8-2016

Investigation of CNT-induced Escherichia coli Lysis and Protein Release

Abdollah Mosleh
University of Arkansas, Fayetteville

Investigation of CNT-induced *Escherichia coli* Lysis and Protein Release

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Microelectronics-Photonics

by

Abdollah Mosleh
Shahid Bahonar University of Kerman
Bachelor of Science in Materials Science and Engineering, 2012
Tarbiat Modares University
Master of Science in Nanotechnology/Nanomaterials Engineering, 2014

August 2016
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Dr. Robert Beitle Jr. Thesis Director	Dr. Jin-Woo Kim Committee Member
Dr. Ralph Henry Committee Member	Dr. Rick Wise Ex-Officio Member

The following signatories attest that all software used in this thesis was legally licensed for use by Abdollah Mosleh for research purposes and publication.

Abdollah Mosleh, Student	Dr. Robert Beitle Jr., Thesis Director

This thesis was submitted to http://www.turnitin.com for plagiarism review by the TurnItIn company's software. The signatories have examined the report on this thesis that was returned by TurnItIn and attest that, in their opinion, the items highlighted by the software are incidental to common usage and are not plagiarized material.

Dr. Rick Wise, Program Director	Dr. Robert Beitle Jr., Thesis Director

Abstract

This research investigated the use of carbon nanotubes (CNTs) as a treatment to increase the permeability of a bacterial cell wall. Recombinant *Escherichia coli* BL21 (DE3) containing a plasmid that expressed Green Fluorescent Protein (GFP) and β-lactamase were exposed to CNTs under various levels of agitation for different times. Fluorescence assay for GFP, optical absorbance for β-lactamase activity, and Transmission Electron Microscopy (TEM) were used to determine the amount of released protein, and visually examine the permeability enhancement of the cells, respectively. It was found that more β-lactamase was present in the culture fluid after treatment with CNTs in a dose dependent manner. Indeed, CNTs can lyse the cells up to 90% of maximum when compared to lysozyme treatment. Based on TEM, it is believed that this treatment damaged the cell walls to make *E. coli* permeable, causing periplasm proteins and enzymes to leak out into the medium. Consequently, CNTs can be used as lysis agents when it is undesirable to add an additional enzyme (lysozyme) to cause the release of intracellular proteins.

Keywords: Carbon nanotubes, Membrane damage, Cell wall permeability, *Escherichia coli*

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor, Dr. Beitle, for the continuous support of my Master's study, for his patience, motivation, and immense knowledge. His guidance helped me in the research and writing of this dissertation. I could not have imagined having a better advisor and mentor for my Master's study. His advice on research as well as on my life has been priceless. I would like to thank the rest of my thesis committee: Dr. Jin-Woo Kim, Dr. Ralph Henry, and Dr. Rick Wise, for serving as my committee members and for their insightful comments and encouragement.

Last but not the least, I would like to thank my family: my parents, my brother, Salman and my sisters, Somayyeh and Leila, for supporting me spiritually throughout writing this thesis and my life in general. Your prayer for me was what sustained me thus far. At the end I would like express appreciation to my beloved wife, Negin, who spent sleepless nights with me and was always my support in the moments when there was no one to answer my queries.

Dedication

To my parents and my wife.

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

1.1. Motivation

In this chapter, first, we will review the structure of *Escherichia coli* cell envelope. Then uptake across the outer membrane via hydrophilic and hydrophobic pathways will be reviewed. Also, different chemicals and antibiotics that can enhance the outer membrane permeability will be introduced. Then, the effect of physical damage on permeability of cell membrane and the nanoparticles that have been used to damage the cell walls will be presented. At the end, properties of carbon nanotubes (CNTs) that will be relevant for this thesis will be reviewed. Next, CNTs structure and the different methods of producing them, including the method that was used for the experiments will be illustrated. Then, functionalization of nanotubes will be examined. Lastly, the way functionalized nanotubes can be used to make cells permeable in order to extract proteins will be covered.

1.2. Structure of the *E. coli* cell envelope

The *E.coli* cell (Figure 1) is composed of an inner and an outer membrane which are separated by peptidoglycan layer. Also, it has an aqueous phase between the outer membrane and the inner membrane which is known as periplasm. Cytoplasmic membrane proteins are able to generate energy and enzymatic synthesis. They can also translocate the cell components, export the toxic byproducts and transport nutrients[1]. In the absence of a utilized transport system, the cytoplasmic membrane works as an enormous obstacle for hydrophilic molecules. On the other hand, even moderately hydrophobic molecules are able to get in or even cross the lipid bilayer at certain temperatures[2].

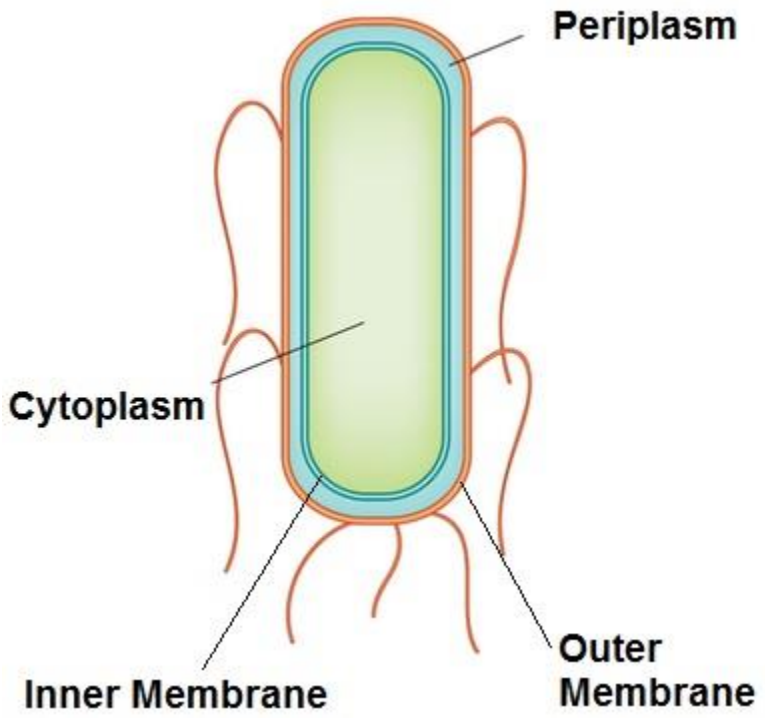


Figure 1. Schematic of *Escherichia coli* structure[3].

The periplasm is a negatively charged section which consists of polypeptides and saccharides. Many enzymes such as processing enzymes for conversion of non-transportable metabolites to transport substrates exist in the periplasm. The peptidoglycan of the periplasm has barrier function and is vital for the cell.

The inner layer contains phospholipids while the outer membrane contains lipopolysaccharide (LPS)[4]. Because of the LPS which carries a net negative charge, gram-negative cells have negative surface charge[5]. They can be treated with ethylenediamine tetra acetate (EDTA) in order to loose divalent cations and subsequent disturbance of the outer membrane.

The combination of divalent cation cross-bridging of LPS and negative charge provides gram-negative cells with many of properties, such as resistance to hydrophobic antibiotics, bile salts, detergents, proteases, lipases, and lysozyme[6]. Some so-called "major" proteins exist in

the outer membrane. They may have important roles in their anchor of the outer membrane and peptidoglycan.

1.3. Uptake across the outer membrane

1.3.1. Hydrophilic pathway

Using porins(the water-filled channels of proteins), hydrophilic compounds can pass the outer membrane [6]. Organisms have different exclusion limit of porin pores of membranes and area of the channels. For instance, *E. coli* can pass the trisaccharides [6] or tetrapeptides [7], whereas *Pseudomonas aeruginosa* porins only allows those saccharides with molecular weights of greater than 6000 to pass. The molecular properties of the porin channel including channel size and ionic selectivity, and also the total number of available channels per cell, determine the rate of uptake of variable hydrophilic compounds [4].

1.3.2. Hydrophobic pathway

Many lipid bilayer membranes allow the amphiphilic molecules and hydrophobic compounds can pass across the many lipid membranes[8], but the outer membranes of *E. coli* cells are not permeable to the hydrophobic or amphiphilic molecules.

It would seem that the hydrophobic uptake pathway is very inefficient in *E. coli* as a gram-negative bacterium compared to some gram-positive bacteria [9]. Furthermore, in *Salmonella* with heptose-deficient LPS, divalent cations can block the pathway [9]. It can be suggested that hydrophobic uptake pathway in most gram-negative bacteria such as *E. coli* and *Salmonella* does not exist due to high-surface negative charge and the effects of divalent cation bridging of LPS molecules.

1.4. Enhancement of outer membrane permeability by chemicals and antibiotics

1.4.1. Ethylenediamine tetra acetate (EDTA)

In order to enhance the permeability of outer membrane of many gram-negative bacteria especially *E. coli*, EDTA can be used due to its strong divalent cation-chelating function [10]. The mechanism of making holes on outer membrane is unknown yet. It seems that EDTA causes the loss of 30% to 67% of substantial amounts of LPS for *E. coli*. After EDTA treatment, the outer membrane seems to remain continuous and there no noticeable holes in the outer membrane [10].

The number of particles and holes can be decreased through freeze crack and it will be the main change in outer-membrane substructure. Ultracentrifugation can separate the released LPS into two fractions [11]. The first fraction which is called F1 contains linked protein and lipid and the second one, F2, is apparently pure LPS. F2 may have different sugar composition than bulk LPS and because of the release of F2, the outer membrane will become permeable. In *E. coli*, during EDTA treatment, a larger amount of protein is secreted[10].

1.4.2. Polymyxins

The major cause of rodlike pits of the membranes of gram-negative bacteria is Polymyxin[12]. The polycationic part of polymyxin is able to displace divalent cations. On the other hand, using chelation, EDTA is able to remove divalent cations. This is the main difference between polymyxin and EDTA that it seems to release little LPS into the medium. Moreover, the outer membrane of polymyxin-treated cells seems to be unstable because they can secrete high amounts of proteins. On the other hand, EDTA-treated cells due to their thicker membrane, can save more periplasmic proteins inside the cells. The hydrophobic ends of polymyxin can

facilitate the release of periplasmic proteins. This can destabilize bilayers by interacting with both the polar and nonpolar regions of lipids[13].

1.4.3. Aminoglycosides

Polycationic aminoglycoside antibiotics (e.g., streptomycin, gentamicin) are able to permeabilize the outer membranes of the organisms by interacting with divalent-cation binding sites. A great majority of evidence suggest that aminoglycosides can act like EDTA and polymyxin at the same site [14].

1.5. Protein release of the outer membrane

Some gram-negative bacteria such as *E. coli* have few proteins that are released out of the cell. Some organisms can naturally release proteins across their outer membrane. *P. aeruginosa* of phospholipase C has unique secretion among all organisms. The secretion of this organism is not accompanied by the release of phosphate-binding protein or periplasmic β-lactamase [15]. Furthermore, during phospholipase release, no β-lactam nitrocefin can secrete across the outer membrane[15]. On the other hand, after phosphate limitation, the amounts of phospholipase C and alkaline phosphatase enhance in the periplasm.

1.5.1. Physical damage of outer membrane

The cells also would be permeable by mechanical damage treatments. For instance, *E. coli* cells exposed to their reagent grade water become susceptible to deoxycholate and lysozyme[16]. According to the electron microscopic examination of these cells, outer membrane blebbing had occurred. Likewise, *E. coli* outer membrane could be more permeable to lysozyme through aerosolization. Mg^{2+} can reverse the sensitivity of aerosolized *E. coli* to

lysozyme. Also, it is important to know that energy metabolism should be applied to fully repair the outer membranes. Similarly, freeze-thawing *E. coli* strains leads to secrete of cyclic phosphodiesterase as well as improved vulnerability to dyes, enzymes, and detergents[17].

Recently, nanostructured materials have been used as an agent to increase the permeability of the cells through physical damage of outer membrane. The most applicable nanostructured materials are silver and nanostructured materials based on silver, gold nanoparticles, aluminum nanoparticles, zinc oxide nanoparticles, and carbon nanotubes.

Silver nanoparticles

Silver nanoparticles have high tendency to react with sulfur and phosphorus. Silver nanoparticles can damage the cell membrane because of the high amount of sulfur-containing proteins on the cell walls. Silver nanoparticles with small diameter (< 20 nm) can attach to these proteins of cell walls because they have high particle penetration and greater antibacterial activity due to their larger surface-to-volume ratios[18]. Consequently, they can make the membrane permeable, and finally kill the bacteria[19]. Low concentration of Ag^{+} ions can inhibit respiratory chain enzymes and enhance the membrane permeability[20].

The effect of silver nanoparticles on the cell morphology of *E. coli* has been shown in Figure 2 and Figure 3 using TEM. In Figure 2, the structure of the untreated *E. coli* cells has been depicted. As shown, the cells are intact and DNA molecules are randomly presented in the cells. According to Figure 3, the cytoplasmic membrane separates from cell walls. The inhibitory activity of silver ions is high because of the thick layer of the peptidoglycan in *E. coli* cell wall. Figure 3(a) gives an overview of the structure of the silver treated *E. coli* cells. The condensed form of DNA shown in Figure 3(b) and Figure 3(c)–(f) are enlarged magnifications of cells. As shown in Figure 3(e) and (f), there is no cell wall in that cell and just cytoplasm and many small

particles are in a region which may be the last life stage of the Ag^+ ion treated *E. coli*[20]. Silver ions interacted with nucleic acids and formed the particles containing silver ions and sulfur ions. These particles have been suggested to be the main cause of cell damage[21].

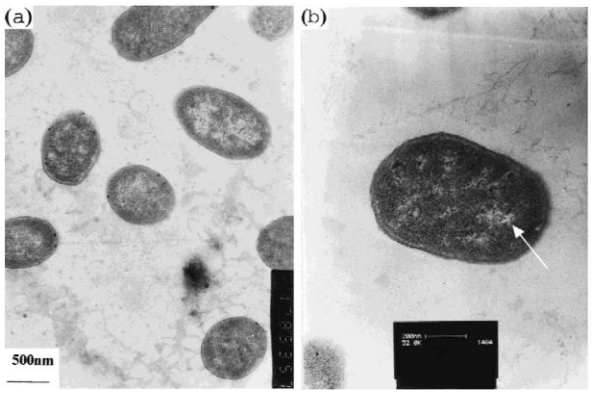


Figure 2. Structure of the intact *E. coli* cells.[20]

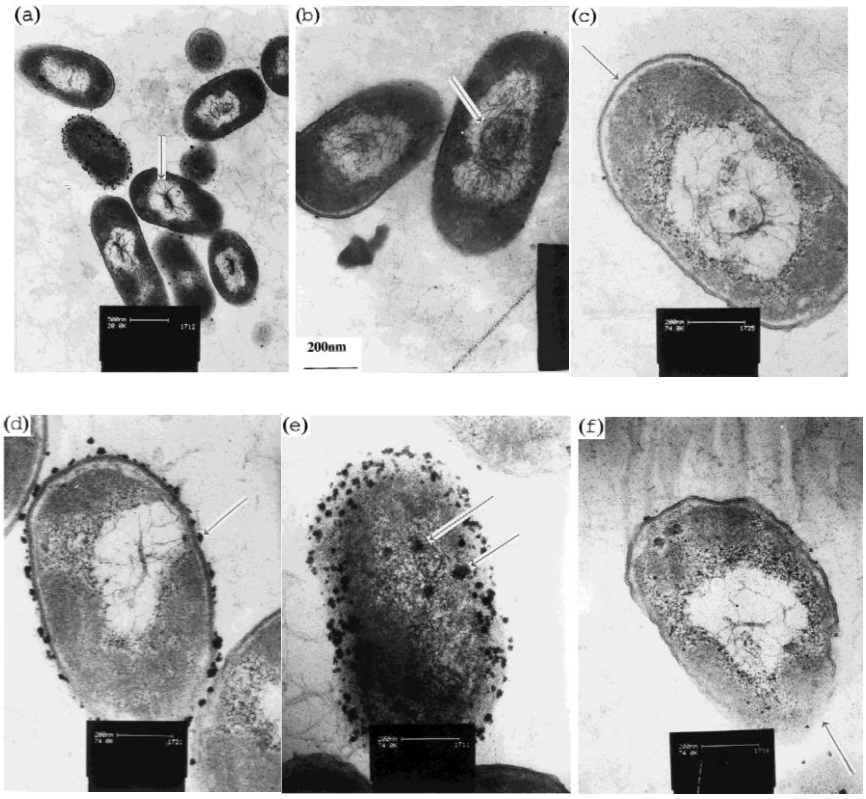


Figure 3. Structure of the silver treated *E. coli* cells. (a) A significant bright region in the center of the cell. (b) Compressed form of DNA. (c) A remarkable space between the cytoplasm and the cell membrane. (d) Dark particles containing silver ions and sulfur ions around the cell. (e) A cell contained many dark particles. (f) The damaged cell membrane[20].

Gold nanoparticles

Gold nanoparticles have high antimicrobial efficacy when they have been mixed with antibiotics[22]. The antibacterial effect of aminoglycosidic antibiotics on a range of gram-negative bacteria especially *E. coli* could increase by mixing with gold nanoparticles. Compared to Cefaclor, which is a second-generation β-lactam antibiotic, and to gold nanoparticles alone, a mixture of Cefaclor and gold nanoparticles have effective antimicrobial activity on *E. coli*[23]. Cefaclor can damage the cell walls by making pores and prevents the production of the peptidoglycan layer. Also, the gold nanoparticles can make the cell wall permeable by generating holes and cause cell death[24].

Alumina nanoparticles

The alumina nanoparticles have surface positive charge at pH 7. The adhesion of nanoparticles on the bacterial cell walls may occur due to electrostatic interaction between the *E. coli* cells which have negative charge and the alumina nanoparticles [25]. By increasing the concentration of the particles in the medium, the adhesion has been increased. Increasing the adhesion led to a negative effect on growth. Bacterial adhesion may be caused by the electrostatic interaction between bacteria and alumina nanoparticles surface. Generation of reactive oxygen species (ROS) is the main cause of cell wall damage and consequently cell death [26].

Zinc oxide nanoparticles

Zinc oxide nanoparticles have been found to be extremely toxic among antimicrobial metal oxides which have been studied so far. This is the active ingredient in most sunscreen, demonstrating low toxicity to humans in contrast to their potent antimicrobial efficacy [27]. Also, zinc oxide nanoparticles display negligible effect on human cells and have selective

toxicity to bacteria [28]. Nanostructured zinc oxide particles with average diameter of 12 nm can disintegrate the cell membrane and enhance the membrane permeability and consequently inhibit the growth of *E. coli* [29].

The antibacterial activity of zinc oxide nanoparticles can be illustrated by many mechanisms. One of the methods for the stoppage of growth of bacteria is the production of hydrogen peroxide from zinc oxide nanoparticles surface [30]. It is assumed that the number of zinc oxide nanoparticles per unit volume increases with decreasing particle size. Subsequently, the surface area increases which leads to an increase in the production of hydrogen peroxide. Also, releasing Zn2+ ions may be another mechanism for zinc oxide antibacterial activity which can damage the cell membrane [28].

Up to this point, several methods have been discussed to increase the permeability of cells. The most appropriate method is the one in which the efficiency is relatively high and it does not make any trouble for the following purification processes. Lysozyme could lyse the cells by damaging the cell walls and has the highest efficiency among the methods. However, the presence of lysozyme may require separation of it from the product, which is resource and time consuming and could be undesired. According to the aforementioned cases, CNTs have been proposed as an appropriate method to damage the cell walls and enhance the permeability of the cells without using any enzyme which might be less resource and time consuming in comparison with lysozyme. CNTs and their properties, structure and growth, functionalization and toxicity will be reviewed in the following section.

1.6. Carbon nanotube

Carbon nanotubes are nanoscale hollow cylinders of carbon which basically consist of "rolled" sheets of graphene (Figure 4). Carbon nanotubes can be found in single-walled (SWCNT) and multi-walled carbon nanotube (MWCNT) types and can be produced with very large aspect ratios. These two different types of CNTs (SWCNT and MWCNT) may vary in length, in some cases being up to hundreds of micrometers[31]. The carbon nanotubes may differ in shape, physical characteristics, dimension, chemical composition, surface coatings, or surface functionalization. CNTs usually contain residual metal catalysts. On the other hand, purified CNTs are the ones which have minimum amount of residual metal catalysts. Single-walled carbon nanotubes (SWCNTs) have a diameter of approximately 1-2 nm because they consist of a single rolled graphene sheet. MWCNTs, depending on the number of rolled sheets of graphene, have a diameter in the range of 2-100 nm. Many single-walled tubes arranged one inside the other in order to make a MWCNT.

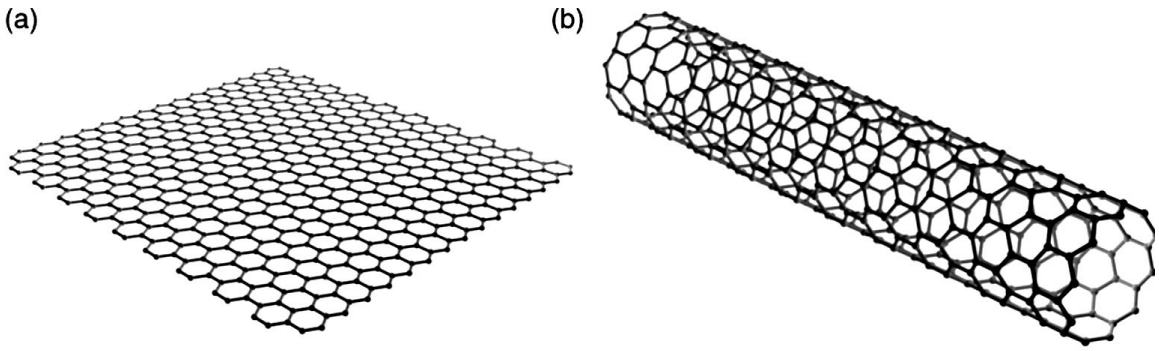


Figure 4. Schematic of graphene sheet and single-walled carbon nanotube (SWCNT). (a) Carbon atoms that covalently bonded in a honeycomb lattice formed the graphene sheet. (b) An equivalent single-walled carbon to a sheet of graphene. This sheet has been rolled into a cylinder shape[31].

In order to synthesize CNT, a carbon source and an energy source is required. Several methods exist that are used to produce or manufacture CNTs. A metal catalyst can play an

important role in enhancing sample homogeneity and yield, and in decreasing the synthesis temperature, based on synthesis methods materials. Also, catalysts characteristics (e.g. shape, and crystallinity) can play an important role in determination of the diameter of the tube. In order to make the product as pure as possible, sometimes post-synthesis treatments are used to remove residual catalyst which may exist after synthesis. Also, some purification techniques are used to remove the catalyst. Selective oxidation of the amorphous carbon is the main goal of the typical purification technique. In this technique, first, at a controlled temperature, the amorphous carbon is washed and then sonicated the in an acid (HCl, HNO_3, H_2SO_4) or base (NaOH) [32].

Due to their structure, high surface energy, and relatively small size, CNTs can be difficult to discrete in the bulk form, and they have a tendency to agglomerate quickly when released in the medium.

Individual carbon nanotubes have been used as test systems for one-dimensional electron theories, as photonic devices, and as nano-electromechanical resonators. The small size of carbon nanotubes also make them very good lysis agent by making a mechanical rupture to the cell walls. This application of nanotubes as lysis agents will be the focus of this thesis.

1.6.1. Nanotube structure and growth

The carbon atoms in a nanotube are covalently bonded in a honeycomb lattice, so a nanotube is equivalent to a graphene sheet that has been rolled into a cylinder, as seen in Figure 4. Carbon nanotubes are extremely stable, and they can form whenever a source of carbon is heated in the presence of catalyst particles to help initiate the structure. Multi-walled nanotubes (many concentric graphene cylinders) with many defects have been found naturally in 10,000 year-old ice core samples and in common flames[33], as well as in a 400-year-old Damascus

saber [34], but more controlled fabrication methods are used to make nanotubes for laboratory study. The first reported nanotubes were formed by arc discharge, in which graphite was heated with a large current[35]. Graphite can also be heated by shining a laser on it inside a hot (around 1000 °C) furnace; a technique known as laser ablation. In both arc discharge and laser ablation, the catalysts are metal particles — such as iron, cobalt, or nickel — that are mixed with the graphite.

Another method for growing carbon nanotubes is chemical vapor deposition (CVD) in which a hydrocarbon gas like methane or ethylene is flowed through a 700-950 °C furnace[36]. CVD is currently the best method for the growth of single-walled nanotubes with minimal defects, and the locations of the nanotubes can be controlled by first photo-lithographically patterning the substrate.

There has also been recent progress in selectively destroying only the metallic nanotubes on a sample with a plasma reaction, and in using pieces of one nanotube as a template for growth of identical nanotubes[37]. Further improvements will need to be made in these directions before individual nanotubes can be integrated into large-scale circuits and used in commercial applications.

CNTs, as synthesized, contain impurities that include amorphous carbon and catalyst. Also, as synthesized, their structure dictates a high degree of hydrophobicity, making them virtually insoluble in aqueous media. In order to purify the fabricated CNTs, different acid refluxing methods have been used. Also, different hydrophilic functions can be used to functionalize the produced CNTs and subsequently overcome the hydrophobicity issue of CNTs.

1.6.2. Purification and functionalization of carbon nanotubes

Proceeding to be used as an agent to make the cells permeable, CNTs should be soluble and purified in a physiological environment. There are several methods used to purify CNTs. A widely used method relies on sonication in a mixture of sulfuric and nitric acid which removes catalyst impurities [38]. In addition, a variety of materials have been tested as modifiers to keep CNTs in solution. They include ionic materials that can form a salt bridge, proteins, surfactants, and natural materials that include gum. Adding these modifiers may lead to an increase in the size and diameter of CNTs which may not be desired because these additives could reduce the functionality of CNTs. On the other hand, functionalization of CNTs with hydrophilic functions can be more applicable. Coincidentally, as the mixture is treated in this manner, the solubility of the tubes in water increases due to introduction of –COOH at defects in the CNT. Other materials can be chemically or physically added to the CNTs to improve both solubility and biocompatibility.

Covalent Functionalization

Covalent modification is preferred when it is desired for the chemical moiety to be strongly associated with the CNTs. The covalent binding depends on the linking of molecules which are chemically reactive onto their inert sp^2 carbon structure. Covalent modification has been accomplished with the use of oligomers, biologicals (e.g. cholesterol), and long chain organics (e.g. PEG)[39]. Acid oxidation introduces defects on the side and end of the CNT. These cause decoration of tips and sidewalls of the CNTs with oxygenated functionalities such as carboxylic, carbonyl, and hydroxyl groups. Modification via surface –COOH is very attractive because there is a plethora of chemical crosslinking agents that can use this feature [39]. These include carbodiimides, esters, and thionyl. Subsequently, they covalently attached to various

types of biomolecules. Other methods include treatment with hydrogen, fluorine, and azomethine [39].

Noncovalent Functionalization

sp^2 bonding is preserved in noncovalent functionalization in comparison with covalent functionalization. Therefore, it conserves the functional properties and original structure of CNTs more efficiently than covalent approaches. Uses of noncovalent techniques are vastly simpler in comparison to covalent methods, and include a combination of mixing, sonication, filtration, and centrifugation. Nonetheless, noncovalent bonds are susceptible environmental factors such as salt concentration and pH[40].

1.6.3. Toxicity of carbon nanotubes

The first and most important requirement of every experiment is safety. Over past decades, many research groups have focused their research on side effects of CNTs. They have shown that CNTs may be toxic based on their type (SWCNT and MWCNT) and functionalization approaches. The results of scientific research and cell culture experiment showed that functionalized carbon nanotubes have no toxicity [40]. On the other hand, after inhalation into the lungs, unfunctionalized carbon nanotubes were depicted to be poisonous to mice [41]. Using carbon nanotubes for biomedical applications is currently a controversial issue for both the public and research communities. Clarification of the carbon nanotubes toxicity issue is very serious and urgent. It is proved that geometry and surface functionalization of carbon nanotubes may affect the level of their toxicity. It is reported that biocompatible surface coated carbon nanotubes that are well-functionalized are nontoxic *in vitro* to cells[41]. Also it is

reported that carbon nanotubes which have been functionalized did not show any toxicity *in vivo* in mice [41].

1.7. Scope of research

In this research, the effect of shear and CNT exposure on bacterial cultures used to make recombinant DNA proteins was investigated. To explain more explicitly, the following points will be discussed:

1. By increasing the amount of CNTs, the amount of release will increase.
2. By increasing the agitation rate, more materials will be released to the medium.
3. CNTs will damage the cell walls and cause the leakage of peripalsmic materials
4. CNTs could damage the cytoplasm and may cause its material to leak out to the medium.
5. It will be shown that CNTs can lyse the cells close to the lysozyme treatment.

Chapter 2. Materials and Methods

In this chapter, all chemicals and materials that were used to accomplish the project, parameters that have been affect the procedure and finally assays will be discussed.

2.1. Materials

2.1.1. Carbon nanotubes

Carbon nanotubes used in this experiment were prepared in Dr. Jin-Woo Kim's lab. They were purchased from Carbon Nanotechnologies Inc. (Houston, TX). Acid refluxing technique using 2.6 M nitric acid was used to purify the purchased CNTs, and then purified CNTs were reconstituted in 5 mM potassium phosphate buffer (pH of 8) which contained 0.2% sodium dodecylbenzenesulfonate (NaDBS). In order to remove nondispersed CNT aggregates, the CNT solutions were also centrifuged at 14000 G at 25 °C for 40 min. At the end, the supernatant contained CNTs that were well-dispersed, shortened, and oxidized at the tips. The average length (Table I) of shortened SWNT was 186 nm with a standard deviation of 47 nm and the average diameter of 1.7 nm with a standard deviation of 0.14 nm. The initial concentration of $1 mg \cdot mL^{-1}$ for the SWCNTs was used. Finally, the processed SWNTs were reconstituted in water[42].

Table I. Specifications of used carbon nanotubes in this research.

Specification	Value
Type	Single walled
Average length	186 ± 47 nm
Average diameter	1.7 ± 0.14 nm
Purity	99.99%

2.1.2. Cells

E. coli BL21 (DE3) bacteria containing a plasmid that expresses green fluorescent protein (GFP) and β-lactamase were used in this experiment. Cells were grown in LB medium (5 $g \cdot L^{-1}$ yeast extract, 10 $g \cdot L^{-1}$ NaCl, 10 $g \cdot L^{-1}$ Bacto-trypton, and DI water and PH = 7.5) at 37 °C via fed batch fermentation and they contained approximately 50% saturated dissolved oxygen.

2.2. Design of experiment

In order to investigate the use of CNTs that artificially cause a bacterium to release a protein, this experiment was designed. There were two assumptions for release of protein in this context. One was that CNTs might cause the periplasm materials and proteins to leak out to the medium. The second one was that CNTs may destroy the cell and mechanically cause not only the preplasmic but the cytoplasmic materials to leak to the medium. Tris base was used as a buffer in this experiment to prevent any unwanted increase or decrease of pH in the system. $2g \cdot L^{-1}$ of recombinant *Escherichia coli* BL21 (DE3) bacteria was used to study the amount of protein released using a fluorometer. Single-walled carbon nanotubes functionalized with –COOH function were used to enhance the release of protein. Hydrophilic tips of these functionalized CNTs could have caused mechanical rupture and consequently damaged the cell walls. Agitation rate of reactor and amount of CNTs were the effective parameters in this experiment.

2.3. Sample preparation

Figure 5 is a schematic of the experimental setup. The reactor consisted of a motor, a motor controller, a glass flask, a metallic lid, a stirrer, a syringe, and a belt which transferred the

mechanical energy from the gearwheel of the motor to the stirrer. One gram of cell pellet was added to 500 ml of 25 mM Tris base with pH = 7.5 in the glass flask. After mixing for 20 minutes at 200 rpm, different amount of SWCNTs were added to the flask. Samples were taken from the reactor using the installed syringe every 30 minutes for 2.5 hours and were stored in microtubes. The values of these parameters are listed in Table II. The samples were then centrifuged using Eppendorf minispin® plus machine (Figure 6) at relative centrifugal force (rcf) of 14000 for one minute for separation of proteins from dead cells. The effect of exposing 2 $g \cdot L^{-1}$ of *E. coli* BL21 (DE3) to SWCNTs and reactor agitation rate on protein release of cells by passage of time was then studied.

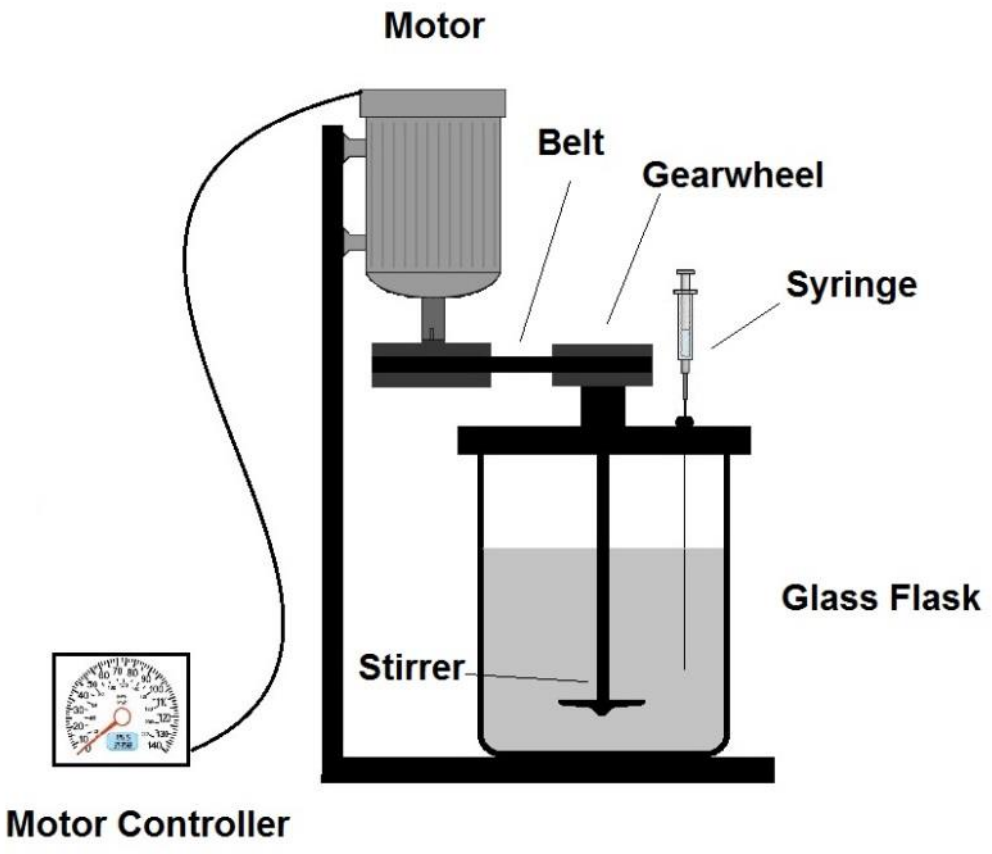


Figure 5. Schematic of experimental setup.

Table II. Values of parameters used in this research based on design of experiment.

Value	1	2	3	4	5	6	7
Time (hours)	0 (As mixed)	0.5	1	1.5	2	2.5	-
Amount of CNTs ($\mu g \cdot L^{-1}$)	0 (Control)	200	400	800	1600	8000	32000
Agitation Rate (rpm)	200	300	600	-	-	-	-

Figure 6. Eppendorf minispin® plus machine.

Eight samples were prepared and the amount of CNTs and the agitation rate for each of the samples are listed in Table III.

.

Table III. Specification of each prepared sample.

Sample Number	Agitation rate (rpm)	Amount of CNTs ($\mu g \cdot L^{-1}$)
1	200	200
2	200	400
3	300	800
4	600	800
5	300	1600
6	300	8000
7	600	8000
8	600	32000
Control	300	0
Lysozyme	300	0

2.4. Enzyme assay

2.4.1. Fluorescence assay for GFP

In order to determine the amount of released GFP, a fluorescence assay was performed. For this purpose, after centrifugation and decantation, 1 ml of sample was added to a micro cuvette. Then, using Shimadzu RF-Mini 150 Recording Fluorometer (Figure 7) at wavelength of 509 nm, fluorescence assay was carried out.

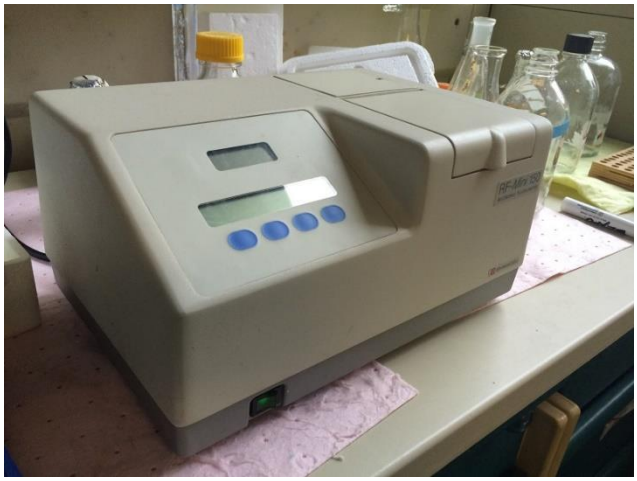


Figure 7. Shimadzu RF-Mini 150 Recording Fluorometer.

2.4.2. Optical absorbance assay for β-lactamase

Optical absorbance is a complementary assay to fluorescence. For this purpose, 0.1 M sodium phosphate with pH = 7 was used as a buffer and 0.5 $g \cdot L^{-1}$ penicillin G was added to it. Then, 20 µl of sample was added to 2 ml in a quartz cuvette. Then, the quartz cuvette was placed in Beckman Coulter DU® 800 UV/Vis Spectrophotometer (Figure 8) to measure its optical absorbance at a wavelength of 240 nm at which Penicillin G absorbs.

Figure 8. Beckman Coulter DU® 800 UV/Vis Spectrophotometer.

2.4.3. Protein gel electrophoresis

There are different types of proteins in the medium that can be distinguished and sorted in many distinct ways. One of the common methods is to sort them based on their molecular weights. Thus, protein gel electrophoresis assay was performed. In order to run this assay, 990 µl of 25 mM Tris base with pH = 7.5 was used as a buffer and 10 µl of sample was added to it in a quartz cuvette. Then, the cuvette was placed in Beckman Coulter DU® 800 UV/Vis Spectrophotometer to measure its optical absorbance at a wavelength of 280 nm at which proteins absorb. Next, the samples were concentrated 100 times before performing the electrophoresis test. 24 ml of every Sample were stained using 6 ml of 6XUREA SDSPAGEDYE dye. Every sample was placed in boiling water for five minutes in order to denature its existing proteins. Lastly, the stained samples were added to the gel. The composition of the lower and stacking gel are listed in Table IV and Table V, respectively.

Table IV. The composition of 10% SDS page lower gel.

Content	Volume (ml)
8X lower buffer	0.65
30% 0.8% Acrylamide	1.65
75% Sucrose	0.65
Deionized water	2
5% ammonium persulfate	0.05
TEMED	0.003

Table V. The composition of stacking gel.

Content	Volume (ml)
4X lower buffer	0.625
30% 0.8% Acrylamide	0.375
Deionized water	1.5
5% ammonium persulfate	0.0225
TEMED	0.011

In order to have a better idea about the molecular weights of the proteins, 3 µl of Precision Plus Protein ™ standard was added to the first and last lanes of the gel. The gel was placed in the PowerPac™ BIO-RAD electrophoresis machine (Figure 9) and the container was filled with the SDS page buffer to conduct the electricity to the gel. The electricity causes the proteins to be sorted based on their molecular weights. In order to run the test efficiently, 120 volts was applied to the prepared gel. After about 90 minutes, the proteins were sorted but they were invisible. In order to make them visible, they were developed using Comassie blue dilute acetic acid containing 20% methanol overnight. The prepared gel then was rinsed using 7.5% acetic acid containing 7.5% methanol twice for an hour and two hours, respectively, to wash any

remaining dye from the Comassie blue dilute acetic acid. It is important to note that the prepared gel is very sensitive so it was carried with the maximum care.

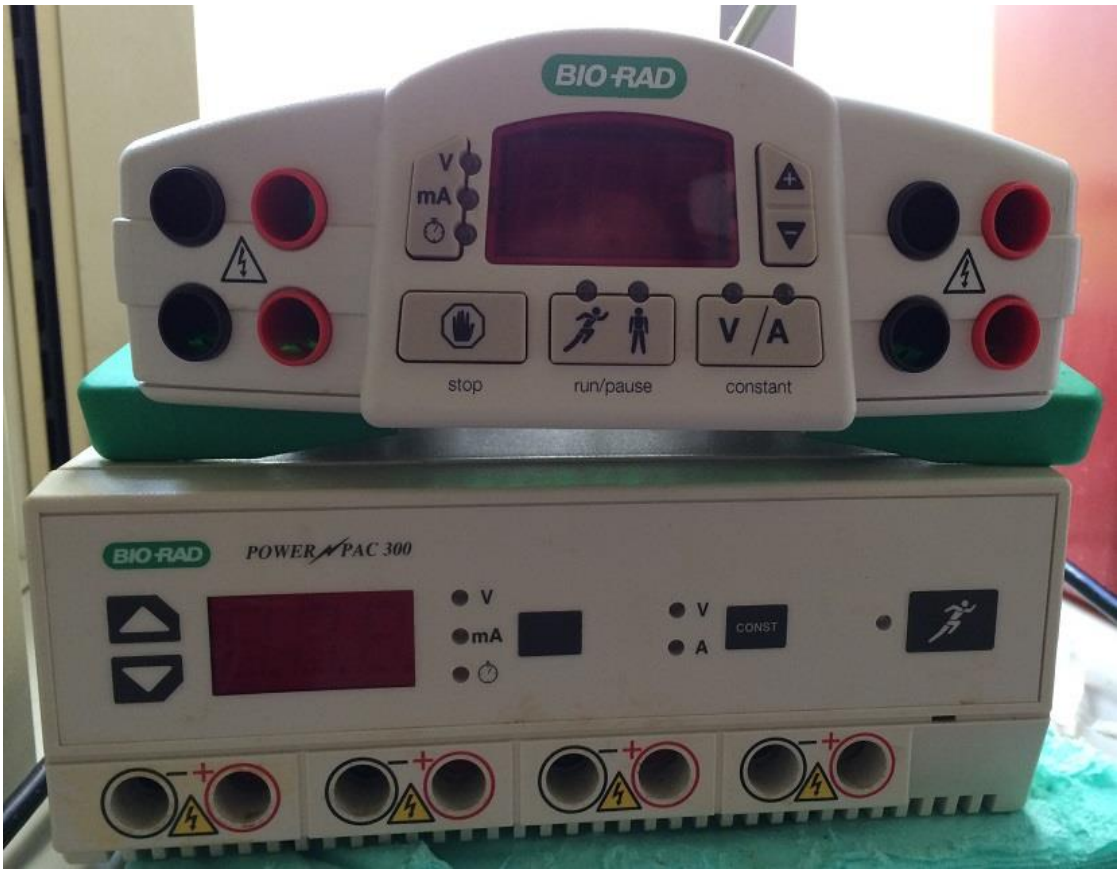


Figure 9. PowerPac™ BIO RAD protein gel electrophoresis machine.

2.4.4. Transmission Electron Microscopy (TEM)

Transmission electron microscopy images were used to prove the claim that CNTs are able to enhance the permeability of the cells. For this purpose, a drop of each cell pellet was placed on a pink dental wax-nu-base plate wax using borosilicate glass disposable pipet. Using sterilized forceps, a 300 mesh carbon-coated copper grid was soaked in each drop to take the

sample and then it was placed on filter paper in a glass petri dish for 10 minutes to dry out. Then, grids were placed in a JEOL-1011 electron microscope to take TEM images of the samples.

Chapter 3. Results and Discussion

In this chapter, fluorescence results will first be presented and discussed. The amount of release GFP could be determined using fluorescence assay. Second, in order to determine the amount of released protein to the medium, optical absorbance assay results for β-lactamase will be discussed. At the end, the results of transparent electron microscopy as evidence for the ability of CNTs to enhance the permeability of the cells will be provided.

3.1. Fluorescence assay

The first assay that was used in this research was fluorescence assay which is a pretty easy to measure, cost-saving, and routine test. Because the used cell pellets in this experiment contained GFP, fluorescence assay was carried out to measure the effect of different amount of CNTs and agitation rates on the amount of released proteins. The fluorescence values of samples are listed in Table VI. In this assay, 25 mM Tris (pH = 7.5) was used as a reference. In order to determine the related fluorescence of released GFP, fluorescence values of the same concentration of CNTs for all the samples were measured. According to the fluorescence value at wavelength of 509 nm, CNTs had no fluorescence at those concentrations which means that the fluorescence values were related only to the amount of released GFP.

As listed in the table for sample 1, which contained 200 $\mu g \cdot L^{-1}$ CNTs and was prepared at agitation rate of 200 rpm, the fluorescence values decreased in the first hour and then increased to reach to 128 after 2.5 hours. However, the fluorescence value after 2.5 hours was less than the initial value for the as-mixed sample. One of the reasons for this might be related to the amount of CNTs which was not quite high enough to make the cell walls permeable.

Although the amount of CNTs was increased for sample 2, the results of fluorescence for this sample were pretty similar to sample 1.

Table VI. Fluorescence values for samples at wavelength of 509 nm.

	Fluorescence			
Time(hour)	Sample 1	Sample 2	Sample 3	Sample 4
0	143	142	141	143
0.5	112	135	160	138
1	102	116	133	118
1.5	114	122	127	138
2	112	127	130	165
2.5	128	126	128	150

For sample 3, the fluorescence value increased within 30 minutes but decreased after that and reached 128 at 2.5 hours. According to the data in Table IV, the average fluorescence value for sample 3 was 137 which again supports the hypothesis that the average fluorescence value is a function of the amount of CNTs. The results for sample 3 were different from the results of sample 1 and 2 though. In sample 3, fluorescence values increased in 30 minutes and decreased after that while sample 1 and 2 fluorescence values decreased in the first hour and then increased again.

The average fluorescence value for sample 4 which contained 800 µg CNT and was prepared at an agitation rate of 300 rpm was 142. Fluorescence values for sample 4 did not have

any pattern in changes, although the average fluorescence values again increased by increasing the amount of CNT.

It is hard to draw a conclusion from the fluorescence assay data because they did not follow a pattern. After passage of time, the amount of released protein was expected to increase because the CNTs would have more chance to make the cell walls permeable, but the data did not support this assumption. Also, it is possible that the amount of GFP in the cell pellets was not high enough and may have caused inaccuracy in the fluorescence values. In summary, fluorescence assay was not a reliable test for measuring the amount of released proteins.

3.2. Optical absorbance assay

The second assay that was used in this research was optical absorbance assay which is an accurate assay to measure the released β-lactamase in medium. β-lactamases are enzymes that some bacteria produce to resist β-lactam antibiotics like penicillin. Thus, in order to measure the amount of released β-lactamase in medium, as mentioned in chapter 2, 20 µl of each prepared sample was added to 2 ml of penicillin G containing buffer.

3.2.1. Optical absorbance of sample 1

Figure 10 shows optical absorbance of sample 1 at different times. The wavelength of 240 nm was chosen because at this wavelength penicillin G absorbs. According to Figure 10, at t = 0, the absorbance of penicillin G was high which means that at the beginning of this assay for sample 1, the amount of penicillin was at the highest level. After passage of time, the absorbance of penicillin G decreased which indicates that penicillin was used gradually to inhibit the existent β-lactamase in the medium. Consequently, after seven minutes, the absorbance of penicillin G

was at the lowest level. In this assay, the slope of each line indicates the concentration of β-lactamase in the medium. The slopes of different lines are listed in Table VII and the amount of release increased gradually with the passage of time.

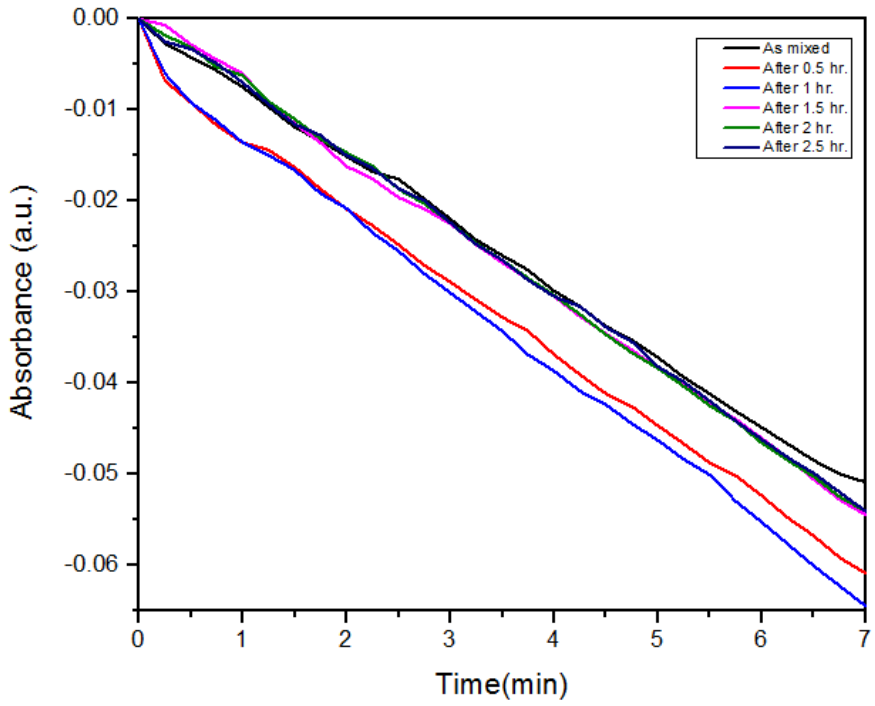


Figure 10. Optical absorbance of sample 1 at different times at a wavelength of 240 nm.

In order to understand the effect of two parameters (amount of CNTs and agitation rate), a control sample was prepared without adding any CNTs to the medium. Also, another sample was prepared using lysozyme and ethylenediamene acetic acid (EDTA). Lysozyme is an enzyme which is able to damage the bacterial cells and has the highest efficiency among the damage methods. EDTA can detach the ions that are connected to the cells membrane and facilitate the lysis of the cells.

Table VII. Slopes of different lines for the sample 1.

Time (hours)	Slope
As-mixed (0)	-0.0074
After 0.5	-0.0075
After 1.0	-0.0076
After 1.5	-0.0078
After 2.0	-0.0079
After 2.5	-0.0079

The slopes of different lines for different samples are listed in Table VIII. In the control sample there were no CNTs and the slope of the lines pertained to the effect of agitation rate and may be natural β-lactamase release. On the other hand, in the lysozyme sample, the slopes are related to maximum release of β-lactamase in the medium. As listed in Table VIII, the slope values increased from sample 1 to sample 6 as the amount of additional CNTs and agitation rates increased. In order to understand the increased amounts of release, the percentages of release based on the lysozyme treatment for different samples at different times are listed in Table IX.

.

Table VIII. Slopes of different lines for different samples.

	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Lysozyme
as-mixed	-0.0072	-0.0074	-0.0079	-0.0082	-0.0083	-0.0086	-0.0095	-0.0098	-0.0119	-0.0156
0.5	-0.0072	-0.0075	-0.0080	-0.0084	-0.0084	-0.0086	-0.0095	-0.0099	-0.0123	-0.0156
1	-0.0072	-0.0076	-0.0081	-0.0085	-0.0087	-0.0087	-0.0095	-0.0101	-0.0130	-0.0156
1.5	-0.0074	-0.0078	-0.0082	-0.0086	-0.0089	-0.0088	-0.0096	-0.0102	-0.0136	-0.0156
2	-0.0075	-0.0079	-0.0083	-0.0086	-0.0089	-0.0089	-0.0097	-0.0103	-0.0140	-0.0156

Table IX. The percentages of release based on the lysozyme treatment.

Sample No.	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Lysozyme
Agitation rate	-	200 rpm	200 rpm	300 rpm	600 rpm	300 rpm	300 rpm	600 rpm	600 rpm	-
Amount of CNTs	0	0.2 $mg \cdot L^{-1}$	0.4 $mg \cdot L^{-1}$	0.8 $mg \cdot L^{-1}$	0.8 $mg \cdot L^{-1}$	1.6 $mg \cdot L^{-1}$	8 $mg \cdot L^{-1}$	8 $mg \cdot L^{-1}$	32 $mg \cdot L^{-1}$	0
as-mixed	46	47	50	52	53	55	61	63	76	100
0.5	46	48	51	54	54	55	61	64	79	100
1	46	49	52	54	55	56	61	65	83	100
1.5	47	50	52	55	57	56	62	65	87	100
2	48	50	53	55	57	57	62	66	90	100

According to Table IX and Figure 11, the minimum amount of release occurred in control sample means that the bacterial cells can secrete protein naturally around 46% of lysozyme treatment. The amounts of release almost remained unchanged for an hour, then increased to 47%. Comparing the as-mixed sample with two hours-treated sample could give the clue that the amount of protein release in the control sample has been increased. Agitation rate may play an important role in this enhancement because the two hours-treated cells had more chance to leak-out into the reactor than 30 minutes-treated cells. They also have been contacted more with the impeller and this may enhance the protein release within the last taken samples.

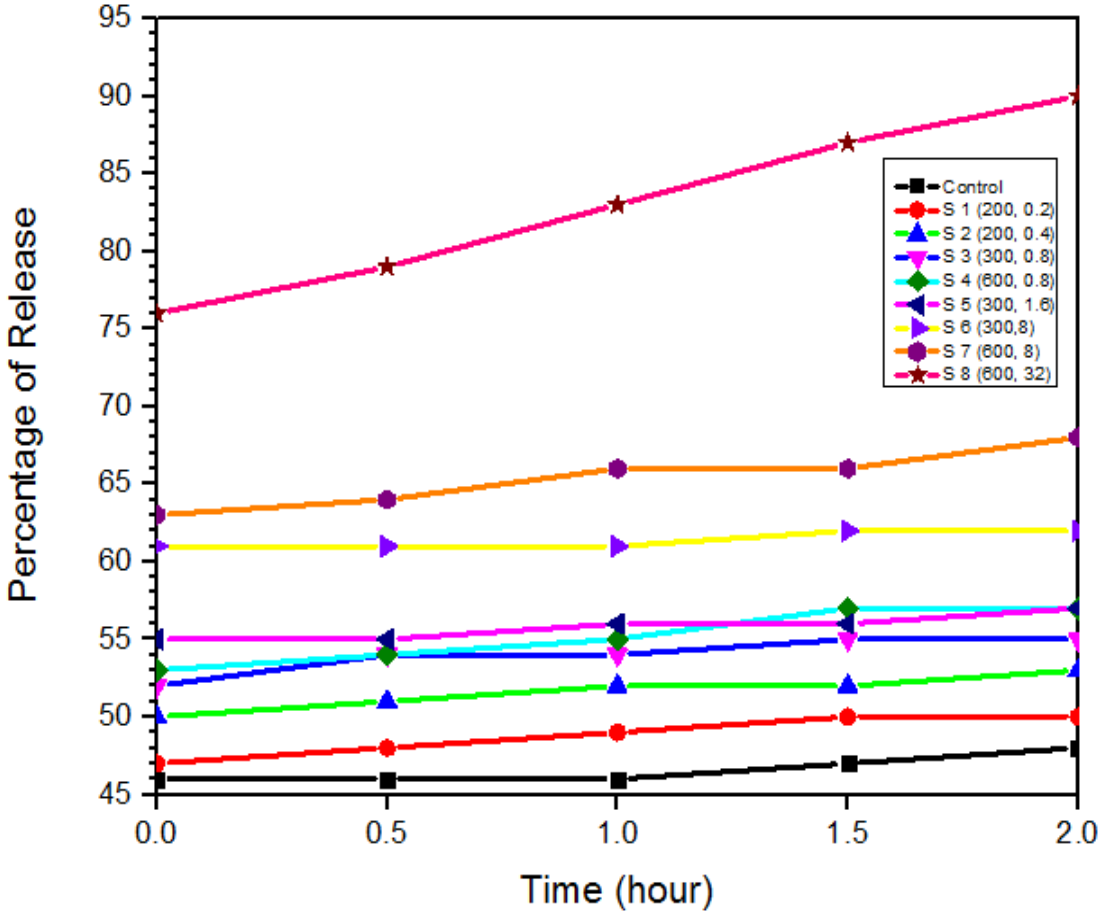


Figure 11. The percentage of release based on the lysozyme treatment.

3.2.2. Samples with agitation rate of 200 rpm

Sample 1 contained about 200 $\mu g \cdot L^{-1}$ CNTs and it was prepared at an agitation rate of 200 rpm. As listed in Table IX, the minimum amount of release in this sample, at 47%, was slightly more than the control which was 46%. CNTs may affect the relatively higher amount of release because in as-mixed sample 1, the only effective parameter for release was additive CNTs. For sample 2 through sample 8, agitation rate also was counted as the other parameter but due to relatively low agitation rate, it could not impressively increase the permeability of the cells. Also, the percentage of release based on lysozyme treatment was still low which means that a higher amount of CNTs should be added to the cells to make them leaky.

In order to investigate the effect of higher amount of CNTs as an effective parameter for increasing the permeability of the cells, 400 $\mu g \cdot L^{-1}$ CNTs were added to the medium for preparation of sample 2. Sample 2 was also prepared under the agitation rate of 200 rpm. Compared to sample 1, the amount of released protein for the as-mixed sample increased by 3% to 50% of maximum protein release. Hence, with the agitation rate of sample 1 and 2 being the same, the amount of additive CNTs increased the permeability of the cells. After 2 hours treatment, the released protein increased to 53%, 3% above the as-mixed sample 2. This increase was a factor of agitation rate and relatively higher amount of additive CNTs. By increasing the amount of CNTs and also by the passage of time, CNTs could mechanically damage the cell walls. They could rupture the cell walls using their hydrophilic functionalized ends allowing the cells to leak their proteins and enzymes out into the medium. Although the amount of released protein in sample 2 was higher than that of sample 1, it still needs to be closer to that of lysozyme treatment. One way to get more released protein would be increasing the agitation rate

and/or the amount of CNTs. Thus, for the sample 3, the amount of CNTs and agitation rate has been increased to 800 $\mu g \cdot L^{-1}$ and 300 rpm, respectively.

3.2.3. Samples with agitation rate of 300 rpm

The amount of additive CNTs and agitation rate were increased to 800 $\mu g \cdot L^{-1}$ and 300 rpm, respectively, to determine if it could enhance the amount of released protein. As listed in

Table IX, released protein was 52% for the as-mixed sample. After two hours, the amount of release had increased by 3%. Although both agitation rate and amounts of CNTs added had increased, the amount of released proteins has not changed drastically.

For sample 5 and 6, 1600 $\mu g \cdot L^{-1}$ and 8000 $\mu g \cdot L^{-1}$ CNTs were added to the reactor, and the as-mixed protein released by the cells was 55% and 61%, respectively. Furthermore, the amount of as-mixed release was 9% and 15% greater compared to the control sample, respectively. Since the applied agitation rates for samples 3, 5, and 6 were the same, it can be concluded that adding more CNTs to the medium was the main cause of increase in the permeability of the cells. Moreover, after two hours, the cells leaked proteins out into the medium up to 62% of lysozyme treatment.

3.2.4. Samples with agitation rate of 600 rpm

In preparation of the next three samples, agitation rates were increased to 600 rpm. This increase could lead to higher protein release because functionalized CNTs could damage the cell walls more intensively and cause higher permeability of the cells compared to lower agitation

rates. Moreover, a higher amount of CNTs were added to these samples to further investigate the effect of CNTs on permeability of the cells.

For sample 4, compared to sample 3, the amount of additive CNTs remained unchanged while the agitation rate was increased from 300 rpm to 600 rpm. As given in Table IX,

the amount of released protein for the as-mixed sample increased by one percentage point to 53% of maximum protein release. After two hours elapsed time, the amount of release was 57% which is two percentage points more than that of sample 3.

In preparation of sample 7, the amount of 8000 $\mu g \cdot L^{-1}$ CNTs were added to the medium, five times more than the amount added to sample 5. The percentage of protein release for sample 7 as-mixed was 63%, 17 percentage points more than the protein release of the as-mixed control sample. Compared to sample 5, the amount of release for as-mixed samples have increased by eight percentage points. Also, after two hours elapsed time, the amount of release was 66% which is 18 percentage points and seven percentage points more than that of control sample and sample 5 for the same elapsed time, respectively.

Up to this point, the results of experiments showed that agitation rate and additive CNTs could increase the protein release of the cells up to 66% of lysozyme treatment. In order to try to get the desired outcome of equivalency to lysozyme treatment, 32000 $\mu g \cdot L^{-1}$ CNTs were added to the medium of sample, this was four times the amount added to sample 7. As given in Table IX, the amount of protein release drastically increased for this sample and was 76% as-mixed. This indicated that the permeability of the cells increased 30 percentage points more than that of control sample by adding 32000 $\mu g \cdot L^{-1}$ CNTs. Also, compared with sample 7, sample 8 released 13 percentage points more proteins into the medium due to the relatively higher amount of additive CNTs. Thirty minutes after the 600 rpm agitation, the amount of release increased to

79%. Suggesting that more CNTs had a chance to collide with cells and make them permeable. Also, due to high agitation rate, the collisions were very intensive and more proteins leaked out of the cells. After one hour, the number of collisions had increased, and more CNTs had mechanically ruptured the cell walls. The amount of release one hour after treatment of sample 8 was 83% of lysozyme treatment. 1.5 and two hours after treatment, the level had increased to 87% and 90%, respectively, which indicated that by increasing the time after agitation, CNTs would have more chance to collide with the cells. Due to the high agitation rate, the energy of collisions between CNTs and cell walls increased and could be a primary reason for the high amount of as-mixed release. The effect of 32000 $\mu g \cdot L^{-1}$ CNTs on the high amount of release was also inevitable.

As some of the released proteins in lysozyme treatment are cytoplasmic, it can be suggested that the relatively high level of release in sample 8 was because of the mechanical damage of cytoplasm membrane. Thus, some of the released proteins in sample 8 were cytoplasmic. On the other hand, most of the released proteins in sample 1 to sample 7 were periplasmic. A correlation between the amount of released periplasmic proteins, agitation rate, and the amount of additive CNTs, can be expressed as:

$$P = k(A)^a(C)^c \qquad \text{Equation (1)}$$

Where P is the amount of released periplasmic protein is, k is constant, A is agitation rate, C is the amount of additive CNTs, and k and c are the exponents of agitation rate and the amount of additive CNTs, respectively. In order to find k, a, and c, least square analysis approach was used. In this approach, residuals can be defined as:

$$Residuals = \sum(P_{Exp} - P_{Calc})^2 \qquad \text{Equation (2)}$$

Where P_{Exp} and P_{Calc} are the experimental and calculated amount of released periplasmic protein, respectively. In order to find the best correlation, the sum of residuals was minimized subject to $k, a, c > 0$. By minimizing the residuals, the resultant equation, P is defined as:

$$P = 27(A)^{0.045}(C)^{0.064} \qquad \text{Equation (3)}$$

Where k is 27, a is 0.045, and c is 0.064. As shown in Figure 12, the obtained percentage of release in experiment was approximately equal to the calculated one.

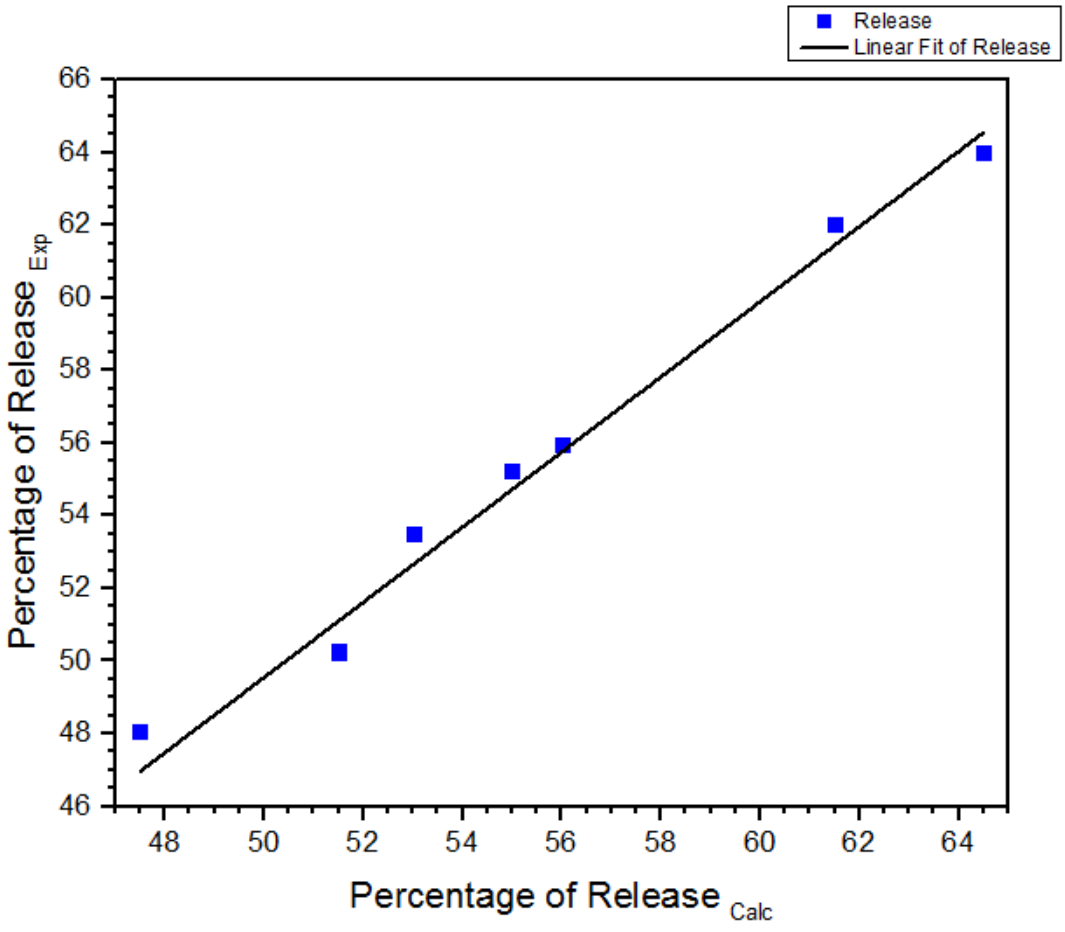


Figure 12. The experimental percentage of release versus the calculated percentage of release.

3.3. Transmission electron microscopy of treated samples

In order to visually examine the permeability enhancement of the cells, TEM images were used. The TEM image of *E. coli* cells before exposing to CNTs is shown in Figure 13. The cells were intact and the average width of bacteria was 1.1 μm and the thickness of periplasm layer was 50-200 nm.

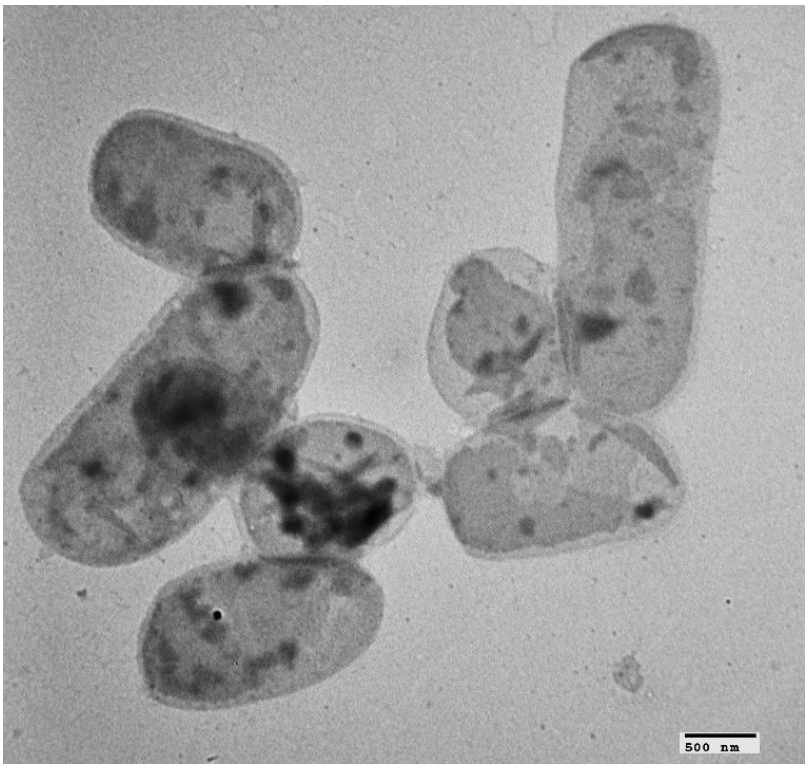


Figure 13. TEM image of *E. coli* cells before exposing to CNTs.

As mentioned in Chapter 2, there are two assumptions for release of protein. The first one is that CNTs may cause the periplasm materials and proteins to be released into the medium. A TEM image of *E. coli* cells exposed to CNTs from sample 8 which had 32000 $\mu g \cdot L^{-1}$ CNTs and was prepared with agitation rate of 600 rpm is shown in Figure 14. The bacterium is surrounded with many CNTs and they mechanically damaged the periplasm because they were functionalized with –COOH. This function made the CNTs surfaces hydrophilic in order to be

applicable in aqueous media and make the bacterium leaky. The average length of the CNTs was 186 nm with a standard deviation of 47 nm. As the thickness of periplasm layer was 50-200 nm, the CNTs were able to do mechanical damage to the cells. Because a high amount of CNTs were added to this sample, the chance of collision with bacteria had increased. Also, by applying high agitation rate, CNTs more intensively destroyed the periplasm by piercing and penetrating the layer. As a result, the periplasm materials and proteins leaked out to the medium and CNTs successfully made the cell permeable.

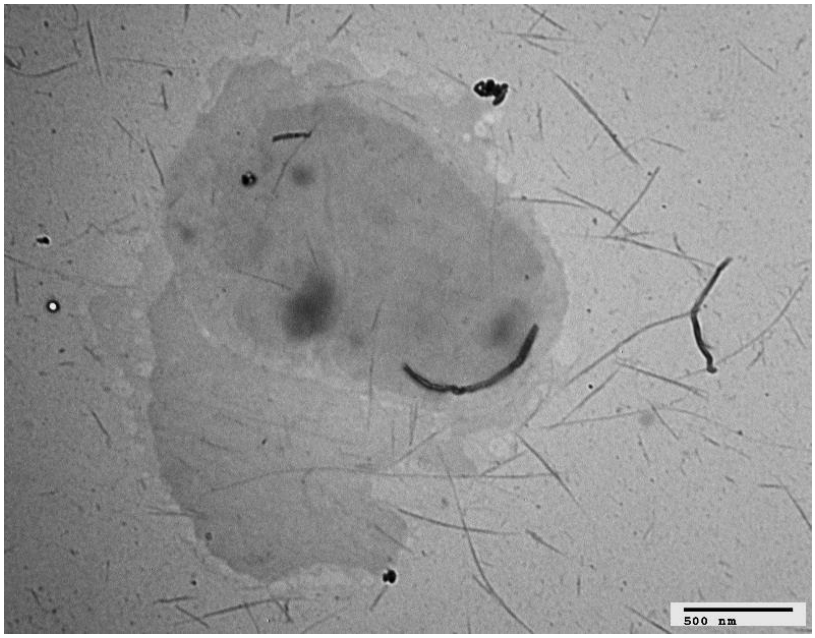


Figure 14. TEM image of a bacterium after exposing to CNTs. The periplasm of this *E. coli* cell is destroyed.

The second assumption is that CNTs may destroy the cell and, mechanically, cause not only the periplasmic but the cytoplasmic materials to leak to the medium. As shown in Figure 15, the cell was totally damaged by the CNTs. There is a possibility that CNTs first damaged the membrane then, by passage of time, and due to high agitation rate, came into the cytoplasm and destroyed the cell from inside. This could cause the cell to fall apart and the materials inside the

periplasm and cytoplasm to be released to the medium and finally, the cell to be killed by carbon nanotubes.

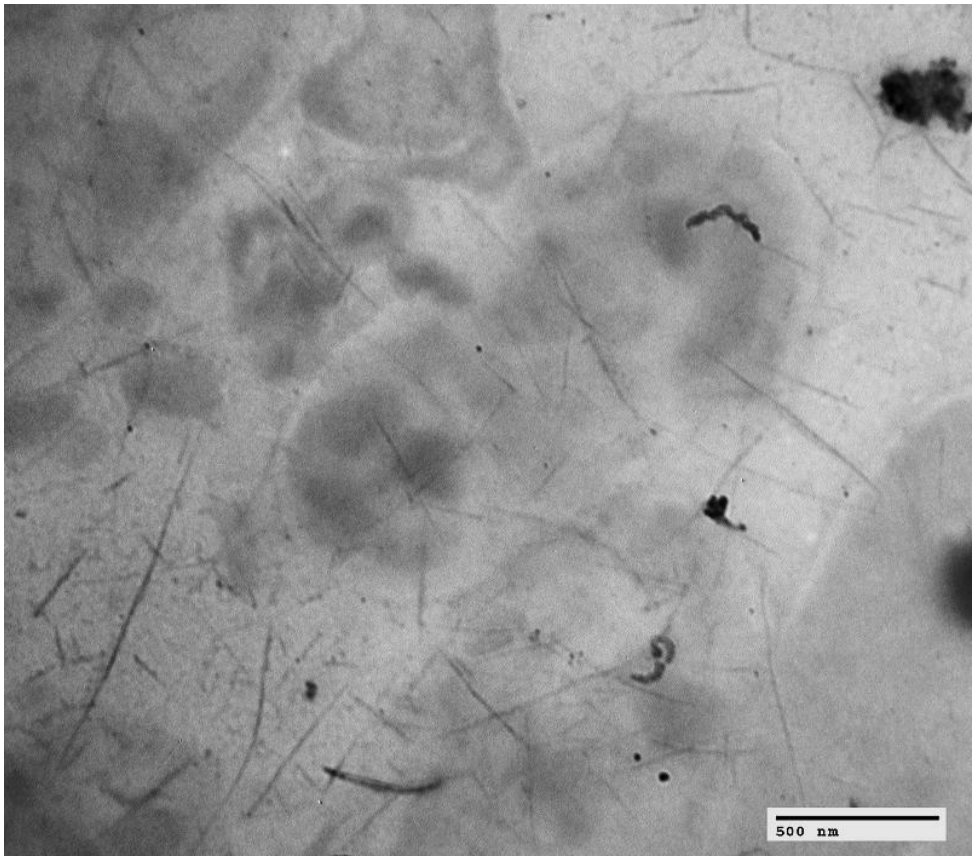


Figure 15. TEM image of a CNT treated cell. *E. coli* cell has fallen apart after exposure to the CNTs.

3.4. Gel electrophoresis of proteins

In order to study the mixture of the proteins in the sample, protein gel electrophoresis technique was performed. As discussed before, there are different types of proteins in the medium that should be distinguished using protein gel electrophoresis approach. As shown in Figure 16, there are many proteins in the samples. Based on the results of the optical absorbance assay and the percentage of released β-lactamase of the samples, sample 5, 7, and 8 were chosen for this assay. The first and last lanes are allocated to the Precision Plus Protein ™ standard to

express the range of different proteins based on their molecular weights from 20 kD to 250 kD, as shown in the right side of the gel. Also, some proteins have molecular weight of less than 20 kD which can be seen in the sample lanes. The proteins in the sample 5 lane was more detectable than the other lanes because sample 5 was two and four times more concentrated than sample 7 and 8, respectively.

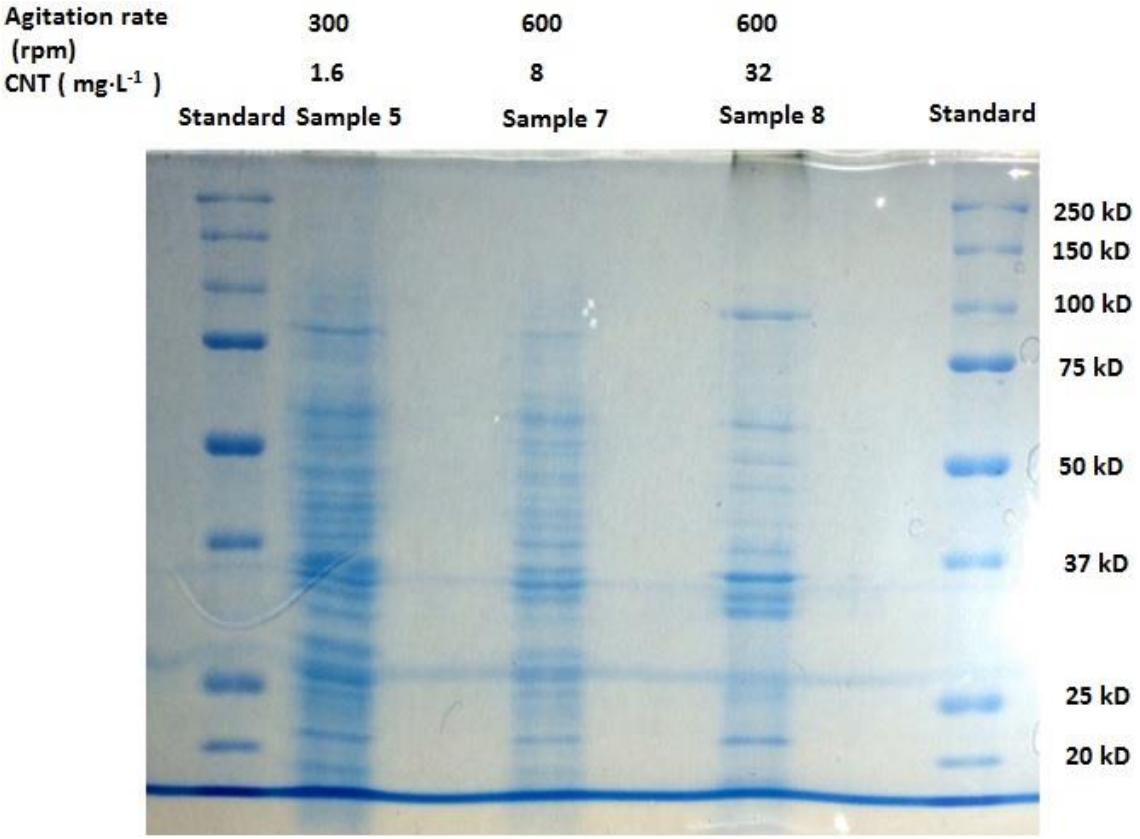


Figure 16. Proteins release into growth media in response to treatment with CNTs. The amount of CNTs and agitation rate during CNT exposure is shown above each lane. The bands express the presence of different types of proteins.

β-lactamase protein has the molecular weight in the range of 25 kD – 37 kD. According to the sample lanes, there are three lines closed to the standard of 37 kD molecular weight for all the samples. These lines indicate the presence of the β-lactamase in the sample. The presence of β-lactamase in the samples is because of the released protein caused by mechanical rupture of the

Escherichia coli cells. As shown in Figure 16, although the sample 8 was less concentrated than the other two samples, the lines in the range of 25-37 kD were almost as thick as the sample 5 and thicker than sample 7. This could be an indicator of more β-lactamase in sample 8 which was prepared under higher amount of CNTs and agitation rate than the others.

Chapter 4. Summary

In this research, the use of carbon nanotubes (CNTs) as a treatment to increase the permeability of a bacterial cell wall was investigated. For this purpose, the structure of *Escherichia coli* and methods of enhancing outer membrane permeability was reviewed. Also the use of nanoparticles, as well as CNTs as lysis agents, was studied. CNTs and their structure, growth, functionalization, and toxicity properties were reviewed. For this research, recombinant *Escherichia coli* BL21 (DE3) containing Green Fluorescent Protein (GFP) plasmid and β-lactamase were exposed to CNTs under various levels of agitation for different times. In order to determine the amount of released protein, fluorescence assay for GFP and optical absorbance for β-lactamase activity were used. Also, Transmission Electron Microscopy (TEM) was used to visually examine the permeation of the cells. The results of optical absorbance for β-lactamase activity indicated that, for agitation rate and CNT concentration evaluated, CNTs could lyse the cells up to 90% of maximum compared to lysozyme treatment. Based on TEM study, it is believed that this treatment damaged the cell walls to make *E. coli* permeable, causing periplasm, and in some cases cytoplasm, proteins and enzymes to leak into the medium. Consequently, CNTs could be used as lysis agents when it is undesirable to add an additional enzyme (lysozyme) to cause the release of intracellular proteins.

References

[1] A. Netik, N. V. Torres, J. Riol and C. P. Kubicek, "Uptake and export of citric acid by Aspergillus niger is reciprocally regulated by manganese ions," *Biochimica Et Biophysica Acta (BBA)-Biomembranes,* vol. 1326, pp. 287-294, 1997.

[2] H. Nikaido, "Outer membrane of Salmonella typhimurium: transmembrane diffusion of some hydrophobic substances," *Biochimica Et Biophysica Acta (BBA)-Biomembranes,* vol. 433, pp. 118-132, 1976.

[3] W. C. DeLoache and J. E. Dueber, "Compartmentalizing metabolic pathways in organelles," *Nat. Biotechnol.,* vol. 31, pp. 320-321, 2013.

[4] G. Sherbet and M. Lakshmi, "Characterisation of Escherichia coli cell surface by isoelectric equilibrium analysis," *Biochimica Et Biophysica Acta (BBA)-Biomembranes,* vol. 298, pp. 50-58, 1973.

[5] H. Nikaido and T. Nakae, "The outer membrane of Gram-negative bacteria," *Adv. Microb. Physiol.,* vol. 20, pp. 163-250, 1979.

[6] J. M. Dirienzo, K. Nakamura and M. Inouye, "The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly, and functions," *Annu. Rev. Biochem.,* vol. 47, pp. 481-532, 1978.

[7] W. D. Stein, *The Movement of Molecules Across Membranes.* Academic Press, 1969.

[8] M. Kong, X. G. Chen, K. Xing and H. J. Park, "Antimicrobial properties of chitosan and mode of action: a state of the art review," *Int. J. Food Microbiol.,* vol. 144, pp. 51-63, 2010.

[9] L. Leive, "The barrier function of the Gram-negative envelope," *Ann. N. Y. Acad. Sci.,* vol. 235, pp. 109-129, 1974.

[10] S. B. Levy and L. Leive, "An equilibrium between two fractions of lipopolysaccharide in Escherichia coli," *Proc. Natl. Acad. Sci. U. S. A.,* vol. 61, pp. 1435-1439, Dec, 1968.

[11] M. S. Cooperstock, "Inactivation of endotoxin by polymyxin B," *Antimicrob. Agents Chemother.,* vol. 6, pp. 422-425, Oct, 1974.

[12] R. I. Lehrer, A. Barton and T. Ganz, "Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in E. coli by multiple-wavelength spectrophotometry," *J. Immunol. Methods,* vol. 108, pp. 153-158, 1988.

[13] R. E. Hancock, S. W. Farmer, Z. S. Li and K. Poole, "Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of Escherichia coli," *Antimicrob. Agents Chemother.,* vol. 35, pp. 1309-1314, Jul, 1991.

[14] M. E. Kahn and H. O. Smith, "Transformation inHaemophilus: A problem in membrane biology," *J. Membr. Biol.,* vol. 81, pp. 89-103, 1984.

[15] S. Nablo, *Process for Bulk Sterilization, Minimizing Chemical and Physical Damage,* 1973.

[16] S. L. Wilson and V. K. Walker, "Selection of low-temperature resistance in bacteria and potential applications," *Environ. Technol.,* vol. 31, pp. 943-956, 2010.

[17] K. Cho, J. Park, T. Osaka and S. Park, "The study of antimicrobial activity and preservative effects of nanosilver ingredient," *Electrochim. Acta,* vol. 51, pp. 956-960, 2005.

[18] J. R. Morones, J. L. Elechiguerra, A. Camacho, K. Holt, J. B. Kouri, J. T. Ramírez and M. J. Yacaman, "The bactericidal effect of silver nanoparticles," *Nanotechnology,* vol. 16, pp. 2346, 2005.

[19] K. B. Holt and A. J. Bard, "Interaction of silver (I) ions with the respiratory chain of Escherichia coli: an electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag," *Biochemistry (N. Y.),* vol. 44, pp. 13214-13223, 2005.

[20] Q. Feng, J. Wu, G. Chen, F. Cui, T. Kim and J. Kim, "A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus," *J. Biomed. Mater. Res.,* vol. 52, pp. 662-668, 2000.

[21] J. P. Ruparelia, A. K. Chatterjee, S. P. Duttagupta and S. Mukherji, "Strain specificity in antimicrobial activity of silver and copper nanoparticles," *Acta Biomaterialia,* vol. 4, pp. 707-716, 2008.

[22] G. Burygin, B. Khlebtsov, A. Shantrokha, L. Dykman, V. Bogatyrev and N. Khlebtsov, "On the enhanced antibacterial activity of antibiotics mixed with gold nanoparticles," *Nanoscale Research Letters,* vol. 4, pp. 794-801, 2009.

[23] A. N. Grace and K. Pandian, "Antibacterial efficacy of aminoglycosidic antibiotics protected gold nanoparticles—A brief study," *Colloids Surf. Physicochem. Eng. Aspects,* vol. 297, pp. 63-70, 2007.

[24] A. Rai, A. Prabhune and C. C. Perry, "Antibiotic mediated synthesis of gold nanoparticles with potent antimicrobial activity and their application in antimicrobial coatings," *Journal of Materials Chemistry,* vol. 20, pp. 6789-6798, 2010.

[25] B. Li and B. E. Logan, "Bacterial adhesion to glass and metal-oxide surfaces," *Colloids and Surfaces B: Biointerfaces,* vol. 36, pp. 81-90, 2004.

[26] P. K. Stoimenov, R. L. Klinger, G. L. Marchin and K. J. Klabunde, "Metal oxide nanoparticles as bactericidal agents," *Langmuir,* vol. 18, pp. 6679-6686, 2002.

[27] R. Brayner, R. Ferrari-Iliou, N. Brivois, S. Djediat, M. F. Benedetti and F. Fiévet, "Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium," *Nano Letters,* vol. 6, pp. 866-870, 2006.

[28] T. Jin, D. Sun, J. Su, H. Zhang and H. Sue, "Antimicrobial efficacy of zinc oxide quantum dots against Listeria monocytogenes, Salmonella enteritidis, and Escherichia coli O157: H7," *J. Food Sci.,* vol. 74, pp. M46-M52, 2009.

[29] O. Yamamoto, "Influence of particle size on the antibacterial activity of zinc oxide," *International Journal of Inorganic Materials,* vol. 3, pp. 643-646, 2001.

[30] P. Dibrov, J. Dzioba, K. K. Gosink and C. C. Hase, "Chemiosmotic mechanism of antimicrobial activity of Ag(+) in Vibrio cholerae," *Antimicrob. Agents Chemother.,* vol. 46, pp. 2668-2670, Aug, 2002.

[31] E. T. Thostenson, Z. Ren and T. Chou, "Advances in the science and technology of carbon nanotubes and their composites: a review," *Composites Sci. Technol.,* vol. 61, pp. 1899-1912, 2001.

[32] P. Hou, C. Liu and H. Cheng, "Purification of carbon nanotubes," *Carbon,* vol. 46, pp. 2003-2025, 2008.

[33] L. Murr, K. Soto, E. Esquivel, J. Bang, P. Guerrero, D. Lopez and D. Ramirez, "Carbon nanotubes and other fullerene-related nanocrystals in the environment: A TEM study," *JOM,* vol. 56, pp. 28-31, 2004.

[34] M. Reibold, P. Paufler, A. Levin, W. Kochmann, N. Pätzke and D. Meyer, "Materials: Carbon nanotubes in an ancient Damascus sabre," *Nature,* vol. 444, pp. 286-286, 2006.

[35] S. Iijima, "Helical microtubules of graphitic carbon," *Nature,* vol. 354, pp. 56-58, 1991.

[36] J. Kong, A. M. Cassell and H. Dai, "Chemical vapor deposition of methane for single-walled carbon nanotubes," *Chemical Physics Letters,* vol. 292, pp. 567-574, 1998.

[37] R. E. Smalley, Y. Li, V. C. Moore, B. K. Price, R. Colorado, H. K. Schmidt, R. H. Hauge, A. R. Barron and J. M. Tour, "Single wall carbon nanotube amplification: En route to a type-specific growth mechanism," *J. Am. Chem. Soc.,* vol. 128, pp. 15824-15829, 2006.

[38] I. D. Rosca, F. Watari, M. Uo and T. Akasaka, "Oxidation of multiwalled carbon nanotubes by nitric acid," *Carbon,* vol. 43, pp. 3124-3131, 2005.

[39] B. Zhao, H. Hu, A. Yu, D. Perea and R. C. Haddon, "Synthesis and characterization of water soluble single-walled carbon nanotube graft copolymers," *J. Am. Chem. Soc.,* vol. 127, pp. 8197-8203, 2005.

[40] M. L. Schipper, N. Nakayama-Ratchford, C. R. Davis, N. W. S. Kam, P. Chu, Z. Liu, X. Sun, H. Dai and S. S. Gambhir, "A pilot toxicology study of single-walled carbon nanotubes in a small sample of mice," *Nature Nanotechnology,* vol. 3, pp. 216-221, 2008.

[41] C. W. Lam, J. T. James, R. McCluskey and R. L. Hunter, "Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation," *Toxicol. Sci.,* vol. 77, pp. 126-134, Jan, 2004.

[42] J. Kim, N. Kotagiri, J. Kim and R. Deaton, "In situ fluorescence microscopy visualization and characterization of nanometer-scale carbon nanotubes labeled with 1-pyrenebutanoic acid, succinimidyl ester," *Appl. Phys. Lett.,* vol. 88, pp. 213110, 2006.

Appendix A: Description of Research for Popular Publication

Nanoparticles are small objects that are smaller than human hair and cover a range between 10^{-9}-10^{-7} meter (1-100 nanometer). These particles exhibit some unique properties due to their small size. There are many types of nanoparticles such as nanoclusters, nanowires, and carbon nanotubes. Carbon nanotubes (CNTs) are nanoscale hollow cylinders of carbon with diameters in the range of 1-100 nanometer. In this research, the use of carbon nanotubes (CNTs) as a lysis or rupture agent was examined. *Escherichia coli* is a model bacterium and due to its simple structure is widely used in the biology lab by researchers and scientists. Researchers for many years use the proteins and materials made by this organism in order to make products that include medicine.

Carbon nanotubes (CNTs) have many impurities that might lower their efficiency. In order to make CNTs usable, their residual metal impurities must be washed. In laboratories acids or bases are used to wash out the impurities from carbon nanotubes. After completing the washing process, purified CNTs were prepared and most of the metal impurities removed from them. Another important process that needs to be done with CNTs is functionalization. CNTs, due to their small size, tend to aggregate and become as big as they can. The size of agglomeration may reduce their functionality. Also, they intrinsically tend to repel or fail to mix with water which makes them unuseful in aqueous media. Thus, in order to overcome this issue, they should be functionalized with these chemical compounds, which enable them to mix with water-containing solutions.

In this research, carbon nanotubes functionalized with –COOH were used to release protein from the *Escherichia coli* bacteria. Also, in order to increase the chance of extracting proteins form the cells, CNTs should have more chance to have collision with cells. Thus, in this

research, the bacteria were exposed to CNTs under various levels of agitation in the reactor for different times and the amount of released protein was determined. It was found that treatment with CNTs and agitation rate contributed equally to damage which means that by increasing the amount of CNTs and agitation rate, more proteins were released in the culture fluid. Also, it is suggested that this treatment damaged the cell walls to make *E. coli* permeable, causing proteins and materials to release into the medium. Consequently, CNTs can be used as lysis agents to cause the release of intracellular materials.

Appendix B: Executive Summary of Newly Created Intellectual Property

1. Using CNTs as lysis agent to release the protein from the bacteria under various level of agitation rate
2. A mathematical relation was developed to describe the release of protein as a function of CNT concentration and agitation rate
3. Based on the correlation, it was determined that for the geometry of a shake flask that the concentration of CNTs and the agitation rate contribute equally to lysis

Appendix C: Potential Patent and Commercialization Aspects of Listed Intellectual Property Items

C.1 Patentability of Intellectual Property (Could Each Item be Patented)

1. Carbon nanotubes cannot be patented as lysis agent, but could be used as trade secret. The problem associated with carbon nanotube is that according to the materials safety datasheet (MSDS), carbon nanotubes are nano-hazardous materials which can affect the human health.

2. Modeling data about kinetic expression between the amount of the CNTs and agitation rate could be patented. In truth, it is likely that someone could treat the kinetic expression as a trade secret which will be rendered moot with publication.

C.2 Commercialization Prospects (Should Each Item Be Patented)

There are no commercialization prospects for intellectual items 1. and 2.

C.3 Possible Prior Disclosure of IP

The following items were discussed in a public forum.

1. On Tuesday, November 24, 2015, public presentation was made to MicroEP students about using CNTs as a lysis agent.
2. The thesis defense was held on Friday, December 4, 2015. This meeting was public and also about using CNTs as a lysis agent.

Appendix D: Broader Impact of Research

D.1 Applicability of Research Methods to Other Problems

Using carbon nanotubes may increase the cell wall permeability of a wide range of gram negative bacteria which have thicker outer membrane. In order to release more proteins from outer membrane of these types of bacteria, the length of CNTs should be diminished. Having smaller length could increase the chance of penetration to the cell membrane. Also, the diameter of the CNTs plays an important role in making mechanical rupture to the cell membrane. As the diameter of CNTs decreases, the chance of releasing proteins will be increased. Thus, using single-walled carbon nanotubes instead of multi-walled ones is a priority of researchers. This can increase the chance of releasing more proteins. For instance, notable groups of gram-negative bacteria including *Salmonella*, *Shigella*, and other *Enterobacteriaceae*, *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella* can be treated by CNTs.

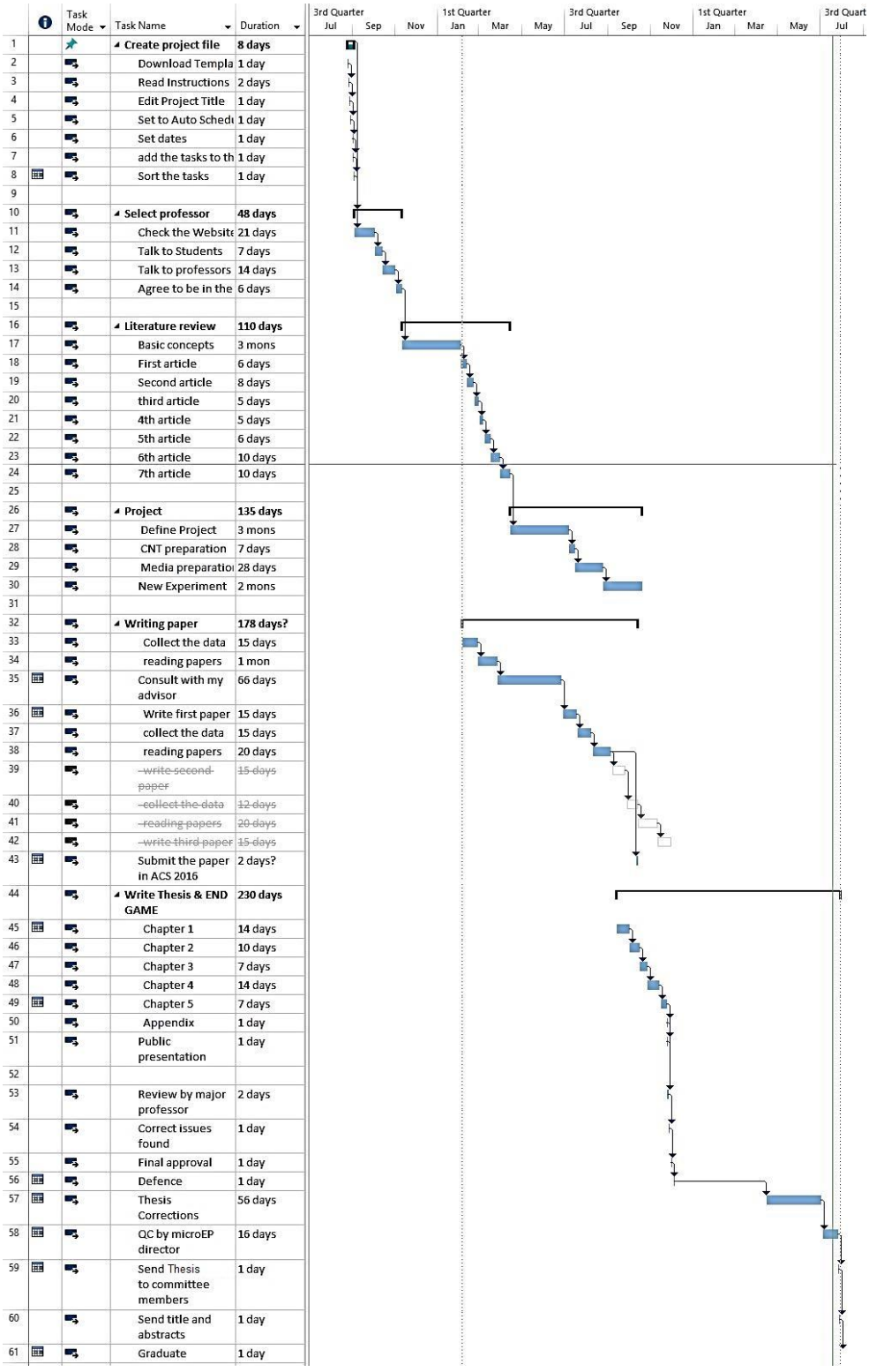
D.2 Impact of Research Results on U.S. and Global Society

Using this method, more proteins can be extracted from the cells. This method might be helpful for scientists to take more proteins out of the cells in order to use for human healthcare.

D.3 Impact of Research Results on the Environment

One of the concerns of using nanoparticles and carbon nanotubes is recycling. It is important to keep the materials which have been used during research in the nano-waste bucket in order not to let them pollute the environment. In this research, all the materials contaminated with carbon nanotubes were discarded into the nano-waste bucket.

Appendix E: Microsoft Project for MS MicroEP Degree Plan



	Task Mode	Task Name	Duration
1		**Create project file**	**8 days**
2		Download Templa	1 day
3		Read Instructions	2 days
4		Edit Project Title	1 day
5		Set to Auto Schedu	1 day
6		Set dates	1 day
7		add the tasks to th	1 day
8		Sort the tasks	1 day
9			
10		**Select professor**	**48 days**
11		Check the Website	21 days
12		Talk to Students	7 days
13		Talk to professors	14 days
14		Agree to be in the	6 days
15			
16		**Literature review**	**110 days**
17		Basic concepts	3 mons
18		First article	6 days
19		Second article	8 days
20		third article	5 days
21		4th article	5 days
22		5th article	6 days
23		6th article	10 days
24		7th article	10 days
25			
26		**Project**	**135 days**
27		Define Project	3 mons
28		CNT preparation	7 days
29		Media preparatio	28 days
30		New Experiment	2 mons
31			
32		**Writing paper**	**178 days?**
33		Collect the data	15 days
34		reading papers	1 mon
35		Consult with my advisor	66 days
36		Write first paper	15 days
37		collect the data	15 days
38		reading papers	20 days
39		~~write second paper~~	~~15 days~~
40		~~collect the data~~	~~12 days~~
41		~~reading papers~~	~~20 days~~
42		~~write third paper~~	~~15 days~~
43		Submit the paper in ACS 2016	2 days?
44		**Write Thesis & END GAME**	**230 days**
45		Chapter 1	14 days
46		Chapter 2	10 days
47		Chapter 3	7 days
48		Chapter 4	14 days
49		Chapter 5	7 days
50		Appendix	1 day
51		Public presentation	1 day
52			
53		Review by major professor	2 days
54		Correct issues found	1 day
55		Final approval	1 day
56		Defence	1 day
57		Thesis Corrections	56 days
58		QC by microEP director	16 days
59		Send Thesis to committee members	1 day
60		Send title and abstracts	1 day
61		Graduate	1 day

Appendix F: Identification of All Software Used in Research and Thesis Generation

Computer #1:

Model Number: Dell Studio 1458
Serial Number: 00196-083-475-832
Location: Personal laptop
Owner: Abdollah Mosleh

Software #1:

Name: Microsoft Office 2010
Purchased by: Abdollah Mosleh

Software #2:

Name: Microsoft Project 2010
Purchased by: MSDN Academy Alliance through Engineering

Software #3:

Name: Write-N-Cite III
Purchased by: Free download available from RefWorks.com, activated by uark student account

Appendix G: All Publications Published, Submitted and Planned

The results have been accepted as presentation in AICHE 2016 conference which will be held in San Francisco in November, 2016.