nowadays, one of the major reasons that the antibacterial results varied from different groups is lack of characterization of CNMs. In chapter 2, the synthesis methods of high purity and well controlled SWCNTs and graphene-related materials (GtO, GO, and rGO) were described. And characterization of these materials was performed in detail as well.

2.1 Materials and methods

2.1.1 Synthesis and purification of SWCNTs.

Co-MCM-41 catalysts were synthesized following procedures described in previous publications.¹⁻³ Typically, 100 mg of catalysts were reduced at 500 °C under hydrogen first, and then carbon monoxide decomposition was carried out at 800 °C for SWCNT growth.⁴⁻⁶ In this study, the centrifugation based purification protocol was used.⁷ Briefly, MCM-41 silica supports were removed from as-synthesized SWCNTs by refluxing in 1 M NaOH twice. Then, SWCNTs were suspended in 2 wt % SC solution. After that, the solution was centrifuged for 1 hour at the force of 120,000 g. SWCNT supernatants were then filtered on filter membranes (25 nm mixed cellulose ester membrane, Millipore). And then the SWCNT sample was heated under air at 350 °C for 30 min to remove adsorbed surfactant molecules from SWCNT surface.

2.1.2 Synthesis and purification of graphene-related materials (GtO, GO and rGO)^{8,9}

2g $K_2S_2O_8$, 2 g of P_2O_5 and 6 mL of 98% H_2SO_4 were mixed in a 50 mL beaker, and then heated to 80 °C in water bath. 1g of graphite (Gt) powder (Aldrich, synthetic, < 20 μ m) was added into the mixture (80 °C, 6 h). Then, the mixture was diluted using deionized water, and filtered through 0.20 μ m nylon membrane, followed by washing and drying. Afterwards, powder was added into 92 mL of H_2SO_4 in ice bath. 15g of $KMnO_4$ was added slowly, and then heated to 35 °C under vigorous stirring, and kept for 2 h. Next, 184 mL of water was added; 15 min later, 560 mL of water and 10 mL H_2O_2 was added. Solid powders were collected by centrifugation from the mixture, and then washed with diluted hydrochloric acid (~3%). Last, GtO powder was suspended in deionized water, and metal ions and acids were removed by dialysis.

GtO powder (100 mg) and 100 mL of water were loaded in a flask to yield an inhomogeneous yellow-brown dispersion. This GtO dispersion was sonicated until it became clear without visible particles to yield GO dispersion. Subsequently, hydrazine hydrate (1 mL) was dropped into GO dispersion, and the dispersion was heated in an oil bath at 100 °C with a water-cooled condenser for 24 h. A homogeneous black suspension was obtained. The reduced GO was collected through filtration, and washed with vast amounts of water.

2.1.3 Characterization

Physical and chemical properties of CNM samples were characterized by various techniques including raman, TGA, PLE, XPS and UV-Vis-NIR, TEM and AFM. Raman spectra of SWCNTs were collected via a Ramanscope using 633 nm and 514 nm laser wavelengths. Raman spectra of GO samples were obtained using a laser excitation of 532 nm at a power of < 1 mW. TGA was performed with PerkinElmer Diamond TG/DTA instrument. PL was conducted on Nanolog-3 spectrofluorometer. AFM measurement was conducted on a

MFP3D microscope. TEM were conducted on Tecnai microscope (Philips). SEM observations were carried out on JSM-6700F, working at 5 kV. The carbon-related chemical component species of GO were characterized by high resolution XPS.

2.2 Results and Discussion

2.2.1 Single-walled carbon nanotubes

For many toxicity or antibacterial activity studies on nanomaterials, the lack of a comprehensive characterization on nanomaterials complicates the interpretation of experimental results.

A novel catalyst, MCM-41 (a siliceous mesopores molecular sieve) supported controllable subnanometer scale Co metal clusters (Co-MCM-41) developed early was used in this study.^{4,6} More recently, a facile centrifugation based purification protocol has been proposed to purify SWCNTs.⁷ By tuning the centrifugation forces,¹⁰ SWCNT samples containing different amounts of metal residues (ranging between 19.28 and 0.17 at. %) can be easily obtained.⁷ Furthermore, surfactant molecules on SWCNT can be moved through heating after removal of metal residues. This allows re-dispersing “naked” SWCNTs in biocompatible surfactants. Finally, a detailed characterization on the tube samples was performed.

Figure 2-1 shows the SWCNT characterization results obtained by various techniques. Raman spectra in Figure 2-1A display strong radial breathing mode (RBM) peaks and G bands along with very weak D bands, suggesting that a minimum amount of amorphous carbon and few defects or functional groups exist on the tube samples. It also demonstrates that centrifugation based purification protocol⁷ does not alter the physicochemical properties of pristine SWCNTs.

Figure 2-1 Characterization of SWCNT samples using various techniques: (A) Raman spectra of a solid SWCNT sample under 514 and 633 nm laser excitations; (B) TGA spectra of a solid SWCNT sample; (C) PLE intensity map for SC dispersed SWCNTs in D₂O; (D) AFM image of SWCNT bundles dried on a mica surface; (E) SEM image of a SWCNT film composed of tube bundles deposited on a filter membrane; (F) TEM image of SWCNT bundles.

Figure 2-1B shows a single peak at 420 °C on the differential thermogravimetric analysis (DTG) curve in TGA, confirming the absence of amorphous or graphitic nanoparticles byproducts exist. Photoluminescence (PL) intensity map in Figure 2-1C readily shows that individually dispersed SWCNT samples have a narrow (n,m) distribution, the most abundant (n,m) species is (7,5) which accounts for 45 % of semiconducting tubes. When diluted SWCNT dispersions are dried on a mica surface, AFM image in Figure 2-1D shows that tubes form small bundles. The length of individual tubes is about 1 μm . SWCNT films were also formed by filtering the SWCNT dispersions on a membrane and inspected by SEM. Larger tube bundles can be observed in the SEM image from Figure 2-1E. TEM image from Figure 2-1F reveals that SWCNT bundles have diameters ranging from 5 to 50 nm in the deposited films. SEM and TEM results also confirm the successful removal of carbon impurities and metal residues.

2.2.2 Graphene oxide and reduced graphene oxide

GO can be chemically exfoliated from graphite oxide (GtO). Thermal annealing or chemical treatment can eliminate functional groups on GO to produce rGO. The GtO dispersion was prepared by oxidation of Gt, and it is opaque yellow in color. Some small GtO particles can also be identified in the GtO dispersion. Significant portion of GtO particles precipitated after the GtO dispersion was identified for 2 h. However, if washed GtO powders were bath sonicated for 6 h, GO nanosheets were exfoliated from the GtO, resulting in the clear and homogenous yellow-brown GO dispersion. The GO dispersion was stable after standing still for several days, due to the large amount of hydrophilic functional groups, such as carboxyl, hydroxyl and epoxy groups, on GO nanosheets.

Figure 2-2A shows an AFM image of GO nanosheets dried on a mica surface. The thickness of them is around 1 nm (see the AFM profile in Figure 2-2B); indicating single-layer

GO sheets were produced. Figure 2-2C is an SEM image of dried GO sheets on a silicon wafer. GO sheets are smooth with small wrinkles at edges.

Figure 2-2 (A) AFM height image of GO nanosheets dried on a mica surface (the scale bar shows 200 nm) and (B) the corresponding height profile of the AFM image. SEM images of GO (C) and rGO (D) nanosheets dried on silicon wafers.

The rGO dispersion was obtained by chemically reducing the GO dispersion using hydrazine.¹¹ After reduction, the surface of rGO nanosheets became hydrophobic, and some black particles started precipitating.¹¹ The strong van der Waals forces among rGO nanosheets would facilitate the aggregation of rGO particles. Thus, plenty of rGO particles precipitate after the rGO dispersion stood still for 2 h. Figure 2-2D shows an SEM image of dried rGO sheets, and the rGO sheets are much rougher compared with GO sheets.

Figure 2-3 Raman spectrum of GO samples

Figure 2-3 shows a representative Raman spectrum of GO samples used in this study after removal of a fluorescent background. Several peaks can be observed, and were assigned to the D, G, G', D+G and 2D' bands. The G band, resulting from the doubly degenerate zone center E_{2g} mode,¹² is at $\sim 1587\text{ cm}^{-1}$, which illustrates the graphitic nature for this material. The existence of the D band at $\sim 1343\text{ cm}^{-1}$ is due to the scattering of a zone-boundary phonon with defects. These defects could be caused by covalent bonds of carbon atoms with oxygen or other functional groups.¹³ The D band is not seen in Raman spectra of defect-free Gt, while a high intensity has been reported in similarly generated GO.

The intensity ratio of the D and G band (I_D/I_G) is 1.36 for our GO samples. The broad peaks (G, D bands) compared to those of standard graphene sheets imply the creation of defects as well. In addition, there are three Raman bands with weaker but recognized features and intensity, called G', D + G, and 2D' bands, locating at $2700\text{--}3200\text{ cm}^{-1}$. The G' band at 2700 cm^{-1} from two phonons with opposite momentum in the highest optical branch near the K (A_{01} symmetry at K),¹³ is Raman active for crystalline graphitic materials. It is sensitive to the π band in the graphitic electronic structure. The combination modes of D + G band at ~ 2900

cm^{-1} and the second order $2\text{D}'$ band at $\sim 3174 \text{ cm}^{-1}$ emphasize the high defectiveness of GO samples.¹⁴ D' band was not observed in our sample. Our Raman results suggest that the material significantly changed upon exfoliation of Gt flakes to more amorphous and defective GO nanosheets.

Figure 2-4 (A) XPS survey scan for the elements and (B) Carbon components in the GO sample

The carbon-related chemical component species in GO samples were characterized by XPS spectrum. Figure 2-4A shows a survey scan for GO samples. Apart from the major elements of carbon and oxygen, there are trace amounts of calcium, sodium, sulfur, and nitrogen. These elements can all be associated with the exfoliation oxidation process.

High resolution XPS analysis of carbon components in the GO is shown in Figure 2-4B. A binding energy of 284.9 eV indicates the existence of $\text{C}=\text{C}$ sp^2 bonds in the GO sheets; while 286.8 eV results from $\text{C}-\text{O}$ bonds (epoxy and hydroxyl groups), and a binding energy of 288.7 eV gives the evidence of $\text{C}=\text{O}$ (carbonyl) group formed during the oxidation. According to the integrated areas, ($A_{\text{C}=\text{C}}/A_{\text{C}-\text{O}}/A_{\text{C}=\text{O}} = 19479.0/8662.3/1370.1$) the ratios of sp^2 -bonded

carbon atoms vs. carbon atoms in epoxy and hydroxyl groups and in carbonyl groups in the GO sheets are approximately 7/6/1.¹⁵ Thus, the ratio of carbon atoms in the perfect graphene sheet to defects in the lattice is 1 to 1. This high defect density agrees with the result obtained by Raman spectroscopy.

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Chapter 3

Antibacterial Activity of Single-Walled Carbon Nanotubes

Since the discovery of CNTs in 1991,¹ a wide range of current and/or future applications of CNTs have been proposed owing to their amazing electronic, mechanical, and structural properties.^{2,3} Recently, the discovery of antibacterial activity⁴ has triggered the exploration of CNT applications on related fields.⁵ The advent of CNTs in applications raised concerns on the impacts of CNTs on human health and environmental safety.⁶⁻⁹ From a scientific point of view, the best strategy for maximizing CNTs' application potentials while minimizing their risks, is to have a better insight in their toxicity mechanism. Based on such a mechanistic understanding, synthesis, purification and functionalization procedures can be modified to avoid the specific features of CNTs that cause toxicity.¹⁰

Due to the strong π - π interaction, CNTs usually bundle together in aqueous or organic solutions. One *in vitro* study has previously showed that the agglomeration affected MWCNT toxicity toward human cells.¹¹ SWCNTs are much smaller in size with the average diameter of 1–2 nm compared to MWCNTs. Aggregation of SWCNTs may play a crucial role in controlling their antibacterial activities. However, due to the difficulty in debundling SWCNTs and characterizing their aggregation state (note that light scattering techniques may have problems in determining the aggregation due to the tiny dimension of SWCNT bundles), previous studies have utilized either suspended or deposited SWCNT aggregates in the antibacterial activity evaluation other than individually dispersed SWCNTs.

In this chapter, a comparative investigation on the antibacterial activity of SWCNTs was reported. Chapter 3 elucidates the effect of aggregation on antibacterial activity of SWCNTs, and subsequently to prove or disprove some key factors that could influence the SWCNT antibacterial effect.

3.1 Materials and Methods

3.1.1 Cell preparation

E. coli, *P. aeruginosa* and *S. aureus* were grown in LB broth at 37 °C, and *B. subtilis* were grown at 30 °C. The cultures harvested in the mid-exponential growth phase were centrifuged (6,000 rpm, 10 min) to pellet cells, then bacteria were washed with saline solution (3 times). The pellets were then suspended in a saline solution. Bacterial suspensions were diluted to obtain cell samples containing 10^6 to 10^7 CFU/mL.

3.1.2 Measurements of bacterial activity

Purified SWCNT solid samples (5 µg /mL) were first dispersed in saline solution (0.9 wt % NaCl), Tween-20 saline solution (0.1 wt % Tween-20 and 0.9 wt % NaCl), and sodium cholate (SC) solution (1 wt % SC) by sonication. Clear SWCNT dispersions were obtained after sonication. 10 mL of SWCNT dispersion (5 µg /mL) was incubated with 1 mL of bacterial suspensions (10^6 to 10^7 cfu/mL) for 2 hours (at 0 to 250 rpm shaking speeds) at 37 °C or 30 °C, respectively. The antimicrobial evaluations were carried out by a colony forming count method and a Live/Dead viability assay (Invitrogen, USA). For the colony forming count method, 100 µL serial 10-fold dilutions with saline solution were spread onto LB plates and culture overnight (at least 3 separate occasions). Death rate % = (counts of control – counts of samples incubated with SWCNTs) / counts of control. The death rates obtained in the colony forming count method were further verified by the Live/Dead viability assay. Propidium iodide (PI) and SYTO® 9 stock solutions from the assay kit were combined with an equal volume of *E. coli* or *B. subtilis* suspension. The mixtures were incubated for 15 min, and then observed under an epifluorescence microscope (Zeiss Axiovert 200). Death rate of bacteria=bacteria stained with PI /total bacteria.

3.1.3 Integrity of cell membranes

After 2 hour incubation with 5 $\mu\text{g/mL}$ SWCNTs, the bacterial suspensions were then immediately filtered with 0.22 μm syringe filters to remove the bacteria and absorbance at 260 nm was measured.¹²

3.1.4 Cell morphology observation

The morphological changes of bacterial cells were investigated by SEM. Bacterial suspensions were concentrated by centrifugation at 6,000 rpm, 4 $^{\circ}\text{C}$ and quickly fixed. Then bacteria were dehydrated with sequential treatment with ethanol (different concentration, from 10% to 100%). 10 μL of dehydrated cells were dropped on a glass slide to dry.

3.2 Results and Discussion

3.2.1 Individually dispersed pristine SWCNT

Pristine SWCNTs would aggregate to form bundle structures other than individually dispersed tubes in aqueous solvent due to the π - π interaction. Charge transfers among tubes in a SWCNT bundle can quench and broaden their absorption peaks on UV-Vis-NIR absorption. We propose that this technique should be applied in SWCNT toxicity studies for monitoring the aggregation state of SWCNT samples. Simple sonication in saline solution or buffers cannot produce stable individually dispersed pristine SWCNTs. Soluble SWCNT sample can be obtained by either covalent functionalization or noncovalent dispersion of SWCNT. In this study, SWCNTs were dispersed by biocompatible surfactants (Tween-20 and SC) because the covalent functionalization introduces new functional groups to SWCNTs which may alter their toxicity and antibacterial properties.¹³ Figure 3-1A shows that clear SWCNT dispersions can be obtained right after sonication in three different solutions (Tween-20, SC, and conventional saline as a comparison). Both Tween-20 and SC are effective surfactants for solubilizing SWCNTs¹⁴⁻¹⁷ because no changes were observed for SWCNT dispersions after standing still

for 2 hours. On the other hand, SWCNTs dispersed in saline solution would aggregate, and carbon residues started depositing out of solution once the sonication was stopped.

Figure 3-1 (A) SWCNTs dispersed in saline solution (NaCl, 0.9 wt %), Tween-20 saline solution (0.1 wt % and NaCl 0.9 wt %), and SC (1 wt %); right after sonication (0 h) and standing still for 2 hours (2 h). (B) UV-vis-NIR spectra of SWCNT dispersions after standing still for 2 hours.

The SWCNT dispersions after the sonication were further characterized using UV-vis-NIR spectroscopy (Figure 3-1B). The SWCNT dispersions in Tween-20 and SC after 2 hours preserve clear and sharp absorption peaks from both E_{11} and E_{22} transitions of SWCNTs, suggesting that most of tubes are individually dispersed in these two solutions. In the contrast, absorption peaks from SWCNTs originally dispersed in saline solution completely disappear, implying that SWCNT bundles are dominate.

3.2.2 Biocompatibility of surfactant solutions

Figure 3-2 Cell viability measurements after incubation with different surfactant solutions

Although the surfactants can enhance the dispersion of SWCNTs, the biocompatibility of surfactant solutions has to be studied first because they may also complicate the SWCNT antibacterial study due to their own antibacterial activities.¹⁸ Bacterial cells were incubated for 2 hours with the following solutions: physiological saline solution (control), Tween-20 saline solution, and SC solutions. Figure 3-2 shows that the survival rates of *E. coli* are 88.6 ± 1.7 %

and 86.9 ± 1.0 % in the control and Tween-20 saline solution, respectively; and those of *P. aeruginosa* are 88.0 ± 2.0 % and 88.5 ± 3.6 %. The survival rates in these two solutions are 87.7 ± 1.8 % and 87.9 ± 3.2 % for *B. subtilis*, and 90.0 ± 3.6 % and 90.4 ± 2.2 % for *S. aureus*. These results show that the Tween-20 saline solution has no significant impacts on the viability of bacteria, which is similar to a previous study¹⁶ claiming that Tween-80 can be used to disperse SWCNTs for toxicity studies. On the other hand, SC solution demonstrated a strong antibacterial activity. The survival rates of *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* are 46.5 ± 2.5 %, 41.1 ± 3.9 %, 42.7 ± 3.6 % and 50.8 ± 2.8 %, respectively. Several previous studies have also reported that the adsorption of SC on cell membrane may change the membrane permeability due to the structural changes of cell membranes.^{19, 20}

3.2.3 Antibacterial activity of SWCNTs dispersed in solutions

The antibacterial activities of SWCNTs dispersed in different solutions were evaluated first by a colony forming count method. Figure 3-3 shows that SWCNTs dispersed in Tween-20 saline solutions exhibit high antibacterial activities; they kill 58.1 ± 5.0 % of *E. coli*, 65.1 ± 1.8 % of *P. aeruginosa*, 87.5 ± 6.5 % of *B. subtilis* and 85.6 ± 5.3 % of *S. aureus*. As a control experimental results, SWCNTs dispersed in saline solutions only kill 33.8 ± 4.0 % of *E. coli*, 27.7 ± 5.9 % of *P. aeruginosa*, 53.9 ± 2.8 % of *B. subtilis* and 50.3 ± 3.5 % of *S. aureus*. Both saline and Tween-20 solutions show very similar biocompatibility as illustrated in Figure 3-2. Therefore, the remarkably difference antibacterial activities presented in Figure 3-3 can be attributed to the distinct SWCNT aggregation states in these two solutions, as evidenced in Figure 3-1B because numerous SWCNT aggregates were observed during the incubation with bacteria in saline solutions but not in Tween-20 saline solutions. We conclude that SC is not a suitable surfactant for investigating the antibacterial activity of SWCNT samples due to the complication originating from the inherent high antibacterial activity of SC itself, although Figure 3-3 suggests that SWCNTs dispersed in SC solutions possess very high antibacterial

activities. Therefore, the following assays in this study will focus on SWCNTs dispersed in Tween-20 saline solutions.

Figure 3-3 Death rate of bacteria after incubation with SWCNTs (Evaluated by a colony forming count method)

In order to verify the reliability of colony forming count method here, *E. coli* and *B. subtilis* were chosen to further examine their death rates after incubation with SWCNT dispersions by a LIVE/DEAD kit (Invitrogen, USA). Similar fluorescence dye methods have been applied in previous CNT toxicity studies.^{4, 21} The results in Figure 3-4 show that the fluorescence-based assay is consistent with the results from colony forming count method. A brief summary can be drawn from these antibacterial activity results: (1) individually dispersed SWCNTs in Tween-20 saline solutions possess a higher antibacterial activity compared to SWCNT aggregates. (2) SWCNT dispersions show higher antibacterial activities toward gram-positive bacteria than gram-negative bacteria.

Figure 3-4 Fluorescence microscope images of bacteria. (A) *E. coli* in 0.9 wt % NaCl control solution without SWCNTs, (B) *E. coli* after incubation with SWCNTs dispersed in saline solution, (C) *E. coli* after incubation with SWCNTs dispersed in Tween-20 saline solution, (D) *B. subtilis* in control solution without SWCNTs, (E) *B. subtilis* after incubation with SWCNTs dispersed in saline solution, (F) *B. subtilis* after incubation with SWCNT dispersion in the Tween-20 saline solution.(All the scale bars are 20 μm)

3.2.4 Destruction of bacterial membrane

One intriguing questions arise from our antibacterial activity results: why individually dispersed SWCNTs have higher antibacterial activities than SWCNT aggregates? To answer this question, we have to first understand how SWCNTs interact with bacteria. Several studies^{4, 21-23} have proposed that the antibacterial activity of SWCNTs is associated to the damage of cell membrane upon the direct contact between SWCNT aggregates and bacteria.¹² Figure 3-5 shows our UV-Vis study on the release of intracellular materials after we incubated the bacteria with SWCNTs. This ratio shows a remarkable increment by a factor of 3.2 times after bacteria interacting with SWCNTs.

Figure 3-5 Releasing of DNA from cells after incubating with SWCNT dispersions.

Two additional features are observed in Figure 3-5: (1) the OD_{260nm} ratios of *E. coli* and *B. subtilis* are 1.5 –1.6 times in Tween-20 saline solutions compared to in saline solutions. (2) The OD_{260nm} ratios of *B. subtilis* are about 30 % higher than those of *E. coli* suspensions. The results from this absorption study at 260 nm are in agreement with cell viability measurements, suggesting that the reflux of DNA and RNA correlates to the bacterial mortality. (Please note that some proteins also absorb at 260nm especially amino acids with rings, however, the protein absorption would not affect conclusions in this thesis because the increase of absorbance at 260 nm indicates the release of intracellular materials (no matter DNA, RNA or protein, which corroborates the cell membrane damages.) It also implies that the bacterium death is directly associated with the damage of bacterial membrane.

The damage of bacterial membrane was further illustrated by a SEM study shown in Figure 3-6. bacteria in saline solutions without SWCNTs maintain their integrity of the membrane structure after 2 hour incubation (Figure 3-6A, 3-6C, 3-6E and 3-6G). In contrast, this cellular integrity disappears when the cells were incubated with SWCNTs dispersed in

Tween-20 saline solutions for 2 hours (Figure 3-6B, 3-6D, 3-6F and 3-6H). Similar SEM images of various bacteria after incubation with CNTs have been reported before.^{4, 13, 21-23}

Figure 3-6 SEM images of (A) *E. coli* after incubation with saline solution for 2 hours without SWCNTs, (B) *E. coli* after incubation with SWCNTs dispersed in the Tween-20 saline solution (0.1 wt % Tween-20 and 0.9 wt % NaCl) for 2 hours, (C) *P. aeruginosa* without

SWCNTs, (D) *P. aeruginosa* with SWCNTs, (E) *B. subtilis* without SWCNTs, (F) *B. subtilis* with SWCNTs, (G) *S. aureus* without SWCNTs, (H) *S. aureus* with SWCNTs.

However, it should be noted that individually dispersed SWCNTs were employed in this antibacterial activity study which is significantly different from the previous SEM studies in the presence of many CNT bundles. The reason why only few SWCNTs are visualized in our SEM images may be due to the fact that individually dispersed SWCNTs with 1–2 nm in diameter are difficult to be observed in SEM.

3.2.5 Concentration dependent antibacterial activity of SWCNT

Figure 3-7 Antibacterial activities of SWCNT at different concentrations. 10 mL of SWCNT (5–80 $\mu\text{g/mL}$ dispersed in Tween-20 saline solutions) was incubated with 1 mL of *E. coli* dispersion (10^6 – 10^7 cfu/mL).

We have demonstrated that the major cause of bacterial death is the physical puncture by SWCNTs. Along this line, enhancing the physical puncture (both frequency and intensity) on bacteria should be able to significantly improve SWCNT antibacterial activity. Individually

dispersed SWCNTs are smaller in size compared to SWCNT aggregates, and they are more mobile in solution as well. Smaller size and higher mobility can enhance the contact opportunities between SWCNT and bacteria, leading to the observed superior antibacterial activity from individually dispersed SWCNT samples. This agrees well with previously published results²¹ on MWCNTs, debundled, short, and dispersed MWCNTs demonstrated high antibacterial activities. Moreover, a recent study has reported that the antibacterial activity of SWCNT aggregates in saline solution is dependent on SWCNT concentration and treatment time.¹³ We hypothesize that the contact chance between bacteria and SWCNTs is higher with the elevated SWCNT concentration and longer incubation time, which may result in higher cell death rates. When the concentration of individually dispersed SWCNTs in Tween-20 saline solutions increases from 5 to 80 $\mu\text{g}/\text{mL}$, the death rate of *E. coli* increases from $58.8 \pm 6.8 \%$ to $89.3 \pm 2.7 \%$. Small changes in the death rate were observed when SWCNT concentrations is above 80 $\mu\text{g}/\text{mL}$, which can be attribute to the fact that most of bacteria were killed at the 80 $\mu\text{g}/\text{mL}$ of SWCNT concentration.

3.2.6 Shaking speed dependent antibacterial activity of SWCNT

The dependence of SWCNT antibacterial activity on the SWCNT physical interaction with bacteria can be further demonstrated by changing the shaking speed during incubation. *E. coli* and *B. subtilis* were incubated for 2 hours at various shaking speeds from 0 to 250 rpm in the presence of SWCNTs dispersed in Tween-20 solutions. Figure 3-8 shows that the death rates of both bacterial cells increase with the increase of shaking speeds. A higher shaking speed remarkably enhances the mobility of both SWCNTs and bacteria in a solution, therefore leading to a higher frequency and intensity of collisions between SWCNTs and bacteria. We propose that more physical interactions between SWCNT and bacterial membranes result in greater chance of membrane damages, resulting in the increased cell death rates. The results in Figure 3-3 also strongly complement our suggestion that individually dispersed SWCNTs have

a better antibacterial activity than SWCNT aggregates because individual SWCNTs are more mobile in the solution which enhances their physical interaction with bacteria.

Figure 3-8 Antibacterial activities of SWCNTs after 2 hour incubation with *E. coli* and *B. subtilis* at different shaking speeds.

3.3 Conclusion

Antibacterial activity of SWCNTs on various bacteria was investigated using pristine SWCNTs produced from Co-MCM-41 catalyst and purified by a centrifugation-based method. High purity SWCNT samples helped to eliminate the possible contamination caused by impurities such as MWCNTs, amorphous and graphitic carbon nanoparticles. UV-vis-NIR absorption spectra confirmed the aggregation state can be regulated by dispersing SWCNTs in different solutions. Both the colony forming count method and the Live/Dead viability assay demonstrated that individually dispersed SWCNTs showed a higher antibacterial activity compared to SWCNT aggregates. UV-Vis absorption spectroscopy study at 260 nm and SEM images revealed that the bacterium death is related to the damage of cell membrane. Lastly, SWCNT antibacterial activity could be further promoted by improving the interaction between

SWCNTs and cells through individually dispersing SWCNTs, increasing SWCNT concentration, and elevating shaking speed during incubation.

3.4 References

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Chapter 4

Oxidative Stress and Metal toxicity Investigation of Single-Walled Carbon Nanotubes

Investigations in Chapter 3 indicated that intensive physical contact between bacteria and SWCNT is the one of the main causes of bacterial death. However, other antibacterial mechanisms also were proposed for CNTs including oxidation of cell components, and production of secondary produces such as dissolved heavy metal ions or reactive oxygen species (ROS).¹⁻⁶ A major reason for the contradictory results obtained on CNTs may be ascribed to the heterogeneous nature of current available CNT samples due to the co-existence of many species. Toxicity of a CNT sample is dependent on its composition along with its geometry and surface functionalization. Several studies have suggested that well functionalized, serum stable CNTs are safe to animal cells, while raw CNTs exhibit severe cytotoxicity to animal or human cells.⁷⁻¹²

On the other hand, the bioavailability of metal residues (*e.g.* Fe^I and Ni¹³) on CNT samples was found to exist at toxicologically significant concentrations despite its apparent encapsulation by carbon, although a recent study showed no correlation between residual metal content and toxicity.¹⁴

Chapter 4 discussed several possible antibacterial mechanism of SWCNT, including inhibiting cell growth or oxidative stress and metal residue toxicity. Results also proved that the antimicrobial properties of SWCNTs were independent of cobalt residues remaining in SWCNT samples and oxidative stress. Influence of physical puncture of SWCNTs on the antibacterial activity will be discussed in the next chapter.

4.1 Materials and Methods

4.1.1 OD growth

For OD growth curve measurements, 100 μL of *E. coli* or *B. subtilis* (10^8 to 10^9 cfu/mL) were mixed with 900 μL of LB broth or Tween-20 saline solutions (0.1 wt % Tween-20 and 0.9 wt % NaCl). Cell samples were then incubated at 37 °C or 30 °C in 24-well cell culture plates. OD at 600 nm were measured every hour on a Benchmark PlusTM microplate spectrophotometer.

4.1.2 Impact of bacteriostatic agent

Sulfadiazine was dissolved in methanol and acetone (1:1) solution at 10 mg/mL.¹⁵ 50 μg of sulfadiazine (5 μL of the 10 mg/mL solution) was added into suspensions with bacteria (*E. coli* or *B. subtilis*, 10^6 to 10^7 cfu/mL) and SWCNT. The bacterial suspensions were incubated for 2 hours (37 °C or 30 °C). In control experiments, 50 μg of sulfadiazine was added into 1 mL of bacterial suspensions (*E. coli* or *B. subtilis*, 10^6 to 10^7 cfu/mL) and 10 mL of Tween-20 saline solutions without the presence of SWCNTs. The antimicrobial death rates were determined by the colony forming count method.

4.1.3 Oxidation-reduction potential of SWCNT dispersions

The ORP of SWCNT dispersions were measured using an ORP tester (Oakton Instruments, ORPTestr 10). Each tested sample was purged using N_2 for 15 min before reading. SWCNT dispersions at various concentrations in saline solutions (0.9 wt % NaCl) and the Tween-20 saline solutions (0.1 wt % Tween-20 and 0.9 wt % NaCl) were measured 3 to 5 times to obtain the standard deviation.

4.1.4 Monitoring oxidation of cellular components

A Thiol and Sulfide Quantitation Kit (Invitrogen) was applied to measure the level of thiols in proteins. Two sets of assays were performed to evaluate SWCNT oxidation of proteins both in and out of cell membranes. For cytoplasmic proteins, 10 mL of SWCNT dispersions (5 µg /mL in the Tween-20 saline solution) were incubated with 1 mL of *E. coli* or *B. subtilis* (10^6 to 10^7 cfu/mL) suspensions for 2 hours at 37 or 30 °C. The cells were collected by centrifugation. The cell pellets were then washed three times and resuspended in saline solutions. In a glove box (SYS1-2GB, Innovative Technology), cells are lysed by sonication (SONICS, VCX-130, 60s). Supernatants obtained after centrifugation were transferred to fresh tubes. About 2.5 µg of proteins were used for each Thiol assay. The protein concentrations in cell extracts were determined by a Pierce BCA protein assay kit. For cell-free assays, SWCNT dispersions (5 µg /mL) were incubated with bovine serum albumin (BSA) solutions (diluted from 2 mg /mL concentrated solutions by Bio-Rad Quick Start™, 1 thiol group per protein, total 3 µg of proteins). For each assay, a negative control was incubated with Tween-20 saline solutions and a positive control was incubated with 5 mM tert-butylhydroperoxide in Tween-20 saline solutions. All solutions used for thiol determination were degassed in a vacuum oven < 1 Torr (DZF-6053, Shanghai Yiheng Instruments). The thiol assays were conducted in a microplate format as described in the kit manufacturer's protocol. The thiol concentration in samples was shown in nmols.

4.2 Results and Discussion

4.2.1 Impact of bacteriostatic agent

Previous studies have attributed the antibacterial activity of SWCNTs to the physical puncture of SWCNT aggregates, resulting in the physical damages of the outer membrane of the cells.^{2, 14} Findings in the present study (Chapter 3) by SEM and UV absorption at 260 nm

and SEM support such an argument. However, other factors may also contribute to the bacterial membrane destruction, such as inhibiting cell growth, oxidative stress and metal residues, controlled experiments were carried out to prove or disprove their roles on the antibacterial activity of SWCNTs.

Figure 4-1 OD growth curves of *E. coli* and *B. subtilis* in LB broth and the Tween-20 saline solution at 37 °C or 30 °C. 100 μ L of bacterial dispersions (10^8 to 10^9 cfu/mL) were mixed with 900 μ L of LB broth or the Tween-20 saline solution.

The bacteria used here are viable and capable of growth. Thus, other than the direct physical puncture, SWCNTs may also cause the bacterial death by inhibiting the cell growth during the 2 hour incubation. To evaluate this possibility, the cell growth rates in LB broth and the Tween-20 saline solution were firstly compared. Figure 4-1 shows that bacterial cells can grow in LB broth, while no observable cell growth in the Tween-20 solution can be discerned. The survival rate of bacteria (*E. coli* and *B. subtilis*) is about 88 % after incubation with the Tween-20 saline solution for 2 hours (Figure 3-1), indicating that most cells can survive

without growth in the 2 hour incubation period used in our SWCNT antibacterial activity assays. We also investigated the effect of a typical bacteriostatic static agent on the cells while they were exposed to SWCNTs. Sulfadiazine is a bacteriostatic agent at low concentration.^{16, 17} It can interfere with bacterial synthesis of folic acid needed by cell growth.¹⁸ Sulfadiazine can inhibit growth and reproduction of bacteria without killing them. We compared the death rate of bacteria after incubation of SWCNT dispersions with and without the presence of sulfadiazine, as well as after incubation with sulfadiazine alone.

Figure 4-2 The impact of a bacterial static agent (sulfadiazine at 5 $\mu\text{g/mL}$) on the death rates.

Figure 4-2 shows that bacteria incubated with sulfadiazine in the absence of SWCNTs had a very low death rate (< 10 %). The remarkably high death rates of bacteria after incubation with SWCNTs and sulfadiazine are the same as incubating the bacteria using SWCNT alone. These control results indicate that inhibiting cell growth by SWCNTs is not a major cause responsible for the death of cells here. It should be noted that the medium in this experiment is tween-20 saline solution without nutrient substance. Bacteria in control sample also cannot grow, so bacteria in the sulfadiazine (can only inhibit the growth and reproduction of bacteria without killing them) sample showed lower death rate compared with control sample.

4.2.2 Oxidative stress induced by SWCNTs

Figure 4-3 Oxidation-reduction potential of SWCNTs dispersed in saline solutions, and Tween-20 saline solutions.

Another possible mechanism for SWCNT antibacterial activity is the oxidative stress. A SWCNT can be sometimes considered as an extended fullerene (C_{60}). They are both made of pure carbon, and their diameters are at a similar size. Because the oxidative stress was identified as a major mechanism for C_{60} toxicity, it is reasonable to suspect that oxidative stress can be one of the mechanisms of SWCNT antibacterial activity. Lyon et al. have showed that fullerene exerts ROS independent oxidative stress on bacterial cells.^{19, 20} Moreover, previous results implied that the antibacterial activities of CNTs may be partially contributed by oxidative stress.²¹ We modified those two assays applied in the previous C_{60} studies^{19, 20} The oxidation-reduction potentials (ORP) of SWCNT suspensions were measured using an ORP tester (Oakton Instruments, ORPTestr 10). A significant increase in the ORP values is expected when the SWCNT concentration is increased, if SWCNT is a strong oxidant. The

ORP of SWCNT dispersions at various concentrations in saline solutions and Tween-20 saline solutions was depicted in Figure 4-3. Surprisingly, no increase of ORP values was observed with the increase of SWCNT concentration, suggesting SWCNT is not a strong oxidant in our tests, which is completely different from the results obtained on C₆₀ dispersed in water.²⁰

Following the same protocol applied the previous C₆₀ study,¹⁹ the loss of thiol groups (–SH) on the proteins upon exposing cells to SWCNTs was measured. Thiol groups exist on both bacterial membrane proteins and cytoplasmic proteins. Upon exposure to ROS or other oxidants, thiols can be oxidized. The level of thiols in proteins is an indication of oxidative damage to cellular components. The SWCNT oxidation of proteins both inside and outside of cell membranes was assessed.

As shown in Figure 4-4, the cell extracts obtained from *E. coli* and *B. subtilis* after incubating with SWCNT dispersions were tested. The *E. coli* extract has 0.137 ± 0.016 nmoles of thiol in the control sample and 0.134 ± 0.008 nmoles in the SWCNT-exposed sample. Similarly, the *B. subtilis* extract has 0.069 ± 0.014 and 0.073 ± 0.016 nmoles in the control sample and in the SWCNT-exposed sample, respectively. The similar thiol concentrations for both bacteria indicate that no oxidation occurs after incubated with SWCNTs. Comparing with the previous C₆₀ results,¹⁹ we suggest that SWCNTs remain primarily outside the cell and cannot easily enter the cell membrane to oxidize cytoplasmic proteins. Therefore, if SWCNTs can apply a strong oxidative stress on the bacteria, the oxidation would only primarily occur at the membrane interface. Along this line, we examined the oxidation of a free protein bovine serum albumin (BSA) by SWCNTs in a cell-free assay. Figure 4-4 shows that the thiol concentrations are 0.047 ± 0.010 and 0.042 ± 0.007 nmoles in the control sample and in the BSA and SWCNT mixture, respectively, which is fairly close to the theoretically calculated 0.045 nmoles (3 μ g of BSA) of the thiol in this assay. No oxidization of BSA by SWCNTs was observed, suggesting that the proteins on the bacterial membrane are unlikely to be oxidized by

SWCNTs. Although we cannot exclude the possibility that oxidative stress induced by SWCNTs may partially contribute to SWCNT antibacterial activity, it appears not to play a major role in this study. Furthermore, it is reasonable to suggest that SWCNTs are remarkably different from C₆₀ in terms of the toxicity due to the great differences of the physiochemical properties between them.

Figure 4-4 Protein oxidation in cell extracts of *E. coli* and *B. subtilis*, and BSA by measuring thiol concentration. 10 mL of SWCNTs (5 μg /mL, dispersed in the Tween-20 saline solution) were incubated with 1 mL of cell suspensions (10^6 to 10^7 cfu/mL). Cell extracts containing about 2.5 μg of proteins determined by a Pierce BCA protein assay kit were used for thiol assays, while 3 μg of BSA (diluted from 2 mg/mL concentrated solution) was used for cell-free assays.

4.2.3 Influence of cobalt residues on SWCNTs

Figure 4-5 Antibacterial activities of SWCNTs obtained after centrifugation at different forces. SEM-EDS results indicated that SWCNT samples marked as “0 g” contained 19.28 at. % Co and samples marked as “120,000 g” contained 0.17 at. %. 10 mL of SWCNT dispersion (5 μ g/mL) was incubated with 1 mL of bacterial suspensions (10^6 to 10^7 cfu/mL) for 2 hours at 250 rpm shaking speeds, and at 37 $^{\circ}$ C or 30 $^{\circ}$ C.

The bioavailability of metal residues on CNT samples sometimes complicates CNT toxicity studies.^{1, 13} In this study, we are capable of looking into this issue in a systematic way because the advantages of our synthesis and purification methods of SWCNT are obvious: only monometallic Co was employed as the catalysts for growing SWCNT and centrifugation based purification allows us to obtain pristine SWCNTs containing a controllable amount of Co metal residues.²² The antibacterial activities of SWCNT samples obtained after centrifugation at different speeds were studied on both *E. coli* and *B. subtilis* (Figure 4-5).

Cobalt content ranging from 19.28 to 0.17 at. % was determined by SEM-Energy Dispersive Spectroscopy (EDS). The cobalt residue does not display impact on the SWCNT antibacterial activity in this study because no significant differences on their antibacterial activity were observed among different SWCNT samples.

Figure 4-6 Releasing of DNA from bacteria after incubating with SWCNTs obtained after centrifugation at different forces. SEM-EDS results indicated that SWCNT samples marked as “0 g” contained 19.28 at. % Co and samples marked as “120,000 g” contained 0.17 at. %. 10 mL of SWCNT dispersion (5 μg /mL) was incubated with 1 mL of bacterial suspensions (10^6 to 10^7 cfu/mL) for 2 hours at 250 rpm shaking speeds, and at 37 $^{\circ}\text{C}$ or 30 $^{\circ}\text{C}$.

As shown Figure 4-6, various SWCNT samples show similar OD_{260nm} ratios. This negligible effect of cobalt residue may be due to the fact that a low SWCNT concentration (5 μg /mL) was used. It is converted into a maximum Co concentration at approximate 1 μg /mL (for SWCNT samples containing 19.28 at. % Co). Antibacterial activity of saline solution

containing different amounts of $\text{Co}(\text{NO}_3)_2$ was measured. The increase of bacterial death rate can be observed only when the Co^{2+} concentration is 40 $\mu\text{g}/\text{mL}$ or above, which is way higher than the Co residue left in SWCNT dispersions in this antibacterial activity study.

4.3 Conclusion

In this chapter, three main possible antibacterial mechanisms were examined. Results evidenced that inhibition of bacterial growth and oxidative stress were not the major reasons responsible for the SWCNT antibacterial activity. Co remained on SWCNT had no detrimental effects on the antibacterial activity.

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Chapter 5

Physical Interaction between Single-Walled Carbon Nanotubes and Bacterial Cells

Results of Chapter 3 and Chapter 4 have suggested that individually dispersed nanotubes showed strong antibacterial activity, however, oxidative stress and metal residue are both not the major cause of the death of bacterial cells. Until now, the interactions between SWCNTs and bacterial cells still remain unclear. Such an information about SWCNT-bacterium interactions are essential in the development of novel SWCNT-based materials for their potential applications, such as water treatment filters¹ and antibacterial coatings.^{2,3} Moreover, a good understanding on SWCNT–bacteria interactions may also help to elucidate the toxicology of SWCNTs, which are critical for their applications in imaging and therapeutics.⁴

Although SEM has been utilized to visualize physical damages of bacterial cells upon contact with SWCNTs,⁵⁻⁹ AFM may provide better opportunities for studying SWCNT–bacteria interactions. This is because AFM can image nanoscale ultrastructure of bacterial surfaces and measure nanomechanical properties of bacterial cells.^{10,11} Bacteria can be imaged in air or buffer solutions¹² without complex pretreatments (such as conductive film coating required by SEM).¹⁰ Nanomechanical properties of bacteria can also be measured in air,¹³⁻¹⁵ but their properties may change during air drying processes.¹⁶ To prevent such alterations to properties, *in situ* measurement under physiological conditions should be conducted.^{15, 17, 18} Numerous studies have demonstrated the effectiveness of AFM in exploring surface structure and mechanical property changes of bacterial cells upon treatment with β -lactam,¹⁹ cefodizime,²⁰ chitosan,^{13, 14} nitric oxide,²¹ and antimicrobial peptides.^{17, 22-25}

The aim of this chapter is to investigate dynamic changes induced by SWCNTs on bacterial envelope and mechanical properties. First, a super sharp AFM tip was used to create piercings on bacterial cells, mimicking real collisions between SWCNTs and bacteria. Subsequently, AFM was employed to image bacterial cells that were exposed to SWCNTs over different time periods. We also measured the bacteria mechanical properties under various pseudo-physiological conditions (0.9 % NaCl solution) to avoid changes in mechanical properties during air drying process.

5.1 Materials and Methods

5.1.1 Bacterial cell preparation

E. coli K12 and *B. subtilis* were grown in LB broth at 37 °C and 30 °C overnight, respectively, and harvested in the mid-exponential growth phase. Cells were collected by centrifugation (6000 rpm, 10 min) and washed three times. Tween-20 was added to enhance the dispersion of SWCNTs.⁷ Next, cell pellets were suspended in the saline solution. Bacterial suspensions were further diluted to obtain cell samples containing 10^6 to 10^7 colony forming units (cfu/mL). To accurately investigate the antibacterial action of SWCNTs, we employed high purity SWCNT samples with a narrow chiral structure distribution.²⁶⁻²⁹ Nanotubes were purified by a centrifugation-based method.³⁰ To yield individually dispersed nanotube suspensions, solid samples (5 $\mu\text{g/mL}$) were dispersed in the Tween-20–saline solution by sonication.⁷ SWCNT suspensions (10 mL at 5 $\mu\text{g/mL}$) was incubated with 1 mL of bacterial suspensions for up to 2 h under 250 rpm shaking speed at 37 °C for *E. coli* or 30 °C for *B. subtilis*.

5.1.2 Cell piercing experiments

A super sharp silicon probe (SSS-NCH from Nanoworld, typical tip radius of curvature of 2 nm) was used in cell piercing experiments to examine nanoscale collisions between

individually dispersed SWCNTs and bacteria. Fresh bacterial suspensions were drop-casted on cleaned glass slides and dried in air. We carried out the piercing experiments in air because it is easier to observe any structural changes on bacterial surface by AFM imaging in air than in liquid. The cantilever deflection sensitivity was calibrated on a hard surface (a glass slide). AFM images were first obtained in a tapping mode at 256 pixels $\times$ 256 lines with scanning speed at 1 Hz. After imaging, the tip was aligned above bacterial cells and converted to contact mode. Four different positions on the bacterial cell were continuously punctured for 200 times each, with forces of 10, 100, 1000, and 2000 nN, respectively. The cell was eventually imaged in tapping mode to illustrate morphology changes induced by physical punctures at different force loads.

5.1.3 Estimating mass and momentum of dispersed SWCNTs

Nanotube with (7,5) chiral structure is the main species in our SWCNT samples.^{7, 30} Considering the fact that C–C bond length is 0.142 nm, one unit length of the (7,5) nanotube (4.448 nm) contains 436 C atoms (simulated by Materials Studio, Accelrys). The average length of nanotubes is assumed to be 500 nm; accordingly, the mass of one individual nanotube is $m = (500 \text{ (nm, length)} \times 436 \text{ (C atoms)} / 4.448 \text{ (nm/unit length)}) \times (0.012 \text{ (kg/mol)} / (6.02 \times 10^{23} \text{ (atoms/mol)})) = 9.8 \times 10^{-22} \text{ kg}$. During incubation with bacteria, the stirring speed of 250 rpm in a flask of 5 cm in diameter could result a velocity of $v = 2\pi \times 0.025 \text{ (m)} \times 250 \text{ rpm} / 60 \text{ (sec)} = 0.65 \text{ m/s}$. Thus, the momentum of an individual nanotube is approximately at $mv = 6.37 \times 10^{-22} \text{ kg}\cdot\text{m/s}$.

5.1.4 Cell imaging by AFM

Cover glass slides were rinsed with alcohol, acetone, and deionized water in sequential steps. A cell sample of 10 μL was extracted from bacterial cell suspensions after incubation with SWCNTs for different time periods, and drop-casted directly on cleaned glass slides. To

obtain AFM images of bacterial cell wall morphology, we dried and immobilized cells on glass slides under ambient air for 10 min. All samples were characterized within 30 min after drying. To minimize lateral forces on cells during imaging, a silicon non-contact high resonance frequency cantilever (NCH) was used to image cells during tapping mode. Then, AFM height profiles were analyzed by the Igor Pro MFP-3D software to obtain root-mean-squared (rms) roughness on $200\text{ nm} \times 200\text{ nm}$ areas. Five AFM height profiles were scanned within 30 min, and five different areas on the height profiles of each cells were randomly selected to calculate their rms roughness values.

5.1.5 Force measurement on immobilized bacteria

E. coli and *B. subtilis* were immobilized on poly(L-lysine) coated cover glass slides.⁷ In a netshell, 10 μL of poly (L-lysine) solution (Sigma, 0.01% solution) was dropped on a clean glass slide, and the slide was dried at room temperature. Bacterial suspension of 50 μL of was drop-casted on the poly(L-lysine) coated surface. The bacterial suspension liquid drop stood still for 10 min to anchor cells on the surface. Then, the slide was rinsed in 0.9 wt % NaCl solution to remove unattached cells. Cell attached slides were imbedded in $\sim 100\text{ }\mu\text{L}$ of saline solution during force measurement. The spring constant (k_c) of AFM tips was measured in saline solution by a thermal method in the Igor Pro MFP 3D software. The location of bacterial cells was identified by AFM imaging in contact mode ($10 \times 10\text{ }\mu\text{m}^2$ with 128×128 pixels or $15 \times 15\text{ }\mu\text{m}^2$ with 256×256 pixels) using Silicon probes (ContAI-G, Budget Sensors). Reference force curves were first obtained on bare spots on the poly (L-lysine) coated surface (not covered with bacteria). Next, AFM tips were aligned at different positions on bacterial cells. The positions were all set near the center of bacteria to avoid variations induced by the edge effect.³¹ The force trigger point was set to 3 nN, and the tip approaching and retraction rates were 500 nm/s. At least five random locations on the same cell and five different bacterial cells on each glass slide were chosen to record force–distance (f–d) curves. Subsequently, one

additional AFM image was taken to confirm that the structure and location of the cells remain unchanged during the force measurement. Spring constants (k_f) of bacterial cells were calculated using slope (s) of the linear portion of approaching branch of f–d curves using the expression: $k_f = k_{cs} / (1-s)$.³²⁻³⁴

5.2 Results and Discussion

5.2.1 Multiple piercing of bacterial cells

Figure 5-1 AFM amplitude images of (A) *E. coli* and (B) *B. subtilis*. (C) and (D) are, respectively, *E. coli* and *B. subtilis* after piercing by a 2 nm AFM tip for 200 times at different locations. (All scales in A, B, C, and D are 1μm) Small images on the extreme right are enlarged images of areas punctured by the tip. The deflection set points of the AFM tip are 10, 100, 1000, and 2000 nN. (All scales in small images are 0.1μm)

When bacteria are incubated with SWCNT dispersions, cells may collide with numerous SWCNTs in solution. The collision between bacterial cells and SWCNTs may damage bacterial cells. Physical piercing on cell walls by a nanoscale tip can be used to simulate such collisions, and it is interesting to find out the force needed by the tip to induce damages on cell surfaces. We chose a super sharp tip with 2 nm radius to mimic the physical structure of SWCNTs, which would interact with bacteria in solution. As described in the previous section, the tip continuously pierced the cell surface under four different force loads (10, 100, 1000, and 2000 nN).

Figure 5-1 shows images of bacterial cells before and after pierced. When the force is higher than 100 nN, clear holes can be found on cell surfaces after punctures; the holes remain unchanged after the piercing experiment has ended for 30 min. For both *E. coli* and *B. subtilis*, the hole dimensions increase with the raise of applied force from 100 to 2000 nN. Conversely, no damages were noted when the force is at 10 nN. We further lowered the force to 3 nN, and no changes on the bacterial morphology were observed after multiple puncturings. Suo *et al.*³⁵ illustrated that a live gram negative bacterium *Salmonella typhimurium* can survive 20 to 40 puncturings of 4 nN by a sharp AFM tip, and the authors concluded that phospholipid molecules may undergo rapid reorientation in response to AFM tip penetration, sealing holes that are caused by the tip. In the same fashion, the holes, which were caused by force at or below 10 nN, on *E. coli* and *B. subtilis* were covered back. However, the reorientation of phospholipid molecules is unable to reseal holes induced by force above 10 nN.

An intriguing question is whether SWCNTs dispersed in solution could induce large forces (> 10 nN) on bacterial cell surfaces? If we assume that the relative velocity of nanotubes to bacteria would decrease from 0.65 m/s to 0 m/s upon collision, the momentum of one nanotube (6.37×10^{-22} kg·m/s) could induce a force of 10 nN only when the collision impact

time is as short as 6.37×10^{-14} s ($t = \Delta mv/F$), which is too small to be realistic. Even if nanotube–surfactant assemblies in solution can be much heavier than one individual nanotube, the momentum change is still unlikely to generate a force higher than 10 nN. Besides, previous investigation has shown that Brownian motion velocity of a 300 nm nanoparticle is only 4–5 $\mu\text{m/s}$.³⁶ The momentum of SWCNTs attributed to their Brownian motion should be much smaller than that induced by stirring. Therefore, it is unlikely that a single collision between an individual SWCNT and a bacterium would produce a force that can cause direct physical damages to the bacterial surface.

5.2.2 Bacterial cell morphology changes

Studies in Chapter 3 indicated that 58.1 ± 5.0 % of *E. coli* and 87.5 ± 6.5 % of *B. subtilis* were killed after 2 hours of incubation with SWCNTs, and SEM images also showed that bacterial cells are seriously damaged after exposure to SWCNTs. Since a direct single collision has little chance in damaging cells, it is essential to investigate how SWCNTs cause dynamic changes to cell wall architecture and cell mechanical properties, as well as to examine the dependence of such changes on interaction time and intensity.

Figure 5-2 renders AFM amplitude images of *E. coli* and *B. subtilis* observed after incubation with SWCNTs for 0, 10, 60, and 120 min. As illustrated in Figure 5-2A, the surface of *E. coli* is relatively smooth before interacting with SWCNTs. No obvious nano-pores or physical damages were found, implying that both drying in air and tapping mode do not alter cell wall architecture. In this study, no peritrichous flagella structures were observed on *E. coli*. On the contrary, gram-positive bacterium *B. subtilis* (see Figure 5-2B) also possesses distinctive peritrichous flagella structures but with smooth surfaces. After incubation with SWCNTs for 10 min, *E. coli* is winded with nanoscale tube-like structures (see Figure 5-2C). A close examination of the cell surface in Figure 5-3 suggests that these tube-like structures partially cover cell surface.

Figure 5-2 AFM amplitude images of *E. coli* and *B. subtilis* after incubation with SWCNT dispersions over different time periods. (A) *E. coli* before contact with SWCNTs, (B) *B. subtilis* before contact with SWCNTs, (C, E and G) *E. coli* after incubated with SWCNTs for 10, 60 and 120 min, respectively, and (D, F and H) *B. subtilis* after incubated with SWCNTs for 10, 60 and 120 min, respectively All scale bars are 1 μm .

We further measured the height of the structures on the flat glass surface. As clearly depicted in Figure 5-3B, heights of these small bundles range from 4 to 10 nm, which are similar to those of SWCNT bundles. Since no peritrichous flagella structures were found on *E. coli* and no other tube-like materials exist in our cell cultures, we conclude that these tube-like structures are SWCNT bundles.

Figure 5-3 (A) AFM height image of the *E. coli* cell (the same cell shown on Figure 5-2C) after incubation with SWCNT dispersions for 10 min. (B) The height profile along the line in part A. (C) 3D height image of the *E. coli* cell.

The surface of bacterial cells became uneven after incubation for 60 min. Figure 5-2E shows a damaged *E. coli*. Grooves and holes were randomly distributed on the cell surface. The cell wall collapsed and the cell height decreased, releasing its cytoplasm. The cell damages were more apparent and severe after the cell was incubated for 120 min (see Figure 5-2G); the cell was almost flattened, and cytoplasm was found all around the cell. AFM image in Figure 5-2G confirmed that SWCNTs can fragmentize bacterial membranes, leading to a

severe volume reduction. The surface roughness of *E. coli* outer membranes was also monitored. The average rms roughness of *E. coli* before contact with SWCNTs was 3.6 ± 0.5 nm. After incubation with SWCNTs for 10 min, the rms of cell surface with and without SWCNT networks were correspondingly 9.9 ± 1.6 nm and 4.0 ± 0.9 nm. The surface roughness further increased to an average of 16.8 ± 1.8 nm after 60 min of incubation with SWCNTs.

The same cell morphology changes were observed on *B. subtilis*. The cell surface becomes rougher after 10 min of incubation with SWCNTs (see Figure 5-2D). It is difficult to identify tube-like structures on *B. subtilis* due to the existence of peritrichous flagella structures, which look similar to nanotube bundles. Some solid residues can be identified near the edge of the cell. Serious bacterial membrane destruction and leakage of cytoplasm was observed after exposure to SWCNTs for 60 and 120 min (see Figures 5-2F and 5-2H). The morphology observation for *B. subtilis* was consistent with that for *E. coli*. The rms roughness of the untreated *B. subtilis* was 4.9 ± 1.1 nm, and the roughness increased to 7.3 ± 2.0 nm, 15.8 ± 4.6 nm, and 22.3 ± 2.7 nm after 10, 60, and 120 min of exposure, correspondingly.

5.2.3 Mechanical properties of bacterial cells

Apart from morphology changes, mechanical properties of bacteria should also be changed after exposure to SWCNTs. To prevent the mechanical properties from changing during our AFM characterization, we conducted all the following measurements in saline solution. A silicon probe (ContAI-G, Budget) was selected to assess the mechanical properties of bacterial cells,³⁷. Several studies have demonstrated that spring constants of AFM cantilevers would not have significant influences on measurement of bacterial mechanical properties.^{17,33} In this study, the AFM probe spring constant is 0.35 ± 0.03 N/m.³⁸ F–d curves were used to reveal cell hardness changes, as illustrated in Figure 5-4 for *E. coli*, and *B. subtilis* before and after treatment with SWCNTs. For glass surfaces, the slope of f–d curves

equals to one, because no indentation was induced by the AFM tip. Conversely, any deformations in cell membranes can cause f–d curve slopes to be less than one. In line with our previous study,⁷ Figure 5-4 shows that f–d curves of *B. subtilis* exhibit smaller slopes than those of *E. coli*, suggesting that *B. subtilis* are softer than *E. coli*. Although gram-positive cells are generally stiffer than gram-negative cells due to their thick peptidoglycan layers (20–80 nm vs. 7–8 nm), our unexpected outcome may likely be rationalized as follows: (1) *B. subtilis* possesses lesser resistance to the AFM tip than *E. coli* because the former bacterial cell does not have an extra layer, the outer membrane, and/or (2) the strain of *B. subtilis* used in this study is softer.¹⁴ Nevertheless, f–d curve slopes of both *B. subtilis* and *E. coli* decrease with increasing incubation time.

Table 5-1 Spring constants of bacterial cell surfaces after incubation with SWCNTs over different time periods

Incubation time (min)	<i>E. coli</i>		<i>B. subtilis</i>	
	slope	k_b (N/m)	slope	k_b (N/m)
0	0.32 ± 0.06	0.172 ± 0.044	0.23 ± 0.06	0.102 ± 0.036
60	0.24 ± 0.07	0.114 ± 0.034	0.14 ± 0.05	0.059 ± 0.026
120	0.19 ± 0.06	0.088 ± 0.036	0.09 ± 0.06	0.034 ± 0.025

The effective spring constants of bacterial cell surfaces can be calculated by the linear part of f–d curves (Table 5-1). The effective spring constants of *E. coli* and of *B. subtilis* decrease from 0.172 ± 0.044 to 0.088 ± 0.036 N/m and from 0.102 ± 0.036 to 0.034 ± 0.025 N/m, respectively, after incubation with SWCNTs for 120 min. Spring constants of bacterial cells correspond to their turgor pressure.³² Hence, the spring constant decrement observed here can be attributable to cytoplasm leakage;³⁹ The turgor pressure of bacterial cells decreases when the dense cytoplasm has released. As evidenced in Figure 5-2, cell membrane is

damaged and large amount of cytoplasm is visible around the cell body. The significant changes found in spring constants of *E. coli* and of *B. subtilis* - are consistent with their respective morphology changes.

Figure 5-4 Force-distance curves of glass surface, *E. coli* (A) and *B. subtilis* (B), before and after incubation with SWCNTs at different time durations. Slopes indicate the hardness of the measured surface.

In Chapter 3, we demonstrated that the antibacterial activity could be promoted through dispersing nanotube individually, increasing nanotube concentration and elevating the shaking speed during incubation. Well-dispersed nanotubes are more mobile in solution and have higher chance of colliding with bacterial cells. Higher nanotube concentration and shaking speed can also enhance the interaction between SWCNTs and cells. Other studies have observed that CNT diameter⁵, length⁶, aggregation,^{6, 7} concentration,^{7, 8} and surface functional groups⁸; buffer solutions⁸; as well as contact time⁹ and intensity⁷ can affect CNT antibacterial activity. All these factors apparently influence direct collisions between bacteria and nanotubes in solution. According to the earlier experimental results for AFM tip piercing, a single collision of SWCNT and bacterium is not likely to directly disrupt the bacterium.

AFM images in Figure 5-3 show that nanotube networks partially cover cell surfaces, signifying that bacterial death is associated with the accumulation effect of many SWCNTs, which may be explained in the following two scenarios. (1) The SWCNT concentration is 5 $\mu\text{g/mL}$, and the mass of individual nanotube is 9.8×10^{-22} kg; accordingly, there are about 5.1×10^{13} nanotubes in the 10 mL SWCNT suspension interacting with 10^6 to 10^7 bacterial cells. Cell surfaces can be attacked millions of times during incubation. Thus, cell surfaces may be damaged by the increased number of physical piercings induced by numerous nanotubes, even though a single piercing is neglectable. (2) Individual nanotubes may initially stick to cell surfaces, and more nanotubes on the cell surface would bundle together due to strong van der Waals attractions between nanotubes.^{40, 41} A recent theoretical study also highlighted that collisions with bacterial surface and rotational Brownian motion can lead to the accumulation of microswimmers near cell surfaces.⁴² SWCNTs dispersed in solution, therefore, may have high tendency to accumulate near cell surfaces and to be trapped into the SWCNT networks formed on cell surfaces. The interaction between SWCNT networks and cells is expected to be

more intense than that of individual nanotubes, giving rise to the destruction of cell envelope. Once the cell envelope is damaged, it will result in cell death. Experimental parameters, such as dispersion, concentration, contact time and intensity, etc., which enhance the adsorption and accumulation of SWCNTs on cell surfaces, are able to enhance the antibacterial activities of SWCNTs.

Recent literature have shown that when bacteria were filtered through nanotube based membranes, many cells were killed.¹ We reason that the filtration process is different from inculcation. In a filtration process, cells are dragged to nanotube network. The force, which a bacterial cell experiences during filtration, could be much higher than 10 nN. Such heavy forces are capable of directly damaging bacterial cells. The direct physical piecing can be the dominate factor in the antibacterial mechanism under filtration conditions.

5.3 Conclusions

The present study is the first AFM visual demonstration of SWCNT antibacterial effects on living bacterial cells. For both *E. coli* and *B. subtilis*, AFM images showed that bacterial envelopes were severely damaged with leakage of intracellular contents after incubation with SWCNTs. The volume and height of bacterial cells also decreased. The rms roughness of bacterial surfaces increased with incubation time, consistent with observed cell destruction. The spring constants of bacterial surfaces after exposure to SWCNTs over different time periods were measured in saline solution;. The effective spring constants of *E. coli* and *B. subtilis* declined from 0.172 ± 0.044 to 0.088 ± 0.036 N/m and 0.102 ± 0.036 to 0.034 ± 0.025 N/m, respectively, after incubation with SWCNTs for 120 min. The decrement of spring constants can be related to cell envelope destruction and cytoplasm release. Physical piercings on bacterial cells by the 2 nm AFM tip can result in significant damages when the force was higher than 10 nN. The collision force between an individual SWCNT and a bacterium during incubation is much weaker as compared to the tested force under piercing condition. Moreover,

SWCNT network structures were found on *E. coli* surface, further implying that bacterial death is related to the accumulation effect of large amount of nanotubes.

5.4 Reference

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Chapter 6

Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide and Reduced Graphene Oxide

In chapter 3, 4 and 5, antibacterial activity and mechanism of SWCNT were illustrated. In this chapter, we will focus on the 2D CNMs-graphene-based materials. Graphene is a single atomic plane of graphite (Gt),^{1, 2} which was first obtained through micro-mechanical exfoliation of Gt.³ Graphene oxide (GO) is a graphene sheet with carboxylic groups at its edges and phenol hydroxyl and epoxide groups on its basal plane^{4, 5}. GO can be chemically exfoliated from graphite oxide (GtO). Thermal annealing or chemical treatment can eliminate functional groups on GO to produce reduced graphene oxide (rGO). These graphene based materials exhibit unique electronic, thermal and mechanical properties,^{1, 6} and hold great promises in potential applications, such as nanoelectronics, conductive thin films, supercapacitors, nanosensors and nanomedicine.^{2, 7} Compared to other synthetic carbon nanomaterials, such as fullerenes and carbon nanotubes (CNTs), few toxicity studies on graphene based materials are currently available.⁸⁻¹¹ Only recently, it was reported that GO and rGO exhibit strong antibacterial activity.^{9, 10} The antibacterial activity of GO and rGO has been attributed to membrane stress induced by sharp edges of graphene nanosheets, which may result in physical damages on cell membranes, leading to RNA leakage.¹⁰ Besides, it was proposed that graphene may induce oxidative stress on neural pheochromocytoma-derived PC12 cells.¹¹

The current few cytotoxicity studies on graphene-based materials suggest some similarity between graphene and other synthetic carbon nanomaterials. Antimicrobial effect of CNTs is the synergy of both “physical” and “chemical” effects.¹² When bacteria directly contact with

CNTs, intensive physical interactions between CNTs and bacterial cells may cause physical damages on cell membranes, and result in the release of intracellular contents.^{12, 13} At the same time, some “small” CNTs could be internalized by bacterial cells, while other “larger” CNT aggregates may stick on the surface of bacterial cells.¹⁴⁻¹⁶ CNTs may chemically increase cellular oxidative stress, which could disrupt a specific microbial process.^{12, 13} If graphene-based materials share a similar antibacterial mechanism as that of CNTs, material characteristics which influence how graphene-based materials physically interact with bacterial cells, such as solubility, dispersion, and size, should strongly influence their antibacterial activities. Moreover, material properties, which control their abilities in producing cellular oxidative stress, should also have a strong impact on their antibacterial activities.

Antibacterial mechanism of graphene-based materials investigated in this Chapter. The time and concentration dependent antibacterial activities were found. Scanning electron microscopy (SEM) was applied to show different behaviors of GO and rGO dispersions toward bacterial cells. Graphene-based materials were first characterized in aqueous dispersions by dynamic light scattering analysis (DLS), and then quantified their average sizes by SEM. The possibility of reactive oxygen species (ROS) (superoxide anion, $O_2^{\cdot-}$) production was evaluated by the XTT method. *In vitro* glutathione (γ -L-glutamyl-L-cysteinyl-glycine, GSH) oxidation was used to examine the ROS-independent oxidative stress. Based on these results, material characteristics related to their antibacterial activities were identified.

6.1 Materials and Methods

6.1.1 Preparation and characterization of Gt, GtO, GO and rGO dispersions

Gt dispersion was obtained by sonicating graphite powders (Aldrich, synthetic, < 20 μ m) in isotonic saline solution (0.9 w/v % NaCl) using a bath sonicator (Elamsonic, S60H) at 37 kHz under 550 W for 1 h. GtO was prepared as described in the Chapter 2.^{17, 18} As-produced

GtO was first thoroughly washed using deionized water to remove chemical residues. GtO dispersion was then prepared by dispersing washed GtO in saline solution by shaking with vortex. GO was produced by bath sonicating washed GtO powder in water at 550 W for 6 h. rGO was obtained by hydrazine reduction as described in the Chapter 2,¹⁹ and dispersed in saline solution by bath sonication at 550 W for 1 h. Gt, GtO, GO and rGO dispersions with different concentrations were prepared by dispersed specific amount of solid powders in saline solution.

6.1.2 Cell preparation

E. coli were cultured in LB (37 °C), and grow overnight. Cultures were centrifuged (6,000 rpm, 10 min) to pellet cells, and cells were washed three times. The pellets were then re-suspended in isotonic saline solution. Bacterial cell suspensions were diluted to obtain cell samples containing 10^6 to 10^7 CFU/mL.

6.1.3 Cell viability test

E. coli cells were incubated with fresh Gt, GtO, GO or rGO dispersions in isotonic saline solutions at 37 °C under 250 rpm shaking speed for 2 h. The loss of viability of *E. coli* cells was evaluated by colony counting method. Briefly, series of 10-fold cell dilutions (100 μ L each) were spread onto LB plates (culture overnight at 37 °C).

6.1.4 Cell morphology observation

As described in 3.1.4.

6.1.5 Detection of $O_2^{\cdot-}$

The possibility of superoxide radical anion ($O_2^{\cdot-}$) production was evaluated by monitoring the absorption of XTT (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, Fluka).²⁰ XTT can be reduced by superoxide radical anion ($O_2^{\cdot-}$) to form

water-soluble XTT-formazan with the maximum absorption at 470 nm. XTT (0.4 mM) were dissolved in PBS at pH 7.0. GO or rGO dispersion (1 mL) in a PBS buffer (80 $\mu\text{g/mL}$) was mixed with 0.4 mM XTT (1mL) and then incubated in dark (5 h). Afterwards, the mixture was filtered by polyethersulfone filter (Acrodisc[®] Syringe Filters with Supor[®] Membrane, 0.45 μm) to remove GO or rGO. Filtered solution (250 μL) was then dropped in 96-well plate. The changes in absorbance at 470 nm were monitored on a Benchmark Plus microplate spectrophotometer. In this assay, TiO_2 (Degussa, P25, 40 $\mu\text{g mL}^{-1}$) dispersion was exposed to a UV light source as a positive control.

6.1.6 Thiol oxidation and quantification

Following the method used in a previous study,¹² the concentration of thiols in GSH was quantified by Ellman's assay.²¹ Gt, GtO, GO or rGO dispersions (225 μL at 80 $\mu\text{g/mL}$) in 50 mM bicarbonate buffer (pH 8.6) was added into 225 μL of GSH (0.8 mM in the bicarbonate buffer) to initiate oxidation. All samples were prepared in triplicate. The GSH-Gt, GtO, GO or rGO mixtures were transferred into a 24-well plate. The 24-well plate was covered with alumina foil to prevent illumination, and then placed in a shaker with a speed of 150 rpm at room temperature for incubation of 2 h. After incubation, 785 μL of 0.05 M Tris-HCl and 15 μL of DNTB (Ellman's reagent, 5, 5'-dithio-bis-(2-nitrobenzoic acid), Sigma-Aldrich) were added into the mixtures to yield a yellow product. Gt, GtO, GO or rGO was removed from the mixtures by filtration (Acrodisc[®] Syringe Filters with Supor[®] Membrane, 0.45 μm). 250 μL of filtered solutions were then placed in a 96-well plate. Their absorbance at 412 nm was measured on a Benchmark Plus microplate spectrophotometer. After 2 h incubation, 98 % of GSH in the positive control sample was lost, which is consistent with previous studies.^{12, 22}

6.2 Results and Discussion

6.2.1 Gt, GtO, GO and rGO dispersions

Figure 6-1 Photographs of Gt, GtO, GO and rGO dispersions. Images marked “0h” show dispersions immediately after sonication, while images marked “2h” are from dispersions after standing still for 2 h.

Photographs of Gt, GtO, GO and rGO dispersions are shown in Figure 6-1. They look differently because of their distinct structural and physicochemical properties. Black particles are visible in the Gt dispersion after sonication for 1 h. Most of Gt particles precipitated after the Gt dispersion stood still for 2 h. The GtO dispersion was obtained by oxidation of Gt, and it is opaque yellow in color. Some small GtO particles can also be identified in the GtO dispersion. Significant portion of GtO particles precipitated after the GtO dispersion was idle for 2 h. However, if washed GtO powders were bath sonicated for 6 h, GO nanosheets were exfoliated from the GtO, resulting in the clear and homogenous yellow-brown GO dispersion. The GO dispersion was stable after standing still for several days. This is because the large amount of hydrophilic functional groups, such as carboxyl, hydroxyl and epoxy groups, on GO nanosheets.¹⁹ The rGO dispersion was obtained by chemically reducing the GO dispersion using hydrazine.¹⁹ After reduction, the surface of rGO nanosheets became hydrophobic, and

some black particles started precipitating.¹⁹ The strong van der Waals forces among rGO nanosheets would facilitate the aggregation of rGO particles. Thus, plenty of rGO particles precipitate after the rGO dispersion stood still for 2 h.

Figure 6-2 Dynamic light scattering spectra of Gt, GtO, GO and rGO dispersions (all dispersions are at $40 \mu\text{g}\cdot\text{mL}^{-1}$)

Aggregation of engineered nanomaterial exists commonly in aquatic system,²³ including CNMs, such as CNTs^{24, 25} and fullerene.^{26, 27} Different aggregation conditions could significantly influence the interaction between nanoparticles and bacteria.^{16, 28-30} Aqueous dispersions of Gt, GtO, GO and rGO were first characterized by DLS analysis. The standard spherical particle models were used in DLS. As shown in Figure 6-2, the nominal effective diameters of particles in Gt, GtO, GO and rGO dispersions are 5.25, 4.42, 0.56, and 2.93 μm , respectively. Because most of graphene-based materials are not spherical particles, the model derived diameters are not their real sizes. DLS results only show size differences among the four materials. The dispersions were further dropped on silicon wafers, and dozens of SEM

images were taken randomly for each sample. The size distribution of Gt, GtO, GO and rGO was determined by analyzing their SEM images using the Image J software.

Figure 6-3 Size distributions of Gt (A), GtO (B), GO (C), and rGO (D). At least 200 particles were measured for each sample to obtain the size distribution. Inserts show their representative SEM images. Scale bars are at 10 μm.

As shown in 6-3, the nominal size of Gt, GtO, GO and rGO particles are 6.87 ± 3.12 , 6.28 ± 2.50 , 0.31 ± 0.20 , and 2.75 ± 1.18 μm, respectively. Among them, GO nanosheets have the smallest size. Although GtO particles have similar chemical functionality as GO nanosheets, their average size is nearly twenty times larger than that of GO nanosheets. The rGO particles were formed by reducing GO nanosheets, and their size is about nine times larger than that of GO nanosheets because of the aggregation of rGO fragments. It also should be noted that GO is two dimensional carbon sheets. Although the size of GO sheets measured by SEM are nearly

300 nm, their thickness is less than 1 nm, as shown in Figure 2-2B. however, Gt, GtO and rGO would aggregate into three dimensional particles.

6.2.2 Antibacterial activity of Gt, GtO, GO and rGO dispersions

E. coli was used to evaluate antibacterial activities of the four types of graphene-based materials. *E. coli* cells (10^6 to 10^7 CFU/mL) were incubated with the same concentration ($40 \mu\text{g/mL}$) of Gt, GtO, GO and rGO dispersions in isotonic saline solution for 2 h, respectively. The death rate of bacterial cells was determined by colony counting method described in the method section.

Figure 6-4 Cell viability measurement after incubation with Gt, GtO, GO and rGO dispersions. A 5 mL portion of graphene-based materials ($80 \mu\text{g/mL}$) was incubated with *E. coli* (10^6 to 10^7 CFU/mL), 5 mL) for 2 h at 250 rpm shaking speed and 37°C . Loss of cell viability was obtained by colony counting method. Error bars represent the standard deviation.

As shown in Figure 6-4, Gt dispersion exhibits a moderate cytotoxicity with the cell death rate at $26.1 \pm 4.8\%$. GtO dispersion shows a slight weaker antibacterial activity compared with

Gt, having the cell death rate at $15.0 \pm 3.7\%$. GO have a much stronger bacterial activity compared with GtO. The loss of *E. coli* viability increases to $69.3 \pm 6.1\%$, which is more than fourfold compared with that of GtO. rGO has a lower antibacterial activity compared with GO with a bacterial inactivation rate of $45.9 \pm 4.8\%$. Significant differences were found in their antibacterial activities among the four materials. In particular, GO and rGO have much higher bacterial inactivation rates compared with those of Gt and GtO. It also be noted that the shaking speed of 250 rpm was used in all antibacterial assays. Although some Gt, GtO and rGO particles precipitate when dispersions stand still for 2 h, all particles are suspended in saline solution, interacting with cells under the shaking condition in all antibacterial assays.

6.2.3 Time-dependent and concentration-dependent antibacterial activity

Next, we examined the time-dependent antibacterial behavior of two materials (GO and rGO), which showed higher activities in our early tests. GO or rGO dispersions ($40 \mu\text{g/mL}$) were incubated with *E. coli* for 4 h. The inactivation rate of bacterial cells was counted at hourly intervals. Figure 6-5A indicates the inactivation rate of bacterial cells steadily increase with extending incubation time. For GO dispersion, the loss of *E. coli* viability increases from $49.1 \pm 6.0\%$ after 1 h incubation to $69.3 \pm 6.1\%$ after 2 h, and further increases to $81.5 \pm 3.9\%$ after 3 h and $89.7 \pm 3.1\%$ after 4 h. rGO dispersion displays a similar trend. The loss of *E. coli* viability is $35.6 \pm 2.5\%$ after 1 h, and increases to 47.4 ± 4.6 , 67.8 ± 5.6 , and $74.9 \pm 4.8\%$ after 2, 3 and 4 h, respectively. For both materials, large fraction of cell death occurs in the first hour of incubation. Comparing GO and rGO dispersions, GO dispersions have much higher antibacterial activities than rGO dispersions at all tested incubation intervals. Furthermore, the concentration dependence of antibacterial activities on graphene-based materials was studied. GO or rGO dispersions at different concentrations were incubated with *E. coli* cells ($\sim 10^6 - 10^7$ CFU/mL) for 2 h at 37°C under 250 rpm shaking speed. The loss of *E. coli* viability progressively goes up with the increases of GO or rGO concentration (Figure 6-5B).

Figure 6-5 (A) Time-dependent antibacterial activities of GO and rGO. 5 mL of GO or rGO (80 $\mu\text{g/mL}$) was incubated with *E. coli* (10^6 to 10^7 CFU/mL, 5 mL) for 4 h. The loss of visibility was measured at 0, 1, 2, 3 and 4 h, respectively. (B) Concentration-dependent antibacterial activities of GO and rGO. 5 mL of GO or rGO (at 10, 20, 40, 80, and 160 $\mu\text{g/mL}$) was incubated with *E. coli* (10^6 to 10^7 CFU/mL, 5 mL) for 2 h.

The loss of *E. coli* viability jumps from $10.5 \pm 6.6\%$ at the GO concentration of $5 \mu\text{g/mL}$ to $91.6 \pm 3.2\%$ at $80 \mu\text{g/mL}$. Majority of *E. coli* was killed after incubation with GO at of $80 \mu\text{g/mL}$. In a similar manner, rGO dispersion at the $5 \mu\text{g/mL}$ kills only $8.4 \pm 7.3\%$ of *E. coli*, while $80 \mu\text{g/mL}$ rGO dispersion kills $76.8 \pm 3.1\%$ of *E. coli*. These results suggest that antibacterial activities of graphene-based materials are also concentration dependent.

6.2.4 Destruction of bacterial membrane

To find out how graphene-based materials kill bacteria, SEM was used to illustrate interactions between graphene-based materials and *E. coli* cells. Figure 6-6 shows most of *E. coli* cells become flattened, and lose their cellular integrity after exposure to GO or rGO dispersions. The destruction of cells in SEM images is consistent with previous images obtained by transmission electron microscope.⁹ It highlights that irreversible damages can be induced on bacterial cells after direct contact with graphene-based materials. This is similar to CNTs, which induce membrane stress on bacterial cells, resulting in destruction of cell structures. Besides, we observed that thin layers of nanosheets from GO dispersion, while rGO dispersion mainly contains large aggregated particles. A comparison of cells interacting with GO and rGO shows that *E. coli* mingle with GO and rGO in different manners. Figure 6-6B and E show most of *E. coli* cells were individually wrapped by thin layers of GO nanosheets. In contrast, *E. coli* cells were usually embedded in large rGO aggregates (Figure 6-6C, F). The different behavior of GO and rGO observed in SEM images suggests the aggregation/dispersion of graphene-based materials play an key role on their antibacterial activities.

Figure 6-6 SEM images of (A, D) *E. coli* after incubation with saline solution for 2 h without graphene-based materials, (B, E) *E. coli* cells after incubation with GO dispersion (40 $\mu\text{g/mL}$) for 2 h, (C, F) *E. coli* cells after incubation with rGO dispersion (40 $\mu\text{g/mL}$) for 2 h.

6.2.5 Oxidative stress mediated by Gt, GtO, GO and rGO

Other than membrane stress mediated by direct physical contacts, a previous study on graphene cytotoxicity has cited oxidative stress as its toxicity mechanism toward neural cells.¹¹ Oxidative stress is also often suggested as a key antibacterial mechanism of other CNMs, such as fullerene^{31, 32} and CNTs.^{12, 13} Because of the similarity in their structural and physiochemical properties among these carbon nanomaterials, it is necessary to find out what cellular oxidative stress may be produced by Gt, GtO, GO and rGO.

Figure 6-7 Production of superoxide radical anion ($O_2^{\bullet-}$) by GO and rGO dispersions. The $O_2^{\bullet-}$ production was monitored during the incubation of XTT (0.2 mM) with Gt, GtO, GO or rGO (40 $\mu\text{g/mL}$) dispersions at pH 7.0 in dark. Incubation with TiO_2 (40 $\mu\text{g/mL}$) under UV radiation was carried out as a positive control.

In general, oxidative stress mediated by graphene-based materials may come from several paths, one is ROS mediated oxidative stress, in which oxidative stress is induced by ROS generated by Gt, GtO, GO and rGO. This is the mechanism proposed in the previous graphene

toxicity study.¹¹ The other possible path is ROS-independent oxidative stress, in which graphene-based materials may oxidize a vital cellular structure without ROS production. This path has been observed in fullerene (C₆₀).³²

To better clarify different oxidative stress paths, we first measured the possibility of ROS (superoxide anion, O₂^{•-}) production using the XTT method as described in the method section.²⁰ Shown in Figure 6-7, no noticeable absorption is detected during the entire 5h incubation period, which indicates that no O₂^{•-} is produced. TiO₂ under UV radiation as a positive control validated our XTT tests. Based on the XTT results, we conclude that graphene-based materials mediate little ROS-dependent oxidative stress. Although trace amount of ROS may be produced, it plays a minor role in the antibacterial activity of graphene-based materials.

Figure 6-8 Oxidation of glutathione by graphene-based materials (a) Loss of GSH (0.4 mM) after in vitro incubation with 40 µg/mL of Gt, GtO, GO and rGO dispersions for 2 h. H₂O₂ (1 mM) is a positive control.

Next, we used *in vitro* GSH oxidation to examine the possibility of ROS-independent oxidative stress mediated by Gt, GtO, GO and rGO dispersions. GSH is a tripeptide with thiol groups. It is an antioxidant in bacteria at a concentration ranging between 0.1 and 10 mM.³³ GSH can prevent damages to cellular components caused by oxidative stress.³⁴ Thiol groups (–SH) in GSH can be oxidized to disulfide bond (–S–S–), which converts GSH to glutathione disulfide. GSH has been used as an oxidative stress indicator in cells.^{12, 31, 35} Ellman’s assay is able to quantify the concentration of thiol groups in GSH.²¹ As described in the method section, we employed Ellman’s assay to evaluate the oxidation of GSH when it was incubated with Gt, GtO, GO or rGO dispersions (40 $\mu\text{g/mL}$).

As showed in Figure 6-8, noteworthy fraction of GSH is oxidized after its exposure to Gt ($29.9 \pm 0.7 \%$), GtO ($21.4 \pm 1.1 \%$), GO ($22.2 \pm 0.7 \%$) and rGO ($94.2 \pm 1.1\%$). Among the graphene-based materials, rGO has the highest oxidation capacity toward GSH, followed by Gt. GtO and GO have lower GSH oxidation capacities. A previous study has shown that the GSH oxidation by SWCNTs depends on the metallicity of SWCNTs.¹² Different oxidation capacities toward GSH among Gt, GtO, GO and rGO can be also attributed to their different electronic properties. Gt is an electrical conductor, a semimetal. When GtO and GO are oxidized from Gt by introducing functional groups, sp^2 hybridized electron structure of the stacked graphene sheets was broken.³⁶ In contrast, GtO and GO are electrically insulating materials. If GO is reduced, the π electron network of graphene can be partially restored.³⁷ Graphene is a zero-gap semiconductor with excellent electrical conductivity.² Conductivity of rGO is much higher than GtO and GO. Materials with higher conductivity, such as rGO and Gt, do show higher oxidation capacities to GSH, compared with materials with lower conductivity, such as GtO and GO. Our observation suggests that rGO may share the similar mechanism as metallic SWCNTs.¹²

6.2.6 Time-dependent and concentration-dependent oxidative stress

Figure 6-9 (A) Time dependent GSH (0.4 mM) oxidation by GO and rGO dispersions (40 µg/mL) after incubation from 0 to 4 h. (B) Concentration dependent GSH (0.4 mM) oxidation by GO and rGO (at 5, 10, 20, 40, and 80 µg/mL) after incubation for 2 h.

Because cell viability tests have shown that the antibacterial activities of graphene-based materials are time and concentration dependent (see Figure 6-5A, B), we speculate the oxidation of GSH by these materials should be time and concentration dependent as well. GSH oxidation by GO and rGO was compared over different periods of time and under several concentrations. When 0.4 mM GSH was incubated with 40 $\mu\text{g/mL}$ GO or rGO, the oxidation of GSH gradually advance with extending reaction time.

Figure 6-9A shows the fraction of GSH oxidized by rGO increases from 79.9 ± 1.6 to 99.9 ± 1.2 %, when the reaction time increase from 1 to 4 h. Likewise, over the same period of time, the oxidation of GSH by GO goes up from 12.5 ± 2.4 to 37.0 ± 1.5 %. A large fraction of GSH is oxidized in the first one hour of incubation. GSH (0.4 mM) was also incubated with different concentrations of GO or rGO (5 to 80 $\mu\text{g/mL}$) for 2 h.

Figure 6-9B shows oxidizing GSH by rGO and GO is also concentration dependent. rGO oxidized 13.0 ± 4.0 % of GSH at 5 $\mu\text{g/mL}$ and all GSH (99.0 ± 1.1 %) at 80 $\mu\text{g/mL}$. GO oxidized 5.3 ± 2.9 and 22.0 ± 0.1 % of GSH at 5 and 80 $\mu\text{g/mL}$, respectively. Comparably, rGO has significantly higher oxidation reactivity than GO at the same reaction time and concentration. On the whole, more GSH are oxidized with the increase of reaction time and rGO/GO concentration, which is consistent with the time and concentration dependent incremental trend observed in the antibacterial activities of rGO and GO. The oxidation of GSH indirectly confirms that graphene-based materials are capable of mediating ROS-independent oxidative stress toward bacterial cells.

6.2.7 Antibacterial mechanism of Gt, GtO, GO and rGO

The correlation among antibacterial activities, GSH oxidation and aggregate size is summarized in Table 6-1, which can be examined from three aspects. First, comparing GtO and GO, they have similar capacities in oxidizing GSH (GtO at 21.4 ± 1.1 % vs. GO at 22.2 ± 0.7 %); however, GO dispersion can kill much higher fractions of *E. coli* (69.3 ± 6.6 %) than

GtO dispersion ($15.0 \pm 3.7\%$). GtO and GO contain almost the same chemical functional groups. Their difference is that GO is individual nanosheet with average size of $0.31 \pm 0.20 \mu\text{m}$, while GtO is aggregated stacks of GO nanosheets with average particle size of $6.28 \pm 2.50 \mu\text{m}$. Their distinct antibacterial activities suggest the aggregation of graphene nanosheets is important in the antibacterial mechanism. Materials, having smaller size (*e.g.* GO), have higher cytotoxicity than those with larger size (*e.g.* GtO). This size dependent toxicity is similar to what have been observed on CNTs.¹³ Smaller diameter SWCNT has higher antibacterial activity than larger diameter MWCNT.¹³ Second, comparing Gt and GtO, Gt particles ($6.87 \pm 3.12 \mu\text{m}$) are slightly larger than GtO particles ($6.28 \pm 2.50 \mu\text{m}$). However, we found the antibacterial activity of Gt ($26.1 \pm 4.8\%$) is much higher than that of GtO ($15.0 \pm 3.7\%$). This is obviously correlated with their different GSH oxidation capacities. Metallic Gt can oxidize more GSH than insulating GtO, suggesting that the metallicity of graphene materials also plays a role in their antibacterial activities. Third, if we compare GO with rGO, although rGO shows much stronger oxidation capacity toward GSH, smaller size GO has much higher antibacterial activity than rGO.

Table 6-1 The correlation among antibacterial activities, oxidative stress and particle size

	Loss of cells ^a	Loss of GSH ^b	Particle size ^c
	(%)	(%)	(μm)
GtO	15.0 ± 3.7	21.4 ± 1.1	6.28 ± 2.50
Gt	26.1 ± 4.8	29.9 ± 0.7	6.87 ± 3.12
rGO	45.9 ± 4.8	94.2 ± 1.1	2.75 ± 1.18
GO	69.3 ± 6.6	22.2 ± 0.7	0.31 ± 0.20

^[a] data extracted from Figure 6-4; ^[b] data extracted from Figure 6-8; ^[c] data extracted from Figure 6-3.

Overall, results in Table 6-1 suggest the antibacterial activities of graphene-based materials are attributed to their dispersibility, size, and oxidization capacity. Their antibacterial mechanism is likely to be the combination of membrane stress and ROS-independent oxidative stress.

Vecitis *et al.* previously hypothesized a three-step cytotoxicity mechanism for SWCNTs.¹² The first step is direct cell-SWCNT contact by adhesion. The second step is that SWCNT would make intimate, membrane disruptive interaction with bacteria, inducing membrane stress. The third step involves disrupting a specific microbial process by disturbing or oxidizing a vital cellular structure or component. We reason that this three-step toxicity mechanism is also applicable to graphene-based materials. *E. coli* cells may first deposit on Gt, GtO, GO or rGO during incubation. The dispersibility and size of materials should strongly influence the chance of cell deposition. In general, the dispersibility of graphene-based materials depends on functional groups on graphene sheets. When carboxyl, hydroxyl and epoxy groups are introduced on graphene sheets, they form much more stable dispersions compared with hydrophobic pristine carbon planes. GO can form stable dispersions with small nanosheets, thus offers more opportunities to interact with cells for cell deposition. Comparably, Gt and rGO dispersions are unstable, and contain large particles; thus, they have fewer chances to mingle with cells. Similar phenomena have been previously observed on other carbon nanomaterials. For instance, surfactant dispersed individual SWCNTs shows higher toxicity to various bacterial cells than nanotube aggregates. Functionalized and debundled CNTs display stronger toxicity compared with as-synthesized CNT aggregates.²⁹ Small nC₆₀ aggregates also show higher bacterial toxicity than large aggregates.²⁸

After cell deposition on graphene nanosheets, the sharp edge of graphene nanosheets may cause significant membrane stress.¹⁰ Nanosheets serve as “cutters” to disrupt and damage bacterial membranes, result in the release of intracellular contents, and eventually death. SEM

images in Figure 6-6 show the disruption role of graphene nanosheets. A notable difference among different graphene materials is that small GO nanosheets can wrap bacterial cells, while large rGO aggregates would trap cells. A molecular simulation has recently confirmed that small graphene sheets ($\sim 5.9 \times 6.2 \text{ nm}^2$) can be trapped in biological membranes consisting of phospholipid molecules.³⁸ It is likely that some graphene nanosheets may be internalized by bacterial cells, as previously observed on A549 cells.⁹ Although we found that no ROS were generated by graphene-based materials, GSH oxidation (Figure 6-8) indicates that the oxidation capacity of graphene-based materials may play a significant role when nanosheets are in direct contact with cellular components. A comparable case is that metallic SWCNTs can act as a conducive bridge over the insulating lipid bilayer, mediating electron transfer from bacterial intracellular components to the external environments.¹² By analogy, graphene-based materials could also oxidize lipids, proteins, and DNA of cells. The strong oxidation of GSH by rGO observed in our study supports that conductive graphene nanosheets are capable of oxidizing thiols or other cellular components.

6.2.8 Kinetic analysis of cell inactivation and GSH oxidation

In order to further evaluate the aforementioned antibacterial mechanism, the cell inactivation and GSH oxidation were compared through quantitative kinetic data extracted from Figure 6-5 and 6-9. The time dependent rate changes are presented in Figure 6-10A. During the first two hours of incubation, even though rGO has much higher GSH oxidation rate than GO, its cell inactivation rate is still lower than that of GO. This corroborates the importance of the initial deposition of graphene-based materials on cell surfaces. Without direct interactions with bacterial cells, the stronger oxidation capacity of rGO doesn't result in the stronger antibacterial activity. Furthermore, the GSH oxidation rate of GO shows minor changes over the 4 hour incubation, while the cell inactivation rate decreases continuously.

Figure 6-10 (A) The time dependent *E. coli* cell inactivation and GSH oxidation rates after incubated with GO and rGO dispersions, which are extracted from Figure 6-5A and 6-9A with the unit of $d(\%)/d(h)$. (B) The dependence of *E. coli* cell inactivation and GSH oxidation on GO and rGO concentrations, which are extracted from Figure 6-5B and 6-9B with the unit of $d(\%)/d(\mu\text{g/mL})$.

This suggests that the membrane stress induced by graphene-based materials may play more important roles during the first 2 hours, while oxidative stress could become more

prominent when bacterial cells have been covered by graphene-based materials in the last 2 hours. Figure 6-10B illustrates the dependence of cell inactivation and GSH oxidation on GO and rGO concentrations. All incubations were fixed at 2 hours. The GSH oxidation by rGO is more sensitive to the rGO concentration compared with the GSH oxidation by GO. However, the dependence of cell inactivation on concentration is similar between GO and rGO. It provides a circumstantial evidence that the antibacterial activity of graphene based materials is caused by both membrane and oxidation stress. Overall, the kinetic analysis results support the proposed antibacterial mechanism.

6.3 Conclusion

The antibacterial activity of Gt, GtO, GO and rGO aqueous dispersions toward *E. coli* was compared. Colony counting method results show that GO has the highest antibacterial activities, followed by rGO, Gt, and GtO under the same dispersion concentration. Their antibacterial activities are time and concentration dependent. Most of bacterial inactivation happens in the first hour of incubation, and cell death rate increases continuously with the increase of material concentration. The bacterial cytotoxicity may be attributed to both membrane and oxidative stress. A three-step antibacterial mechanism is applicable to graphene-based materials. In general, graphene materials, which contain higher density of functional groups, and are smaller in size, have more chances to interact with bacterial cells, resulting in cell deposition. By direct contact, graphene nanosheets can induce membrane stress by disrupting and damaging cell membranes, leading to cell death. The XTT tests show that no ROS are produced by Gt, GtO, GO and rGO. On the other hand, they display strong time and concentration dependent oxidization capacity toward GSH. The oxidation of GSH suggests that these graphene-based materials are capable of inducing ROS-independent oxidative stress on bacterial cells. If they are in direct contact with cells, conductive rGO and Gt would mediate more intense oxidative stress compared with insulating GO and GtO.

6.4 Reference

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Chapter 7

Effect of Lateral Dimension of Graphene Oxide on Its Antibacterial Activity

Graphene is a two-dimensional single-atom-thick sheet of hexagonally arranged carbon atoms.¹ Graphene oxide (GO) is a graphene sheet with carboxylic groups at its edges and phenol hydroxyl and epoxide groups on its basal plane.^{2,3} GO has attracted great attention, not only because it is a promising precursor to produce graphene-based nanomaterials,^{3,4} but also its excellent water dispersibility⁵⁻¹⁰ and amphiphilic behaviors.¹¹⁻¹⁴ GO is promising for potential applications, such as conductive thin film, biochemical sensing, drug delivery and cell imaging.¹⁵⁻¹⁹ Recently, a number of studies reported the antibacterial activities of GO.^{20,21} Several GO-based composites, such as GO–Ag,²²⁻²⁶ GO–TiO₂,²⁷ GO–polymer,²⁸⁻³⁰ GO–dye³¹, were also fabricated as antibacterial products. The antibacterial activity of GO is found to relate to both membrane puncture²⁰ and oxidative stress. In Chapter 6, I also noticed the similarity between graphene-based nanomaterials and other carbon nanomaterials, such as carbon nanotubes.³²

Researchers have reported that many factors may influence the antibacterial activity of carbon nanomaterials, including material properties, such as electronic structure,³² size,³³⁻³⁵ surface chemical properties,³⁶ incubation time, concentration, medium,³⁷ and light sources.³⁸ For graphene-based nanomaterials, Hurt and Kane *et al.* proposed that the most relevant material properties for their biological effects include surface area, layer number, lateral dimension, surface chemistry, and purity.³⁹ Common GO samples usually contain GO sheets with a broad lateral dimension distribution, spanning orders of magnitude from nano-GO at 10 nm to large GO layers >20 μm .³⁹ The lateral dimension of GO sheets is relevant for many biological phenomena that depend on particle size, such as cell adhesion and spread on large

GO sheets, cell intake of small GO sheets, and cell deformation caused by interacting with GO. Furthermore, recent studies^{40, 41} suggest that antibacterial effects of GO are related to the actual lateral dimension of GO sheets in various suspensions, which is strongly correlated with their aggregation. Well-dispersed GO sheets demonstrate the strongest antibacterial activity among several graphene-based nanomaterials. On the other hand, aggregated GO sheets in LB (Luria-Bertani) medium are found to act as a cellular growth enhancer.⁴¹ The bacterial toxicity mechanism related to the lateral dimension of GO need to be elucidated for their various potential applications.

In this chapter, a model bacterium –*E. coli* was utilized to measure the antibacterial activity of well-dispersed GO sheets with different lateral dimension.

7.1 Materials and Methods

7.1.1 GO preparation

GO were prepared by the modified Hummer's method.^{42, 43} The exfoliation of GO was performed by bath sonication for 30 min. This sample was marked as "GO-0". The GO nanosheets with different lateral dimension were prepared by sonication for 10, 30, 50, 120 and 240 min (SONICS, VCX-130). These samples were marked as "GO-10", "GO-30", "GO-50", "GO-120", and "GO-240", respectively.

GO dispersions (3 μ L) were dropped on freshly cut mica followed by air drying for AFM analysis. AFM analysis was performed on an MFP3D microscope. Lateral dimension distribution of GO samples was determined by analyzing AFM images using the Image J software (USA). SEM images were taken by drying GO dispersions on clean silicon wafers, and then viewed on a JEOL field emission-SEM (JSM-6700F), working at 5 kV.

7.1.2 Cell preparation

E. coli were culture in LB broth (37 °C) overnight. Cultures were collected by centrifugation and washed with deionized water (3 times). The pellets were then re-suspended in deionized water. Bacterial cell suspensions were diluted to obtain cell samples containing 10^6 to 10^7 CFU/mL.

7.1.3 Cell viability test

E. coli cells were incubated with fresh GO dispersions in deionized water at 37 °C under 250 rpm shaking speed for 2 h. The viability of *E. coli* cells was evaluated by the colony counting method. Briefly, series of 10-fold cell dilutions (100 μ L each) were spread onto LB plates (culture overnight at 37 °C).

7.1.4 Cell imaging by AFM

Cover glass slides were rinsed with alcohol, acetone, and deionized water in sequential steps. A cell sample of 10 μ L was extracted from bacterial cell suspensions after incubation with GO for 2 h, and drop-casted directly on cleaned glass slides. To obtain AFM images, we dried and immobilized cells on glass slides under ambient air for 10 min. All samples were characterized within 30 min after drying. To minimize lateral forces on cells during imaging, a silicon non-contact high resonance frequency cantilever (42 N/m) was used to image cells during tapping mode. Then, AFM height profiles were analysed by the Igor Pro MFP-3D software to obtain root-mean-squared (rms) roughness on 200 nm $\times$ 200 nm areas. Five *E. coli* cells and five different areas on the height profile of each cell were randomly selected to calculate their surface roughness.

7.1.5 Estimating quantity of dispersed GO nanosheets

Considering the fact that C–C bond length is 0.142 nm, one unit hexagon of graphene oxide contains 2 C atoms, the area of this hexagon is $a = 6 \times 1/2 \times 0.142 \times 3^{-1/2} \times 0.142 \text{ nm}^2$

= 0.052 nm². If we only consider the GO nanosheets whose area is larger than 9 μm² (3μm×3μm), the average area of these nanosheets is $A = 13.06 \mu\text{m}^2 = 1.306 \times 10^7 \text{ nm}^2$ (Calculated from figure 2A); accordingly, the average number of C atoms in one individual GO nanosheet is $N_C = (A/a) \times 2 = (1.306 \times 10^7 / 0.052) \times 2 = 5.023 \times 10^8$. Assuming the C/O ratio is 5:1 in GO nanosheets,⁴⁴ the number of O atoms in one individual GO nanosheet is $N_O = 5.023 \times 10^8 \times 1/5 = 1.005 \times 10^8$. The average weight of one GO nanosheet is $m = 5.023 \times 10^8 \times 1.993 \times 10^{-26} \text{ kg} + 1.005 \times 10^8 \times 2.657 \times 10^{-26} \text{ kg} = 1.268 \times 10^{-17} \text{ kg}$ (if we neglect the weight of H). The average number of GO nanosheets whose size is larger than 9 μm² (3μm × 3μm) with concentration of 5 μg/mL is $N = 5 \times 10^{-9} \text{ (kg/mL)} \times 21.2\% / 1.268 \times 10^{-17} \text{ kg} = 8.360 \times 10^7 \text{ mL}^{-1}$ (21.2 % is the percentage of GO nanosheets larger than 9 μm² in GO-0 sample).

7.2 Results and Discussion

7.2.1 GO dispersions

As-prepared graphite oxide^{42, 43} was first washed and dialyzed to remove metal ions and acid residues. Then, large GO sheets were produced by sonicating graphite oxide in a bath sonicator for 0.5 h. These GO sheets dispersed in water were marked as “GO-0”. Last, the large GO sheets (GO-0) in aqueous suspension were further sonicated in a cup-horn to yield smaller GO sheets. Sonication was conducted with GO suspensions placed in an ice bath to avoid overheating samples. The sonication time is 10, 30, 50, 120, and 240 min, resulting in samples marked as “GO-10”, “GO-30”, “GO-50”, “GO-120”, and “GO-240”, respectively. We utilized the sonication method to reduce the lateral dimension of GO sheets because this method results in negligible changes to the surface chemical properties of GO sheets. 3 μL of aqueous suspension from each GO sample was dropped on a freshly cut mica for AFM analysis (Figure 7-1A). AFM height profile in Figure 7-1 shows that these samples all have the

thicknesses of around 1 nm, confirming that monolayer GO sheets were prepared. It also indicates no aggregations occurred during the sonication.

Figure 7-1 AFM height images of GO sheets dried on mica surface after tip sonication for 0 (A), 10 (C), 30 (E), 50 (G), 120 (I), and 240 min (K). The scale bars are at 1 μm . The corresponding height profiles along red lines in AFM images: 0 (B), 10 (D), 30 (F), 50 (H), 120 (J), and 240 min (L).

Figure 7-2 Histogram of the weight distributions of GO sheets with different lateral sizes. (A) GO-0, (B) GO-10, (C) GO-30, (D) GO-50, (E) GO-120, and (F) GO-240. 120min (E), and 240min (F). At least 200 GO sheets were measured for each sample to obtain their distributions.

Image J software (National Institutes of Health, USA) was applied to analyze AFM images, and determine the lateral dimension of individual GO sheets. The average size of GO sheets is determined from ~200 GO sheets on at least 10 comprehensive AFM images.

Figure 7-3 SEM images of GO film after sonication for 0min (A), 10min (B), 30min (C), 50min (D), 120min (E), and 240min (F) dried on silicon wafers.

The average size of GO sheets in GO-0, GO-10, GO-30, GO-50, GO-120, and GO-240 samples is 0.753, 0.127, 0.065, 0.035, 0.013, and 0.010 μm^2 , respectively. Lateral dimension distribution of GO samples was also analyzed by the Image J software. Because almost all GO sheets are monolayer, the weight of each GO sheet is proportional to its lateral area.

In order to better compare their concentration dependent antibacterial activities, we quantify their lateral dimension distribution based on the weight percentage of GO sheets with various lateral sizes in a sample, other than just counting the number of GO sheets. The weight distributions of GO sheets in different samples are presented in Figure 7-2. It also should be noted that the average size of GO-0 and GO-10 samples is relatively small due to the existence of many small GO sheets ($<1 \mu\text{m}^2$). The lateral dimension distribution indicates the existence of significant fraction of large GO sheets ($> 1 \mu\text{m}^2$). The size of the largest GO sheets in the GO-0 sample is about 100 times larger compared to that in the GO-240 sample. The weight fraction of GO sheets with smaller sizes also increases significantly with the extension of sonication time. This confirms that GO samples with 6 different lateral dimensions are successfully prepared.

SEM images were taken on GO films, which were prepared by drying GO aqueous suspensions on silicon wafers. As shown in Figure 7-3, GO films compose of multilayer GO sheets forming circular ring structures, which are smooth at the center with small wrinkles at their edges. The diameter of the ring structures decreases with the decrease of GO sheet lateral sizes.

7.2.2 Antibacterial activity of GO

Antibacterial activities of GO sheets with different lateral sizes were evaluated toward *E. coli* cells. 40 $\mu\text{g/mL}$ of GO aqueous suspensions were first incubated with *E. coli* suspensions (10^6 to 10^7 CFU/mL) for 2 h under 250 rpm shaking speed. The viability of *E. coli* was evaluated by the colony counting method. Figure 7-4 shows that the viability of *E. coli*

decreased by 97.7 ± 1.0 , 91.5 ± 0.5 , 87.8 ± 2.09 , 71.2 ± 6.0 , 60.7 ± 8.8 , and 45.5 ± 5.5 % after incubating with 40 $\mu\text{g/mL}$ GO-0, GO-10, GO-30, GO-50, GO-120, and GO-240 aqueous suspensions, respectively. GO samples with larger lateral dimensions show stronger antibacterial activity than smaller ones.

Figure 7-4 The loss of viability of *E. coli* cells (5 mL of 10^6 to 10^7 CFU/mL) after incubating with GO dispersions (5 mL of 80 $\mu\text{g/mL}$) for 2 h with 250 rpm shaking speed at 37 °C.

Previous study in Chapter 6 shows that membrane and oxidative stress play important roles in the biological interactions between graphene-based nanomaterials and bacterial cells. Graphene-based nanomaterials may deposit on cells, which could disrupt and damage cell membranes, resulting in cell death. They may also induce oxidative stress on bacteria. In general, well dispersed GO sheets have more chances to interact with bacterial cells in aqueous suspensions compared with aggregated reduced GO. Conductive graphene or reduced GO would mediate more intensive oxidative stress compared with insulating GO, if they are in contact with cells. However, in the current study, aggregation or dispersion of GO sheets unlikely contributes to the antibacterial activity difference shown in Figure 7-4, because GO

sheets with various lateral sizes are all well dispersed in their mixtures with *E. coli* cells. Figure 7-5 shows clear suspensions are obtained after mixing GO sheets with *E. coli* cells. After 2 h incubation, the suspensions of GO and *E. coli* mixture remain transparent. No significant aggregation or solid particle precipitation were observed during the incubation for all tested GO samples.

Figure 7-5 Photographs of GO and *E. coli* cell mixture suspensions: (A) immediately after mixing, and (B) after incubation for 2 h. Each tube contains GO with various lateral sizes. The concentration of GO is 40 $\mu\text{g/mL}$.

Another possible reason for the observed different antibacterial activities among GO samples could be the different oxidation capacities owned by GO sheets with different lateral sizes. They might induce oxidative stress at different levels on *E. coli* cells. In order to evaluate this hypothesis, the oxidation capacity of different GO samples toward glutathione (GSH) was compared. GSH is a tripeptide with thiol groups serving as a redox state mediator in bacteria. Thiol groups ($-\text{SH}$) in GSH can be oxidized by carbon nanomaterials to disulfide bond ($-\text{S}-\text{S}-$), which converts GSH to glutathione disulfide. The Ellman's assay was used to

quantify the concentration of thiol groups in GSH. GSH (0.4 mM) is oxidized *in vitro* by GO suspensions (40 $\mu\text{g/mL}$). Bicarbonate buffer (50 mM at pH=8.6) without GO was used as a control. Figure 7-6 shows that about 22 % of GSH are oxidized after exposure to GO. The loss of GSH shows no significant difference among GO samples with different lateral sizes, suggesting they have similar oxidation capacity.

Figure 7-6 Oxidation of glutathione by GO sheets with various lateral sizes. Loss of glutathione (0.4 mM) after *in vitro* incubation with 40 $\mu\text{g/mL}$ of GO suspensions for 2 h. The bicarbonate buffer (50 mM at pH = 8.6) without GO was used as a control.

7.2.3 Time and concentration dependent antibacterial activity

In order to find out why GO sheets with different lateral dimensions show different antibacterial activity, we examined the time-dependent interaction between GO samples and cells. *E. coli* cells were incubated with GO-0 or GO-240 dispersions (40 $\mu\text{g/mL}$) for different time up to 4 h, before cell viability testes were carried out by the colony counting method. The loss of cell viability after different incubation periods is showed in Figure 7-7A. It increases with the extension incubation time for both large (GO-0) and small (GO-240) GO sheets.

Figure 7-7 Time-dependent antibacterial activities of GO-0 and GO-240 dispersions. (A) 5 mL of GO-0 or GO-240 (80 $\mu\text{g/mL}$) was incubated with *E. coli* cells (10^6 to 10^7 CFU/mL, 5 mL) for 240 min (the concentration of GO in mixtures is 40 $\mu\text{g/mL}$). The loss of viability was measured at 0, 30, 60, 120 and 240 min, respectively. Deionized water without GO samples was used as control. (B) The time dependent *E. coli* cell inactivation rates after incubated with GO-0 and GO-240 dispersions, which are extracted from Figure 7-7A with the unit of $d(\%)/d(\text{min})$.

Figure 7-8 (A) Concentration-dependent antibacterial activities of GO-0 and GO-240 dispersions. 5 mL of GO-0 or GO-240 dispersion (at 10, 20, 40, 80, and 160 $\mu\text{g/mL}$) was incubated with *E. coli* cells (10^6 to 10^7 CFU/mL, 5 mL) for 2 h. (B) The dependence of *E. coli* cell inactivation rates on GO concentration, which are extracted from Figure 7-8A with the unit of $d(\%)/d(\mu\text{g/mL})$.

However, they change in different trends. For large size sheets, the loss of *E. coli* cell viability increases sharply from 38.7 ± 4.8 % after 30 min to $89.4 \pm 3.3\%$ after 1 h of

incubation. It only increases slightly to 97.7 ± 1.0 % after 2 h and 99.3 ± 0.6 % after 4 h. In contrast, the loss of *E. coli* cell viability for small size GO sheets increases steadily. It is 18.5 ± 3.0 % after 30 min and 33.1 ± 5.1 %, 45.5 ± 5.6 % and 56.2 ± 5.1 % after 1, 2, and 4 h of incubation, respectively. Figure 7-7A further illustrates the inactivation rate of *E. coli* cells. After interacting with large size GO sheets, it is much higher in the first hour, and sharply decreases after 2 h. In contrast, after interacting with small size GO sheets, it displays a moderate decreasing trend.

Next, the concentration dependence of antibacterial activities was examined. GO-0 and GO-240 dispersions at various concentrations (5, 10, 20, 40, and 80 $\mu\text{g/mL}$) were incubated with *E. coli* cells (10^6 - 10^7 CFU/mL) for 2 h with 250 rpm shaking speed at 37 °C. Figure 7-8A shows the loss of *E. coli* cell viability increase as the concentration of GO-0 or GO-240 dispersion increases. For large GO sheets (GO-0), it is 55.7 ± 7.7 % at the concentration of 5 $\mu\text{g/mL}$, and increases to 80.3 ± 4.8 , 90.1 ± 4.9 , 97.7 ± 1.0 , and 99.4 ± 0.4 % at 10, 20, 40, and 80 $\mu\text{g/mL}$, respectively. In contrast, small GO sheets (GO-240) exhibit much weaker antibacterial activities of 7.8 ± 3.1 , 16.7 ± 5.8 , 29.4 ± 5.2 , 45.5 ± 5.5 and 52.5 ± 6.7 % at 5, 10, 20, 40, and 80 $\mu\text{g/mL}$, respectively. The dependence of *E. coli* cell inactivation rates on GO concentration is investigated (Figure 7-8B). The concentration of large GO sheets strongly influences their antibacterial activity at relative low GO concentration, and has fewer effects at high GO concentration, while the concentration of small GO sheets has less impact on their antibacterial activity.

7.2.4 Interaction between GO and *E. coli* cells

Several previous studies have proposed that nanomaterials with high aspect ratio may frustrate certain cells because cells cannot completely ingest long materials.^{33, 45-47} In order to illustrate whether this is the main cause of different antibacterial activity observed, AFM was used to monitor the interactions between GO sheets and *E. coli* cells.

Figure 7-9 AFM amplitude and 3D images of *E. coli* cells after incubation with GO sheets. (A, B) *E. coli* incubation with deionized water for 2 h, (C, D) *E. coli* incubation with the 40 $\mu\text{g/mL}$ GO-0 suspension for 2 h, (E, F) *E. coli* after incubation with the 40 $\mu\text{g/mL}$ GO-240 suspension for 2 h. The scale bars are 1 μm

Figure 7-9 shows AFM images of *E. coli* cells after incubating with large GO (GO-0) and small GO (GO-240) sheets for 2 h. As a control, the cell in deionized water without GO sheets maintains its membrane structure integrity after 2 h incubation (Figure 7-9A and 7-9B). *E. coli* cells were totally wrapped by large GO sheets (Figure 7-9C and 7-9D). We didn't observe any large GO sheets which are partially uptaken by cells. In contrast, small GO sheets (Figure 7-9E and 7-9F) adhere to the surface of cells, and don't fully cover the cell surface. Some small GO sheets seem partially plunged in cell membranes. AFM surface roughness analysis also collaborates with images shown in Figure 7-9. The surface roughness of *E. coli* cells shown in Figure 7-9A, C, and E are 4.78 ± 0.82 , 2.18 ± 0.46 and 14.80 ± 3.19 nm, respectively. After incubation with large GO-0 sheets, the cell surface roughness decreases (Figure 7-9C), which can be attributed to the fact that cell surface is covered by smooth GO sheets. However, after incubation with small size GO nanosheets (GO-240), cell surface roughness significantly increases, which is credited to the adhesion of many small GO fragments. If the frustrated uptake by cells is the main cause of cell death, based on the AFM images and surface roughness, it more likely happens on small GO sheets. Smaller GO sheets (such as GO-240) are more likely to be uptaken by cells because of their small size ($0.010 \mu\text{m}^2$). However, antibacterial activity results shown in Figure 7-4 indicate the opposite trend. Although we cannot exclude the contribution from frustrated uptakes, we conclude that it is not the main cause of cell death in this study.

Monolayer graphene sheets are impermeable atomic membranes to many molecules.⁴⁸ Akhavan *et al.* proposed that graphene sheets can wrap bacteria, biologically isolating them from growth medium.⁴⁰ Thus, cells can neither consume the nutrients nor proliferate. This proposed mechanism is consistent with the findings in our current study. Although GO sheets may have higher permeability compared with graphene sheets, layers of GO sheets can surely isolate *E. coli* cells, as illustrated in Figure 7-9C, D. *E. coli* cells cannot proliferate once fully

wrapped by GO sheets, resulting in the cell viability loss observed in the followed colony counting test. It is still unclear whether the cells would be immediately killed once they are wrapped by GO sheets or over a period of time. Akhavan *et al.* reported that about one-third of the bacteria could be reactivated after 48 h inactivation of the bacteria within the aggregated graphene sheets.⁴⁰ It was attempted to release cells from the confinement of GO sheets by bath sonication as previous researchers have done,⁴⁰ and tested their viability loss again after sonication. As illustrated in Figure 7-10, bath sonication for 1 to 5 min has no effect on the cell viability loss, indicating no live cells are released from the confinement of GO sheets. However, if the sonication time is extended to 15 min, almost all cells are killed, which may be due to mechanical shear forces induced by the excessive sonication.

Figure 7-10 Loss of *E. coli* viability after sonication for different time. *E. coli* cells (5 mL of 10^6 to 10^7 CFU/mL) were incubated with GO dispersions (5 mL of $80 \mu\text{g/mL}$) for 2 h with 250 rpm shaking speed at 37°C . And then *E. coli* cells were sonicated in bath sonicator for 1, 3, 5, 10, 15 min

The different antibacterial activity observed among GO sheets with different lateral dimensions can be attributed to their different wrapping efficiency. *E. coli* cells are easier to be wrapped by larger GO sheets comparing to smaller ones during the 2 h incubation. This wrapping process mainly depends on the lateral dimension of GO sheets, because all GO sheets are well dispersed in the mixture. It should be noted that in our all tests, the number of GO sheets is far more than the number of bacterial cells even at the lowest GO concentration of 5 $\mu\text{g/mL}$. GO-0 dispersion at 5 $\mu\text{g/mL}$ may contain approximate $8.36 \times 10^7 \text{ mL}^{-1}$ large GO sheets ($>3 \mu\text{m} \times 3 \mu\text{m}$) (see the detailed calcination in the Materials and Methods Section), which is 10 times the number of *E. coli* cells at 10^6 to 10^7 mL^{-1} . A GO sheet with size larger than $9 \mu\text{m}^2$ can easily wrap an *E. coli* cells (most of *E. coli* cells is less than $3 \mu\text{m}$ in one dimension as illustrated in Figure 7-9). Once cells are wrapped, they cannot proliferate. The colony counting method measures the proliferation of cells, thus its results are directly correlated with the wrapping of cells. Cells could be eventually killed over a period of time because of (1) membrane stress, (2) oxidative stress, and (3) lack of nutrients.

7.3 Conclusion

Effects of lateral dimension of GO nanosheets on their antibacterial activity toward *E. coli* in deionized water suspension were investigated. The results showed that among the six tested GO dispersions of different sizes (GO-0: $0.753 \mu\text{m}^2$, GO-10: $0.127 \mu\text{m}^2$, GO-30: $0.065 \mu\text{m}^2$, GO-50: $0.035 \mu\text{m}^2$, GO-120: $0.013 \mu\text{m}^2$, GO-240: $0.010 \mu\text{m}^2$), large size GO showed higher antibacterial activity than smaller ones at the same concentration. AFM results reveal that size of GO affect the interaction between GO nanosheets and bacterial cells. Beside the membrane stress and oxidative stress mechanism proposed before,²⁰ the large size GO can wrap the entire bacterial cells, consequently biologically disconnected bacterial cells from environment. Finally the bacterial cells were inactivated without any chance to consume the nutrient substance and proliferate in a culture medium.⁴⁰ However, the small size GO can only adhere

to the surface of bacterial cells, which cannot effectively prevent bacterial cells from the environment. So, small size GO shows weaker antibacterial activity than larger size GO. Our experiment also reveals that antibacterial activities of large size GO (GO-0) and small size GO (GO-240) are time-dependent and concentration-dependent. This study provide a fundamental understanding of factors affecting GO-bacterial cell interactions and antibacterial activity of GO.

7.4 Reference

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Chapter 8

Summary and Future Research

CNMs have been widely and intensively investigated in the past decades. In recent years, the discovery of antibacterial activity has triggered the exploration of environmental and human health applications of CNMs, such as CNT filters for water disinfection. From a scientific point of view, the best strategy for maximizing CNMs' application potentials while minimizing their risks is to have a better insight in their antibacterial mechanism. Based on such a mechanistic understanding, synthesis, purification, and functionalization procedures can be modified to avoid the specific features of CNMs that enhance the antibacterial activities.

Antibacterial activity of SWCNTs on various bacteria was investigated using pristine SWCNTs produced from Co-MCM-41 catalyst and purified by a centrifugation-based method. High-purity SWCNT samples helped to eliminate the possible contamination caused by impurities such as MWCNTs and amorphous and graphitic carbon nanoparticles. UV–vis–NIR absorption spectra confirmed the aggregation state can be regulated by dispersing SWCNTs in different solutions. Both the colony forming count method and the live/dead viability assay demonstrated that individually dispersed SWCNTs showed a higher antibacterial activity compared to SWCNT aggregates. UV–vis absorption spectroscopy study at 260 nm and SEM images revealed that the bacterium death was related to the destruction of bacterial membrane. SWCNTs can be visualized as moving nano darts in a solution, attacking the bacteria, thereby degrading the bacterial cell integrity and causing the cell death. SWCNT antibacterial activity can be further improved by enhancing the physical contact between SWCNTs and bacterial cells through individually dispersing SWCNTs, increasing SWCNT concentration, and elevating shaking speed during incubation.

Then, various possible antibacterial mechanisms of SWCNTs also examined. Results evidenced that inhibiting cell growth and oxidative stress were not the major reasons responsible for the SWCNT antibacterial activity. Co metal residues (up to 1 $\mu\text{g/mL}$) remaining on SWCNT samples had no detrimental effects on the antibacterial activity.

Further, AFM visual demonstration of SWCNT antibacterial effects on bacterial cells was present. For both *E. coli* and *B. subtilis*, AFM images obtained on air dried samples showed that bacterial envelopes were severely damaged with leakage of intracellular contents after incubation with SWCNTs. The volume and height of bacterial cells also decreased. The roughness of bacterial surfaces increased with the incubation time, which was in agreement with the observed cell destruction. The spring constants of bacterial surfaces after exposure to SWCNTs over different time periods were measured in saline solution. The effective spring constants of *E. coli* and *B. subtilis* declined from 0.172 ± 0.044 to 0.088 ± 0.036 N/m and 0.102 ± 0.036 to 0.034 ± 0.025 N/m, respectively, after incubation with SWCNTs for 120 min. The decrement of spring constants can be associated with the destruction of cell envelope and the release of cytoplasm. Physical piercings on bacterial cells by the 2 nm AFM tip can result in significant damages when the applied force was higher than 10 nN. The collision force between an individual nanotube and a bacterial cell during incubation is much weaker as compared to the tested force under piercing condition. Moreover, SWCNT network structures were found on *E. coli* surface, further implying that bacterial death is related to the accumulation effect of large amount of nanotubes.

Next, the antibacterial mechanism of graphene-based materials (Gt, GtO, GO and rGO) toward *E. coli* was investigated. Colony counting method results show that GO has the highest antibacterial activities, followed by rGO, Gt, and GtO under the same dispersion concentration. Their antibacterial activities are time and concentration dependent. Most of bacterial inactivation happens in the first hour of incubation, and cell death rate increases continuously

with the increase of material concentration. The bacterial cytotoxicity may be attributed to both membrane and oxidative stress. A three-step antibacterial mechanism is applicable to graphene-based materials. In general, graphene materials, which contain higher density of functional groups, and are smaller in size, have more chances to interact with bacterial cells, resulting in cell deposition. By direct contact, graphene nanosheets can induce membrane stress by disrupting and damaging cell membranes, leading to cell death. The XTT tests show that no superoxide anions ($O_2^{\bullet-}$) are produced by Gt, GtO, GO and rGO. On the other hand, they display strong time and concentration dependent oxidization capacity toward GSH. The oxidation of GSH suggests that these graphene-based materials are capable of inducing superoxide anion ROS- independent oxidative stress on bacterial cells. If they are in direct contact with cells, conductive rGO and Gt would mediate more intense oxidative stress compared with insulating GO and GtO.

Last, Effects of lateral dimension of GO nanosheets on their antibacterial activity toward *E. coli* in deionized water suspension were investigated. Large size GO showed higher antibacterial activity than smaller ones at the same concentration. AFM results reveal that lateral dimension of GO affect the interaction between GO nanosheets and bacterial cells. Large size GO can wrap the entire bacterial cells, consequently biologically disconnected bacterial cells from environment. Finally the bacterial cells were inactivated without any chance to consume the nutrient substance and proliferate in a culture medium. However, the small size GO can only adhere to the surface of bacterial cells, which cannot effectively prevent bacterial cells from the environment. So, small size GO shows weaker antibacterial activity than larger size GO. In all, cells could be eventually killed over a period of time because of (1) membrane stress, (2) oxidative stress, and (3) lack of nutrients.

Work in this thesis successfully makes two achievements: understanding (1) the antibacterial mechanisms of CNMs (SWCNTs and graphene based materials), and (2)

influence of various factors on antibacterial activity of CNMs, such as concentration and incubation time.

The toxicity of CNMs is an important issue to be carefully studied if any of their environmental and biomedical applications are to be realized. Until now, the toxicity of CNMs has been studied by a number of research groups. The reported results suggested that the physicochemical properties of CNMs can greatly influence their toxicity (see 1.4 Toxicity of Carbon Nanomaterials), although the detailed toxicity mechanism is still missing. A series of experiments should be carried out to study the effects of different physicochemical properties of CNMs on their toxicity in vitro. Furthermore, the in vivo biodistribution of CNMs should also be studied to evaluate the influence of CNMs on human health. If those toxicity studies are successful, we may be able to develop CNMs with optimized structures which can maximize their antibacterial activity while minimizing their toxicity on human health. Based on these findings, the antibacterial devices (such as filter for water disinfection) of CNMs can be eventually realized.

Besides the toxicity issue, in future work, the following studies are proposed.

- (1) Antibacterial effects of CNMs on various bacterial species can be evaluated in the next step. Previous studies have shown that CNMs exhibit different antibacterial activity towards different bacterial models. It is necessary to understand which bacterium is sensitive to CNMs before CNMs can be applied in various industries.
- (2) Although CNMs showed strong antibacterial activity toward some bacteria, the antibacterial effects are not ideal for some applications. Novel methods could be developed to further enhance their antibacterial activity, such as synthesis of targeted CNM composites. First, SWCNTs can be functionalized with chemical groups or molecules which may exhibit some specificity toward bacteria, but not to human cells. Then, various functionalized SWCNTs and CNM composites are screened to determine

the optimum compositions which have strong antibacterial activity together with weak toxicity. Further, research will be carried out to the similarity among these samples. Last, novel synthesis methods can be optimized to further enhance the antibacterial activity of CNMs while minimizing their toxicity

- (3) Further understanding of antibacterial mechanism of CNMs is still needed. Heterogeneous nature of CNMs includes many aspects, such as diameter, length, chirality, and functional groups. More research should be carried out to determine how these different properties influence their antibacterial activities. For example, SWCNTs of different diameters, such as 0.6, 1.0 and 1.4 nm, can be prepared. The antibacterial activities of these samples can be compared to understand the influence of SWCNT diameter.

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Appendix

List of related publications

- [1] **Liu Shaobin**, Wei Li, Hao Lin, Fang Ning, Matthew Wook Chang, Xu Rong, Yang Yanhui and Chen Yuan; *Sharper and faster “nano darts” kill more bacteria: a study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube*, ACS Nano, 2009, 3(12), 3891–3902
- [2] **Liu Shaobin**, Ng Andrew Keong, Xu Rong, WeiJun, Tan Cher Ming, Yang Yanhui, and Chen Yuan; *Antibacterial action of dispersed single-walled carbon nanotubes on Escherichia coli and Bacillus subtilis investigated by atomic force microscopy*, Nanoscale, 2010, 2, 2744–2750.
- [3] **Liu Shaobin**, Zeng Tingying, Hofmann Mario, Burcombe Ehdi, Wei Jun, Jiang Rongrong, Kong Jing, Chen Yuan; *Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide and Reduced Graphene Oxide: Membrane and Oxidative Stress*, ACS Nano, 2011, 5(9), 6971–6980
- [4] **Liu Shaobin**, Hu Ming, Zeng, Tingying, Wu Ran, Jiang Rongrong, Wei Jun, Wang Liang, Kong Jing, and Chen Yuan; *Lateral Dimension-Dependent Antibacterial Activity of Graphene Oxide Sheets*, Langmuir, 2012, 28, 12364–12372
- [5] Chen Fuming, **Liu Shaobin**, Shen Jianmin, Wei Li, Liu Andong, Chan-Park Mary, Chen Yuan; *Ethanol-assisted Graphene Oxide-based Thin Film Formation at Pentane–Water Interface*, Langmuir, 2011, 27, 9174–9181