HELICASE ATTACHMENT TO CARBON NANOTUBES FOR DNA SENSOR

Submitted by:

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And

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Abstract

Purpose:

Current DNA detection techniques require complicated procedures, specialized training, expensive equipment, invasive samples and significant amount of sample collection and processing time. The purpose of this research was to develop a rapid, accurate, non-invasive and electronic method of DNA sensing that harnesses natural unwinding properties of DNA helicase by attaching it to Carbon Nanotubes.

Methods:

a. A literature review on methods of attaching proteins to carbon nanotubes was conducted
b. A design of the biosensor was developed based on previously reported attachment methods for other proteins
c. A part of the sensor was developed by attaching DNA helicase to carbon nanotubes
d. The result was tested for preservation of helicase functionality and carbon nanotube electronic structure integrity

Results:

a. Helicase was successfully attached to carbon nanotubes
b. Helicase was found to retain its NTP hydrolysis function, DNA binding and DNA unwinding ability upon attachment
c. Carbon nanotube electronic structure and function was not compromised upon attachment

Conclusions:

Non-specific attachment of helicase to carbon nanotubes preserves enzyme structure and function, allowing rapid DNA unwinding at an in vitro rate comparable to DNA helicase.
Acknowledgements

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<th>Description</th>
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<tr>
<td>CNT</td>
<td>Carbon Nanotube</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose Oxidase</td>
</tr>
<tr>
<td>LSZ</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>UV-Vis-NIR</td>
<td>Ultraviolet-Visible-Near Infrared</td>
</tr>
<tr>
<td>Fle</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>HEX</td>
<td>Hexachlorofluorescein</td>
</tr>
<tr>
<td>GRAVY</td>
<td>Grand Average of Hydropathy</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonant Energy Transfer</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotide Triphosphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxy Thymidine Triphosphate</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl Formamide</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SET</td>
<td>Single Electron Transistor</td>
</tr>
<tr>
<td>SMRT</td>
<td>Single Molecule Real Time</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithioreitol</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
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<tr>
<td>EDTA</td>
<td>Ethyldiaminetetraacetic acid</td>
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<td>NTPase</td>
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**Relevant Terms and Definitions**

**DNA sequencing:** DNA sequencing is a scientific method that involves a specific technology for determining the precise order of nucleotides (A, T, G and C) in a DNA molecule.

**DNA Helicase:** An enzyme responsible for converting double stranded DNA (dsDNA) to single stranded DNA (ssDNA), making it accessible to other proteins during cellular processes of replication, translation and DNA repair [28].

**NTP hydrolysis:** Nucleoside Triphosphates (NTPs) are building blocks for nucleic acids that provide energy for all cellular functions. Different proteins such as DNA helicase hydrolyze NTPs, converting them to Nucleoside Diphosphate (NDP) and release energy. Bacteriophage T7 GP4 helicase prefers Deoxythymidine Triphosphate (dTTP) over other NTPs [18, 39].

**Carbon nanotubes (CNT):** CNTs are an allotrope of carbon that can be visualized as a graphene sheet rolled into a seamless hollow cylinder [46]. CNTs used in this research are single-walled carbon nanotubes produced by laser vaporization.

**Non-covalent adsorption:** Different methods have been reported in literature for attaching biomolecules to carbon nanotubes [35]. Non-covalent adsorption is one of the simplest chemical methods as it does not require any complicated steps. Protein solution in buffer is incubated with a dispersion of CNT for a set period of time. This allows protein molecules to interact with sidewalls of CNTs using hydrophobic, pi-pi and Vander Waal interactions.

**Pi stacking:** Pi stacking or pi-pi interaction is defined as a non-covalent stabilizing interaction between aromatic molecules due to the presence of pi bonds [49]. These interactions play a major role in protein folding, DNA and RNA interactions, as well as interactions between atoms rich in pi bonds, including proteins and CNTs.
1. INTRODUCTION

This research was conducted at Cardiovascular Devices Division, University of Ottawa Heart Institute as part of a multidisciplinary DNA (deoxyribonucleic acid) detection project which was initiated by Dr. Mussivand in 2010. This chapter provides information about the background, objective, rationale, experimental approach, important results and contribution of this research towards the DNA detection device venture.

1.1 Clinical needs

DNA sequencing is an important tool for obtaining genetic information[1-3]. Rapid and low-cost DNA sequencing can allow early disease identification and development of personalized management and treatment methods. Advanced sequencing techniques can be further exploited for applications in forensics, defense and security, pharmaceutics, agriculture and biotechnology [1]. Despite latest developments in research and commercial DNA sequencing methods, the quest for even faster, cheaper and more sensitive technologies continues; to meet the rapidly growing demand for genome based profiling, prenatal screening, diagnosis, therapy, drug development, preventive and personalized medicine [2-4]. Current cost for whole genome sequencing lies around $10,000 USD, and present worldwide research aims to reduce it down to less than $1000 [3]. Most of the contemporary clinical and commercial DNA sensors rely on complicated sequential steps (Figure. 1.1). These complicated steps and the subsequent complex data processing put a strain on time and sampling requirements much needed for point-of-care (POC) devices, limiting amount of information available to patients and healthcare professionals [5-6]. There is also a need to properly collect, organize and extract meaningful information from the huge amount of data being generated through DNA sequencing.

Figure 1.1 Current DNA detection procedure involves complicated sequential steps

Cell sample collection → Cell lysis → DNA extraction → DNA purification → DNA amplification → DNA sequence detection
1.2 Rationale

Electronic biosensing using nanotechnology can help overcome problems with current DNA sequencing techniques [7-10]. Pre-sequencing steps can be reduced by attaching DNA related proteins to nanoparticles, allowing rapid, specific, more sensitive and scalable detection of DNA[11-13]. Such a device may be used for decentralized, point-of-care and real-time DNA sensing for various applications (Figure 1.2).

![Diagram](attachment:diagram.png)

**Figure 1.2** Simple and rapid DNA testing is needed for many applications

1.3 Hypothesis

Interfacing an enzyme DNA helicase to a carbon nanotube (CNT) transducer can combine natural DNA unwinding properties of helicase, with electronic sensitivity of CNT to sense charges on individual nucleotides. This helicase-CNT conjugate can detect and access the DNA template in a single step. The study is based on the following hypotheses:
1. Helicase can non-covalently adsorb onto CNT due to pi-pi and/or hydrophobic interactions similar to other proteins such as Bovine Serum Albumin (BSA), Glucose Oxidase (GOx) and Lysozyme (LSZ)

2. Different interactions between helicase and CNT may be possible. For preserving DNA binding ability of helicase, binding between primase region and CNT sidewalls is needed (to be verified)

3. Helicase attachment to CNT is stable and retains Nucleotide Triphosphate (NTP) hydrolysis and DNA unwinding function

4. CNT retains its electronic and optical properties upon helicase attachment

5. DNA unwinding by helicase depends on the DNA sequence and may be determined using charges sensed at helicase-CNT junction

1.4 Prior related research

This study is part of a DNA sequencing project currently in progress at Cardiovascular devices division, University of Ottawa Heart Institute under the supervision of Dr. Tofy Mussivand.

Prior related research at the institute has established the use of Bacteriophage T7 gene product 4 DNA Helicase as a bioreceptor for DNA. DNA helicase and helicase-avidin fusion protein were previously purified by a graduate student Leah Labib, in a bacterial host and their ability to bind with DNA was investigated using means that are relatively easy, inexpensive, and rapid and have a potential for large scale commercial production [14]. This recombinant protein is capable of binding to DNA by helicase and to biotin by the avidin region (Figure 1.3).

A rapid sampling and DNA extraction technique using ultrasound technology has also been developed by another graduate student, Bashir Morshed [15]. Electrical cell lysis was employed to release DNA from fingerprint samples with a battery operable voltage within 5 seconds using a microfluidic device[15-16].
Figure 1.3 Previously conducted research related to this project. Helicase-Avidin fusion protein for attachment to DNA. Upper portion of the figure shows the original helicase protein. Lower portion shows the fusion protein that was developed by replacing the primase region by avidin. Adapted from “HELICASE PURIFICATION FOR DNA SEQUENCING”, L. Labib, April, 2014, pp 17.

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Use of nucleotide charges for DNA detection has also been studied by this research group. Molecular Electrostatic Potential (MEP) around a molecule at a point is defined as the force on that point due to intrinsic charge the molecule. Electrostatic potential distribution patterns of nucleotide bases were determined using the molecular dynamics (MD) approach (Figure. 1.4) [17]. Preliminary simulations of three base sequences of DNA show different intensities indicating that charge distribution is different for each nucleotide base at levels within the sensitivity range of CNTs.

DNA detection using intrinsic charge of DNA has been reported previously using CNTs and various transducers [10, 41, 73, 74]. Use of DNA related proteins such as DNA polymerase, exonuclease, topoisomerase and ligase for direct detection of DNA charges has also been proposed [11-13]. The potential of CNTs for biosensing is unparalleled when compared to other nanoparticles due to their unique electrical, mechanical, chemical and optical properties [21]. This research exploits both these principles by combining electronic sensitivity of CNTs with biological specificity of DNA helicase.

*Figure 1.5* Graphene sheet forms a seamless hollow cylinder. The chiral vector, c, forms circumference of the cylinder. Adapted from “Raman Scattering in Carbon Nanotubes”, Christian Thomsen, Stephanie Reich, 1970, *Light Scattering in Solid IX*, pp.115-234.
Figure 1.6 Three types of carbon nanotubes: (6,6) armchair, (8,4) chiral and (10,0) zigzag. Adapted from “Raman Scattering in Carbon Nanotubes”, Christian Thomsen, Stephanie Reich, 1970, *Light Scattering in Solid IX*, pp.115-234.

CNTs are an allotrope of carbon, made up of one (single-walled) or more (multi-walled) hollow cylinders with 0.4 to 2 nm in diameter and 1 to 50 µm in length [86–88]. Single walled carbon nanotubes have simpler geometry than multi walled carbon nanotubes. These can be visualized as seamless hollow cylinders made up of graphene (Figure 1.5). Chirality is defined as the type of connections between hexagonal rings resulting in three main types: armchair, zigzag or chiral (Figure 1.6) [25]. Chirality affects mechanical strength, electrical conductivity, density and optical properties of CNTs. Zigzag and chiral CNTs are semiconducting (S1), while armchair CNTs are metallic (M2). CNTs are produced using three different methods: arc discharge, laser ablation and chemical vapor deposition [26]. These methods generate a mixture of bundled metallic and semiconducting CNTs, with varying properties. Functionalization with proteins can isolate CNTs, and improve solubility, stability and biocompatibility which is needed to fully exploit CNTs for biosensing applications.
1.5 Overview and Proposed research

1.5.1 Objective and scope

The objective of this research was to develop a system that efficiently binds and unwinds double stranded DNA, allowing electronic detection of DNA. The following aims address the hypotheses:

a. To inspect Helicase-CNT attachment using chemical non-covalent methods
b. To analyze DNA binding to Helicase-CNT conjugate
c. To determine NTP hydrolysis and DNA unwinding by Helicase-CNT conjugates
d. To investigate preservation of CNT electronic structure and properties for electronic sequencing

Since use of cell lysis to extract DNA from cell samples has previously been investigated by this lab, the scope of this research was limited to investigating DNA unwinding by helicase upon attachment to CNT. Future work will include developing electronic circuitry for reading the electronic signal and displaying in a readout display. CNTs can be functionalized with helicase and deposited on Field Effect Transistors (FET) for highly sensitive circuitry. The electronic circuitry will generate a unique electronic signature corresponding to each of the nucleotides within the sequence of DNA as DNA is pulled through the central channel of helicase. The electronic data will be analyzed and algorithms developed for programming and developing a portable DNA detector. The data from each nano-biosensor will be reconstructed by either comparing to the known or similar genome.

1.5.2 Proposed DNA detection method

The proposed nano-biosensor is composed of two main components:

1. Helicase is a naturally occurring enzyme within cells which acts as a molecular motor as it unwinds (separate/unzip) the double stranded DNA (dsDNA) and pulls one of the single stranded DNA (ssDNA) through its central hole using energy from nucleotide triphosphate (NTP) hydrolysis [27]. It has been demonstrated that hexameric DNA helicase such as 'bacteriophage T7 gene 4 can operate in vitro achieving an unwinding
rate of up to 130 nucleotides per second, hydrolyzing one NTP per 3 nucleotide movement[28]. This specific helicase is selected due to its small, ring like structure and because it has been studied extensively as a model protein for other helicases in literature[20-25].

2. Single walled carbon nanotubes (CNTs) are an allotrope (different structural form) of carbon, formed by rolling up a single graphite sheet into a tube-like structure [35]. CNTs are highly conductive, and proteins can adsorb onto their surface using pi-pi, hydrophobic and electrostatic interactions [27-29].

![Figure 1.7 Proposed nano-biosensor with helicase conjugated to CNT. It is capable of unwinding DNA by pulling one DNA strand through its central channel using energy released from hydrolysis of Nucleotide Triphosphate (NTP), converting it to Nucleotide Diphosphate (NDP)](image)

Bacteriophage T7 gene 4 helicase is composed of two main regions, helicase and primase [22- 23]. Helicase region is responsible for binding nucleotides and DNA, and for unwinding dsDNA to ssDNA. Primase region is responsible for recognizing primase sites on DNA which are responsible for primer delivery for DNA synthesis by DNA polymerase
Helicase may attach to CNT through interactions between aromatic residues located in the primase region and aromatic sidewalls of CNTs, and has been demonstrated for other proteins [31-33]. The helicase central hole is composed of basic amino acid residues which attract negatively charged phosphate backbone of ssDNA due to natural attractions of acid-base chemistry [43]. Hydrolysis of NTP provides energy for helicase functionality (i.e. to pull the ssDNA through the central hole, and thus unwind the dsDNA). As the ssDNA moves through the helicase, it will come into proximity (~3 nm) with the conductive CNT. Each nucleotide has different charge densities and dipole moments due to slightly different molecular composition. These variations in charge densities within the ssDNA that flow through the helicase central hole may be sensed and analyzed to sequence DNA.

1.5.3 Design Requirements and Challenges

The design requirements considered for the portable DNA detector based in comparison with existing sequencing techniques included:

1) Stability: robust attachment between nanotube and helicase
2) Sensitivity: ability to detect concentrations of DNA as low as 0.025 μM without amplification
3) Rapid: ability to detect DNA in one single step and unwind it at the same time, in the order of milliseconds
4) Sampling: DNA sample does not need to be purified
5) Accuracy: less chances of error due to no modification of original template
6) Higher throughput and read length: can unwind up to 75 kilo base pairs that matches the read length of helicase in vitro [44]
7) Direct-real time: simultaneously unwinding and reading DNA sequence
8) Improved temporal resolution: ability to read around 132 bases per second that matches the unwinding rate of helicase in vitro [44]

Based on these requirements, design requirements for helicase-CNT conjugate were outlined as:

1) Robust and stable attachment between helicase and CNT
2) Reproducibility of attachment method
3) Conservation of enzyme function (DNA binding, DNA unwinding, NTP hydrolysis)

4) Prevention of DNA attachment to CNT (CNT has strong affinity for DNA which can affect DNA unwinding by helicase)

5) Retention of CNT electronic structure and function

Different challenges were encountered during the progress of this project. Problems with CNT purification, isolation, reproducibility and solubility have prevented their use for biosensing [36-37]. These were addressed by investigating helicase attachment using two different solvents, organic and inorganic respectively:

1. Dimethyl Formamide (DMF), and

Retention of enzyme activity and CNT electronic structure are also critical. Non-covalent attachment method was selected due to its simplicity and ability to preserve enzyme and CNT structure and function. Robust enzyme attachment was ensured by allowing sufficient time for adsorption and constant stirring. Non-specific binding of enzyme and DNA to CNT was prevented by using surfactant Tween-20 that coats the surface of CNT, reducing surface area exposed for protein adsorption[47].

1.7 Research Approach and Methods

The research approach for development of this novel biosensor is described in this section.
1.7.1 Literature Review

This literature review covered the following topics necessary background for DNA biosensor development:

1. Current trends in DNA sequencing methods, their advantages and drawbacks
2. Significance of electronic DNA sequencing using CNTs
3. Structure and DNA unwinding mechanism of T7 helicase
4. Methods of attaching proteins to CNTs
5. Applications for DNA sensing

1.7.2 Problem Identification

Current DNA sequencing methods suffer from several drawbacks [2, 3]. Most of these methods require invasive samples such as blood, make use of bulky equipment, require special training and several days to process DNA samples.

1.7.3 Concept formulation

Recent advances in single-molecule DNA detection techniques include Single Molecule Real Time sequencing SMRT, Nanopore technology and Single Electron Transistor (SET) DNA detector. Based on this helicase immobilized CNT DNA detector is proposed. The proposed sensor uses the enzyme helicase similar to DNA polymerase employed in SMRT, which is attached to CNT such as SET DNA detector. It acts as a natural nanopore for DNA translocation. Use of DNA related proteins such as DNA polymerase, exonuclease, topoisomerase and ligase for direct detection of DNA charges have been proposed previously [11-13]. Nanoparticles such as CNTs offer significant advantages over other transducers by directly converting a biological phenomenon into an electrical signal [8, 41]. The proposed sensor aims to overcome problems with aforementioned DNA detection methods such as nanopore clogging, high cost, high error rate and limited temporal resolution with SMRT, and complex fabrication with SET [1, 42].
1.7.4 Structural Comparison

Protein size, structure, amino acid composition and hydrophobicity play an important role in attaching proteins to CNTs. Attachment methods for three proteins lysozyme (LSZ), Bovine Serum Albumin (BSA) and glucose oxidase (GOx) to CNT were studied[28, 43]. BSA is similar to helicase in terms of size, hydrophobicity and aromaticity. Lysozyme has been extensively studied as a model protein for its interaction with CNTs and GOx has been successfully employed in the fabrication of CNT-based glucose sensor [44-47]. Non-covalent attachment was selected due to its potential for preserving enzyme structure and function, and CNT optical and electrical properties.

1.7.5 CNT properties and Testing Helicase attachment

CNTs produced by laser vaporization (1 um in length and 10 nm in diameter) were obtained from National Research Council of Canada (NRC). Helicase was purchased from BioHelix Corporation, Massachusetts, USA. Helicase attachment to CNTs was conducted at Disruptive and emerging Technologies’ Lab at NRC. Confirmation of attachment was carried out using Absorption Spectroscopy [48, 49].

1.7.6 Testing Helicase Functionality

Helicase functionality (NTP hydrolysis, ssDNA binding and dsDNA unwinding) was investigated[35, 50-54]. DNA purchased from GenScript™ available in the lab was used to test for DNA binding and NTP hydrolysis by helicase using MDCC labelled phosphate binding protein (PBP) purchased from ThermoFisher Scientific. DNA unwinding was monitored using Fluorescence Resonant Energy Transfer with fluorescein (Flc) and hexachlorofluorescein (HEX) labelled DNA at 3’ and 5’ ends respectively purchased from Sigma Aldrich.

1.8 Important and Expected Results

1. Helicase can attach to carbon nanotubes using physical adsorption, possibly through pi-pi stacking with aromatic sidewalls, hydrophobic interactions and electrostatic interactions similar to other proteins such as Lysozyme (LSZ), Bovine Serum Albumin (BSA) and Glucose Oxidase (GOx).
2. Non-covalent attachment is stable for up to six months
3. The most stable interaction is predicted to be between helicase-primase region and CNT sidewalls
4. Immobilized helicase retains its NTP hydrolysis function and ability to bind and translocate along ssDNA
5. Immobilized helicase retains its ability to unwind dsDNA
6. CNT electronic structure is not compromised after functionalization

1.9 Contribution and Significance

Helicase was attached to CNT for direct binding and unwinding DNA in a single step. This helicase-CNT conjugate possessed the following attributes:

1. Fast: matches DNA unwinding rate of helicase, up to 130 nucleotides per second
2. Direct DNA unwinding: eliminates the need to purify, amplify and denature DNA to convert it to ssDNA for DNA sequencing
3. Direct sequencing: can be used for directly sequencing DNA in real-time using nucleotide charge sensing

The proposed helicase-CNT conjugate will assist in the development of a rapid, portable, affordable, highly accurate DNA sequencing device. Conjugation of helicase with carbon nanotube for electronic sensing may offer single-base resolution by exploiting speed processivity (~75 kilobases of nucleotides) of a natural biological process (DNA unwinding). Single-atom electronic sensitivity of nanoparticles such as carbon nanotubes can potentially overcome fourth generation sequencing problems such as limited spatial and temporal resolution, and sampling (Small amount of DNA samples can be used without purification and amplification). Based on these findings, enzyme-based electronic transduction using CNTs has the potential to be the next-generation DNA biosensors and sequencing devices.
2. LITERATURE REVIEW AND BACKGROUND

This literature review covers the following topics as necessary background for DNA biosensor development:

1. Current trends in DNA sequencing methods and advantages and drawbacks
2. Significance of electronic DNA sequencing using nanomaterials such as CNTs
3. Structure and DNA unwinding mechanism of T7 helicase
4. Methods of attaching proteins to CNT
5. Potential sensing applications of protein-CNT biosensors

2.1 DNA and DNA sequencing

DNA stands for deoxyribonucleic acid. DNA is made up of two strings of four nucleotide bases (Adenine (A), Guanine (G), Thymine (T) and Cytosine (C)) (Figure. 2.1) [54, 55]. Each nucleotide base is composed of a deoxy ribose sugar, a phosphate group and a unique nitrogen containing base. The nucleotide bases are connected to each other through a covalent bond between the phosphate and the sugar. The two strings wind around each other in a double helical configuration. The complementary base pairing (A binds with T, C binds with G) in each DNA strand further stabilizes the helix though hydrogen bonding. DNA is the blueprint of life: information about which proteins will be expressed in an individual is coded in the DNA. DNA is located in the nuclei of cells. It directs cell function, by performing many essential functions needed for growth and repair at molecular level such as replication (copying of DNA) and transcription (formation of RNA from DNA which is then used to make proteins).

Conversion of dsDNA to ssDNA is the first step for these cellular processes. DNA unwinding takes place when the hydrogen bonds between the complementary nucleotides are broken. The energy required to break these bonds depends on the base pairs and on interaction with neighbouring bases. Inside the cells, separation of two strands takes place with the help of an enzyme called DNA helicase.
DNA is a double helical structure composed of two strings of four individual nucleotide bases with complementary base pairing. Adapted from “Discovery of DNA structure and function: Watson and Crick”, Pray, L., (2008), Nature Education, 1(1):100

The nucleotides in a DNA strand are arranged in sequences of three nucleotides called a codon. Each codon represents a specific amino acid or a start and stop codon for protein synthesis. The complete sequence of all DNA bases inside cells is called a genome. DNA sequencing is a scientific method that involves a specific technology for determining the precise order of nucleotides (A, T, G and C) in a DNA molecule. The sequence establishes the physiological and anatomical uniqueness of an individual. Order of these nucleotides is of critical importance. Diseases are caused by modification in protein synthesis, often a result of addition, deletion or substitution of a nucleotide in the codon. DNA sequencing has numerous applications in health care, forensics, agriculture and biotechnology. Despite latest developments in DNA sequencing methods, even faster, cheaper and more sensitive technologies are needed to meet the rapidly growing demand for genome based profiling, prenatal screening, diagnosis, therapy, preventive and personalized medicine.
2.1.1 Current Sequencing Techniques: Types and Generations

Sequencing techniques employ different transduction mechanisms and can be broadly divided into three generations[3, 57]. Each generation varies in terms of sensing mechanism, read length, throughput, read accuracy and cost per base (figure 2.2) [3]. The first generation began with the classical gel electrophoresis Sanger technique (enzymatic) and Gilbert and Maxim (non-enzymatic) method[62]. Due to limited throughput and lack of scalability, these methods were largely replaced by next generation sequencing methods (NGS) or second generation sequencing methods. These methods aimed to reduce cost per base, time and amount of DNA used while increasing throughput. All NGS methods employ similar DNA modification techniques but differ in how the DNA is read. These
include automated DNA sequencer which uses capillary electrophoresis technique, real
time PCR (sequence by synthesis Illumina) that monitored increase in fluorescence with
each amplification cycle of PCR, pyrosequencing that measured visible light emitted by
luciferase, semiconductor and nanoball sequencing, and DNA microarray that relies on
DNA hybridization with complementary strands of DNA[57–59]. These methods are not
sensitive to individual molecules and require many copies of DNA fragments, rendering
these slow and incapable of handling large read length (over thousand bases)[65]. PCR
requires careful primer design and precise temperature control for detection of single base
variations. Some currently employed NGS techniques include impedimetric DNA
detection, quantum-dot-tagged microbeads, active pixel sensor based on optical
transparency, electronic hybridization sensors and ISFET (Ion selective field effect
transistor). Most of these DNA biosensors use DNA probes as bioreceptor, often labelled
with a reporter molecule.

Third generation methods have replaced DNA probes with other biological molecules such
as enzymes, or allowing DNA to directly bind to an electronic transducer. This allows
single molecule real-time sensing and greatly enhanced sensor response time and signal
transduction. An example is Single Molecule Real Time (SMRT) which uses DNA
polymerase by incorporating labelled nucleotides to a growing DNA strand that emit an
optical signal unique to the nucleotide (figure 2.3 (a))[1, 58, 61].

Third generation techniques have demonstrated the use of intrinsic charge and mass of
DNA molecules for single-molecule detection using various transducers. Some examples
include silicon field effect transistors (FETs), silicon nanowires, gold nanoparticles, carbon
nanotubes and microscopic techniques (scanning tunnelling and electron)[8, 62-63].
Figure 2.3 (a) SMRT (Single Molecule Real Time Sensing) uses an immobilized DNA polymerase which adds labelled nucleotides to a ssDNA strand. Adapted from “Real-Time DNA Sequencing from Single Polymerase molecules”, John Eid, Adrian Fehr, Jeremy Gray, Khai Luong, John Lyle, Geoff Otto, Paul Peluso, David Rank, *Science*, (2009), vol. 323 no. 5910, pp 133-138 (b) Nanopore technology comprises of a synthetic or biological nanopore. Adapted from “DNA Sequencing with Nanopores”, Gregory F. Schneider and Cees Dekker, *Nature Biotechnology*, (2012), 30, 326-328.

Fourth generation techniques have followed the same course with the most recent DNA nanopore technology which uses a biological, synthetic or hybrid bioreceptor[64-67]. DNA
nanopore technology utilizes the differences in nucleotide base sizes and charge for determining DNA sequence (figure 2.3 (b)). An external voltage is applied to a nanopore. DNA passes through the pore, resulting in a change in current flowing across the pore proportional to type of nucleotide. Another DNA-based sensor makes use of the enzymatic activity of topoisomerase for real time measurement [11]. One study has reported DNA sequencing by measuring conductance of DNA polymerase as it adds nucleotides to a growing DNA strand using electron microscopy and tunneling current [13, 67].

Fourth generation techniques may allow further studying of DNA-protein and protein-protein interactions outside and directly inside the cell[66]. These in situ sequencing methods have the potential for localizing specific sequences subcellular, which can help unravel complexity of various tissues and determine specific treatment locations for disease[3,42]. These can allow DNA sequencing of many cells in parallel, helping distinguish tumor differences in neighbouring cells.

2.2 Electronic DNA sequencing based on charge sensing

2.2.1 DNA sequence and Intrinsic DNA charge

Several studies have been reported on DNA detection and characterization based on intrinsic DNA charge[6, 62, 64, 69-70]. DNA charge depends on electronic states that are a result of electronic coupling between neighbouring bases due to sugar-phosphate backbone, and base stacking in the same and across strands[67]. Two different factors contribute to the overall stability of DNA duplex: base pairing between complementary strands and stacking energy between adjacent bases[62-63, 71-72]. Each nucleotide base in a DNA strand possesses a unique charge based on its composition, and position in the strand due to influence from neighbouring bases.

Stacking interactions between complementary base pairs are sequence dependent (different bonds have different stability). Charge transport in DNA has been shown to depend on neighbouring and complimentary bases (Figure. 2.5)[76]. Different effects of stacking and base pairing energies suggest sequence-dependent binding and opening of the DNA double helix. Differences in base composition, such as variations in number of G,C and A,T result in different conductance values in ssDNA and dsDNA[67].
Figure 2.4 Schematic representation of bond strength differences based on influence from neighbouring bases. Different amount of energy is required to break hydrogen bonds between same complementary bases Adenine (A) and Thymine (T) in a DNA strand depending on neighbouring bases.

G and C have proven to have higher electrostatic potential than A and T. Since an addition, substitution or deletion of a base in a DNA sequence greatly alters its conductance, measuring individual charges on nucleotide bases in real time may allow determination of the DNA sequence.

2.2.2 Electronic DNA detection using nanoparticles

Third and fourth generation techniques are aiming towards electronic, label-free and single-molecule techniques. Conductance and field-effect detection techniques exploit changes in electronic parameters when DNA hybridizes to a substrate (complementary DNA, or a transducer itself)[6, 62–64]. Many studies have highlighted use of intrinsic charge for DNA detection using CNTs and various transducers[10, 41, 73, 74].

Nanoparticles offer tremendous advantages over conventional transducers for DNA sensing. DNA samples can be used without modification or amplification. Their high
surface-to-volume ratio increases specificity for DNA molecules. Attachment of proteins and other biomolecules to nanoparticles allows high specificity, sensitivity and signal to noise ratio for electronic transduction. Nanoparticles can be incorporated into scalable, portable and miniaturized devices with low power consumption.

Various electrical DNA detection techniques exploit conductance and field-effect detection techniques to measure chemical changes that occur during DNA-related process such as hybridization, unwinding, amplification or ligation [6, 69]. Charge regulation around a DNA molecule can be amplified using highly sensitive charge transfer properties of nanoparticles. Coupling electronic sensing with proteins can greatly enhance temporal and spatial resolution needed for single base detection by exploiting speed and processivity of proteins, allowing measurement of enzyme kinetics in real-time. It can be carried out by measuring nucleotide specific electrostatic charge distributions, hydrogen bonding and/or mass of nucleotide bases [69, 73, 75]. Use of proteins such as DNA polymerase exonuclease, topoisomerase, ligase and other DNA related proteins for direct detection of electrochemical reactions by DNA nucleotide bases or intercalating molecules that bind to specific nucleotides has been proposed previously [10, 12, 73, 76]. This has been demonstrated by using biological nanopores, coupling a biomolecule with synthetic nanopores and/or nanoparticles, and synthetic biological protein nanowires[10, 59, 64–66]. One of the hindrances in achieving single-base sensitivity with nanopore has been the thickness of the nanopore. Use of nanoparticles such as graphene sheet, carbon nanotube and nanowires can overcome this due to their thinness (one atom thick)[69, 77].

2.3 Carbon nanotubes (CNT) for Biosensing and DNA sequencing

Carbon nanotube (CNT) biosensors are based on electrochemical, mechanical, optical and electronic transduction mechanisms[78–81]. The superiority of CNTs over other nanomaterials has been demonstrated for various biosensing applications, including DNA sensors[78, 79, 82-84]. Despite major advances in nano-biosensor development, problems with biomolecule immobilization, sampling such as transport of analyte molecules to the active sensor site, stability, and steady sensor response remain complex.
Figure 2.5 Measurement of current using nucleotide charge distribution. Adapted from “Nanopore Sensors for Nucleic Acid Analysis”, Bala Murali Venkatesan and Rashid Bashir, *Nature nanotechnology*, (2011), 6, 615-624. (a) Current through nanopore depends on charge distribution of individual nucleotide (b) The four nucleotides can be distinguished for determining DNA sequencing
2.3.1 Protein attachment to CNTs

Selectivity, sensitivity, responsiveness and stability of a biosensor depend on appropriate immobilization method[88]. Proper functionalization with biomolecules, such as carbohydrate, nucleic acids, proteins, antigens, enzymes, peptides and drugs can overcome problems associated with CNTs. Enzymes and other proteins can assist in debundling of CNTs, improving solubility in aqueous and non-aqueous solvents which can transform CNTs into highly specific and sensitive biosensors [94, 97]. Various immobilization methods such as physical adsorption, surfactant assisted, polymer wrapping, endohedral functionalization and covalent attachment can be used to attach proteins to CNTs [10,40,41]. Covalent chemical techniques involve functionalization with acids, salts and ionic liquids which introduce oxygen rich carboxylic ends on the CNT surface. Noncovalent attachment methods preserve the sp$^2$ CNT structure and electronic characteristics as well as enzyme structure and functionality [9, 39, 41, 43, 101, 102].

The first step for enzyme functionalization is dispersing CNTs in a solvent. Different types of CNTs demonstrate varying degree of debundling in organic and inorganic solvents. Organic chemicals can interact hydrophobically, using pi-pi stacking, hydrogen bonding and electrostatic attractions with sites on CNT surface[93]. CNTs undergo limited solubility in most organic solvents, and some polar organic solvents have been used for dispersing CNTs for enzyme conjugation [48, 104-106]. CNTs are generally insoluble in inorganic solvents such as water, salt solutions, or aqueous buffers due to their hydrophobicity [24]. Studies have reported improved wetting fraction of CNTs in human serum and plasma, which can be attributed to the presence of proteins such as fibrinogen, serum albumin and clotting factors [24].

Some techniques that are used for dispersing CNTs in solvents consist of mechanical dispersion techniques, physical adsorption, and/or chemical treatment. Mechanical dispersing techniques include sonication, centrifugation and filtration. These techniques break down large aggregates by overcoming van der Waals forces, introducing molecules on to the surface for higher reactivity, and separating insoluble particles from soluble ones [29, 94, 103, 105, 106]. Addition of a surfactant or polymer after dispersion can prevent reaggregation [84, 99, 105, 107-110]. A surfactant is a substance that lowers surface
tension of two substances using its hydrophilic and hydrophobic part. Hydrophilic part interacts with the solvent, while hydrophobic part remains exposed to interact with CNT surface solvent. Polymers accompanied by ionic liquids, biomolecules and surfactants can also facilitate stable formation of CNT-enzyme complex through hydrogen bonding between CNTs and proteins. Some common surfactants that can substantially adsorb on CNT surfaces using hydrophobic or pi-pi interactions include Tween 20, Triton X-100, biomolecules such as DNA, enzymes, proteins, polymers and ionic liquids [1, 9, 20, 29, 34]. Tween 20 has been used to prevent non-specific binding of proteins to CNT surface [104].

Figure 2.6 Chemical functionalization methods for CNTs include (a) covalent attachment (b) non-covalent adsorption (c) surfactant assisted (d) polymer wrapping and (e) endohedral
CNTs have a natural affinity for proteins and enzymes due to their amphiphilic nature. Hence, a protein can itself act as a surfactant. The hydrophobic tail interacts with CNT sidewalls, while hydrophilic head is exposed on the surface to impart solubility in aqueous media. Enzymes can be adsorbed spontaneously on the surface via this hydrophobic interaction and then further stabilized through electrostatic attractions, π-π stacking interaction between aromatic rings of enzymes and sidewalls of CNTs, or attached covalently by treatment with other compounds [10, 12, 31]. The non-covalent interactions depend on size and structure of an individual enzyme and features of solvent media such as ionic strength, pH and temperature [9, 28, 111, 112]. Various proteins and enzymes have been immobilized onto CNTs using a combination of these functionalization techniques [9, 10, 15, 23, 26, 37, 40, 43, 44].

CNTs can also be directly tip sonicated in protein solution[114, 115]. However, this may damage protein structure which is undesirable for biosensing. Direct bath sonication in protein solution, or bath sonication in buffer followed by incubation with protein solution can preserve protein structure [10, 27, 31]. Incubation ensures that enzyme sufficiently adsorbs on to sidewalls of CNTs and prevents reaggregation. Structural comparison of enzymes such as lysozyme, oxygenase, acetylcholinesterase and lipase reveals that some enzymes tend to get attached to bundled CNTs, while other enzymes are better at binding with individual CNTs or smaller bundles [91, 92]. A number of proteins and biological enzymes have been conjugated using physical adsorption method in physiological buffers [19, 22, 26, 31, 42, 45, 46, 85].

Covalent attachment is carried out by treating CNTs with acids. This introduces reactive groups on the surface. Proteins can be attached using carbon amide coupling chemistry between free amine groups on enzyme surface and carboxylic ends of oxidized CNTs [106]. This method often results in loss of enzyme structure and functionality [29, 92]. Chemical techniques also alter electrical and optical properties of nanotubes by delocalizing the pi network, which is not be suitable for development of electronic biosensors [1, 10, 29, 31, 35].
2.3.2 Protein-CNT interactions

Many proteins and biological enzymes retain their primitive structure and activity when conjugated with CNTs, and some remain stable at higher temperatures compared to isolated proteins in solution[111]. Interactions depend on surface chemistry of CNTs and proteins. Proteins are composed of many different types of amino acids which results in many types of interactions between the protein and CNT. These include pi stacking, hydrophobic interactions and electrostatic interactions. Pi stacking is the most dominant, followed by hydrophobic interactions between hydrophobic chains of amino acids and nanotubes, and then electrostatic interactions.

Aromatic content of enzymes directly correlates with conjugation strength. Residues such as tryptophan (Trp), tyrosine (Tyr), phenylanaline (Phe) and histidine (His) primarily reside on protein surfaces and strongly affect binding process [29, 33, 84, 116]. Tryptophan has the highest binding affinity for individual and bundled CNTs [10, 52, 53]. Tyrosine is more selective for individual CNTs and does not bind to bundled CNTs [23]. Human Serum Albumin (HSA) attaches to CNTs via pi-pi stacking between aromatic tyrosine residues and CNT sidewalls [40]. An increase in the aromatic content of enzymes and other proteins improves solubility of CNTs due to amplified pi-pi interactions between hydrophobic surfaces [32, 43, 116]. Certain amino acids, such as histidine and tryptophan have shown to induce changes in electrical conductance through CNTs.

CNTs bind more strongly to some proteins than others, and undergo changes in structure for some. In one study, CNTs exhibited highest affinity for Bovine Fibrinogen (BFG), followed by Immunoglobulins (Ig), Tissue factor (Tf) and BSA (Figure 2.11) [42]. Slight changes in tertiary structure take place when hydrophobic core of protein gets exposed to form pi bonds with CNTs. Amino acids with hydrophobic side chains undergo least conformational change in tertiary structure, as hydrophobic chains can directly interact with CNT surface. Enzymes and proteins that have similar amino acid content but differ in their secondary and primary structure may respond differently upon adsorption onto the same CNT surface [29, 117].
Different proteins have different amino acids on the surface to interact with CNTs. SBP retains its structure while CT loses its enzymatic activity when attached to CNT using similar procedure [94]. Other proteins that undergo conformational change upon binding include BFG and Ig.

Size of a protein also plays a significant role in binding. Smaller proteins such as streptavidin (60 kDa) adsorb more readily as compared to larger proteins such as fibrinogen (340 kDa) [113]. Proteins, peptides and surfactants have been found to interact more strongly with CNT bundles than with individual tubes, and removal of excess protein from CNT-protein solutions causes proteins to detach from CNTs due to equilibrium between protein-CNT conjugates and free protein [55].
Figure 2.8 (a) Vials containing protein-CNT dispersions at different pH values. Hemoglobin (HBA), Histone (HST), Myoglobin (MGB), Ovalbumin (OVB), Trypsin (TPS). (b) 3D plot showing degree of debundling (DD) as a percentage with respect to the percentage of different amino acids. Adapted from “Proteins and carbon nanotubes: Close encounter in water”, Nepal, Dhriti, Geckeler, Kurt E., Small, 2007, vol 3, no 7, pp 1259-1265.

Regulation of pH values either below or above the isoelectric point (pH at which overall charge on a molecule is zero) of enzyme can also affect attachment [37]. Changes in pH of surrounding medium shifts the isoelectric point towards positive or negative, influencing formation of stable enzyme-nanotube conjugates through electrostatic attractions, reducing hydrophobic interactions between individual tubes and preventing aggregation [28, 84, 112]. Increase in pH activates the polar amino acids in an enzyme, inducing electrostatic attractions between enzyme and CNTs. Decrease in pH accelerates hydrophobic interactions, hydrogen bonding and van der Waals forces [28, 33, 112]. Electrostatic interactions through polar amino acids such as Arginine also stabilize interactions through cation-pi exchanges [42].
Table 2.2.3.2-1 Methods for protein immobilization and their respective effect on protein-carbon nanotube interaction

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dispersing technique</th>
<th>Interaction</th>
<th>Characterization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSZ, HBA, MGB, OVB, BSA, TPS, GOD, HST</td>
<td>Ultrasonication in enzyme solution and double step ultracentrifugation</td>
<td>Vander Waals, cation-pi, pi-pi</td>
<td>Supernatant collected for AFM, Raman, UV-Vis-NIR</td>
<td>[88]</td>
</tr>
<tr>
<td>BSA, HRP, SBP</td>
<td>Ultrasonication in enzyme solution and ultracentrifugation</td>
<td>Pi-pi, hydrophobic interactions</td>
<td>UV-Vis, Raman, AFM</td>
<td>[67, 104]</td>
</tr>
<tr>
<td>SBP, CTR</td>
<td>Sonication in DMF, microcentrifuge, incubation with enzyme</td>
<td>Pi-pi, hydrophobic interactions</td>
<td>AFM, FT-IR</td>
<td>[72]</td>
</tr>
<tr>
<td>ACH</td>
<td>Ultrasonication in PBS, shaking with enzyme solution and centrifugation</td>
<td>Hydrophobic, pi-pi interactions</td>
<td>Enzyme activity test, pellet retained</td>
<td>[83]</td>
</tr>
<tr>
<td>IgG</td>
<td>Mixing protein solution with and without surfactant</td>
<td>Hydrophobic, pi-pi</td>
<td>AFM</td>
<td>[114]</td>
</tr>
<tr>
<td>Lipase</td>
<td>Sonication in PBS, shaking with enzyme solution and centrifugation</td>
<td>Pi-pi, electrostatic attractions</td>
<td>Pellet retained</td>
<td>[115, 116]</td>
</tr>
<tr>
<td>HAS</td>
<td>Incubation in protein solution with recurrent sonication</td>
<td>Pi-pi stacking with Tyrosine residues</td>
<td>AFM, UV-Vis-NIR</td>
<td>[106]</td>
</tr>
</tbody>
</table>

Some proteins such as LSZ and HST dissolve better at basic pH, whereas others such as TPS and GOD show less or no solubility at basic pH (Figure 2.12) [28, 112]. Proteins with higher content of polar residues disperse CNTs best at pH values higher or lower than the isoelectric point. Adsorption of biomolecules such as phospholipids and proteins, and other
surface functionalization approaches shift the isoelectric point of CNTs [118, 119]. Cation-π exchanges further stabilize enzyme-nanotube conjugation [9, 31, 84, 93].

2.3.3 Proteins immobilized on CNTs

Employment of non-covalently enzyme functionalized CNTs for development of numerous biosensors has been reported in literature for healthcare applications and beyond (Table 2.1)[58, 60, 106]. High specificity and sensitivity of enzyme-CNT conjugates can be used for label-free detection of biomolecules, antigen-antibody interactions, DNA hybridization and enzymatic reactions, for profiling and identification in forensics, security and defense [86]. Immunosensors can be used for detection of virus and bacteria for infections. Antibodies attached to carbon nanotube sidewalls have been used for detection of antigens in autoimmune disorders and immunoassaying for detection of prostate specific antigen (PSA) and HRP [60, 120]. Glucose oxidase conjugated carbon nanotubes have been used for glucose sensing [33, 45].

Point-of-care immunoassays have also been investigated for biomarker screening for early cancer diagnosis [124]. Changes in conductance of carbon nanotube field effect transistors (CNTFETs) have been investigated after protein immobilization with DNA, lysozyme, DNA polymerase and kinase [10, 62, 63]. These sensors were reported to have higher sensitivity than current fluorescent techniques and can be fabricated using simple lithography steps, where the carbon nanotube acts both as a sensor and transducer with unprecedented resolution.

Biocatalysis is an emerging field in biotechnology involved with fuel production, environmental protection, health and food supply [85]. Biological enzymes can catalyze reactions at a much higher rate than physical or chemical catalysts and ensure reusability. Protein-carbon nanotube conjugates have proven to be profitable for use in biofuel cells and bioconversion, providing a green alternative to conventional production methods [125]. Biofuel cells convert biomass waste into sugars by hydrolysis for ethanol production. Amyloglucosidase was physically adsorbed on to single-walled and multi-walled carbon nanotubes to assess its functionality for ethanol production [122].
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Analyte</th>
<th>Biosensing area</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Oxidase</td>
<td>Glucose</td>
<td>Field-Effect and Electrochemical</td>
<td>Biocatalysis</td>
<td>[117]</td>
</tr>
<tr>
<td>Lipase</td>
<td>triglycerides, cholesterol, and phospholipids in blood, production of esters and alcohols</td>
<td>Electrochemical</td>
<td>Diagnostics and Biocatalysis</td>
<td>[45, 64, 128]</td>
</tr>
<tr>
<td>Acetylcholinesterase</td>
<td>Organophosphates and other pesticides</td>
<td>Electrochemical</td>
<td>Diagnostics and Biotechnology, pollutant</td>
<td>[119]</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>protein and nucleic acid delivery</td>
<td>Electrochemical</td>
<td>Field-Effect</td>
<td>66[121]</td>
</tr>
<tr>
<td>Inulinase</td>
<td>Inulin hydrolysis</td>
<td>Electrochemical</td>
<td>Biofuels</td>
<td>[111]</td>
</tr>
<tr>
<td>Oxygenase</td>
<td>Organic pollutants</td>
<td>Electrochemical</td>
<td>Biotechnology</td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>Urea, Cancer cells</td>
<td>Electrochemical, Optical</td>
<td>Biotechnology, Drug Delivery</td>
<td>[87]</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>Starch and glucose, ethanol production</td>
<td>Electrochemical</td>
<td>Biocatalysis</td>
<td>69[123]</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>Trace metals, thrombin and phenyl hydrazine, Nitric oxide</td>
<td>Electrochemical</td>
<td>Diagnostics</td>
<td>[22]</td>
</tr>
<tr>
<td>Kinase, polymerase</td>
<td>DNA, dNTPs Kemptide (PKA), ATP</td>
<td>Field-Effect</td>
<td>Diagnostics</td>
<td>[120]</td>
</tr>
</tbody>
</table>
Microbial and biological enzyme-based biofuel cells can be used for the development of self-powered biosensors for detection of energy conversion and biocatalysis. Enzyme-CNT sensors can be developed for detection of minute amounts of pathogens in food and water, and pollutants (organophosphates and pesticides) for environmental monitoring [121]. Recently there has been a growing interest of biosensor applications for tissue engineering and regenerative medicine. Acoustic, optical and electrochemical transduction mechanism can be employed for detection of specific molecules indicative of diseased and damaged tissues and organs. Fluorescence modulation of biocompatible enzyme-CNT conjugates can be used for development of nano-probes for bio-imaging. The unique photoluminescence properties of CNTs in biological media allow biosensing at single particle level. Functionalized CNTs are being used as optical probes in the near-IR (NIR) region for fluorescent imaging of cells [38]. Different proteins and enzymes can be conjugated with carbon nanotubes for imaging of tumors [126].

2.4 DNA unwinding and DNA helicases

DNA helicase is a motor protein responsible for unwinding DNA for many cellular processes such as transcription, replication and DNA repair. Helicases also play a vital role in genome stability, and mutations in these are associated with various diseases, susceptibility to cancer and premature aging [20, 35, 53].

2.4.1 T7GP4 DNA helicase structure and function

Helicases can be divided into five main groups based on seven highly conserved motifs, I, Ia, II, II, IV, V and VI. SFI and SFII are the largest groups discovered to date [20, 130-132]. These contain three highly conserved regions, the core helicase domain N-terminal and the C-terminal (RQC) domain that form the catalytic core, and the helicase and RNase D–like C-terminal (HRDC) domain (Figure 2.14). There are other regions that flank the catalytic core at N and C terminals that are important in subcellular localization, oligomerization and other enzymatic activities. Different helicases possess unique polarity in terms of directionality of translocation along DNA strand, either 5’ to 3’ or 3’ to 5’ and also varying substrate specificity [20, 132].
DNA helicase used for this application is hexameric helicase, bacteriophage T7 gene-4. T7 helicases are ring-shaped motor proteins which belong to SFII family of helicases, with polarity 3’ to 5’, an inner diameter of around 4 nm, and outer diameter about 12 nm [130]. N terminal of helicase is responsible for primase activity (primer synthesis). It is located on the outside of the helicase ring. C terminal takes part in DNA binding and is acidic in nature. The active site of the helicase is located at the interface between the six subunits of the hexamer [131]. It has six nucleotide binding sites at the interface between subunits. Each subunit can accept one nucleotide, while other subunits are nucleotide-ligated and they interact with the DNA to ensure translocation [57]. In the presence of ssDNA (ssDNA overhang or Y-shaped DNA in case of dsDNA), helicase gets activated, pulling single strand of DNA through the central channel of the hexamer. This breaks hydrogen bonds between nucleotide bases of the DNA molecule and energy released from NTP hydrolysis fuels helicase translocation along DNA for further unwinding [29].

2.4.2 Sequence dependent function of Helicase and cycle of DNA unwinding

The process of DNA unwinding goes around in a cycle. The first step is NTP binding, followed by hydrolysis and then release of NDP. This cycle goes around the helicase ring
as helicase translocates along one of the DNA strands. DNA is passed from one subunit to the next, as helicase progresses at a rate of one nucleotide for each hydrolysis reaction and separates the base pairs [19, 24].

Two different models have been proposed for this mechanism: coordinated and uncoordinated [132]. In the coordinated model, NTP hydrolysis occurs as a sequential cycle around the helicase ring. Each subunit binds to a different nucleotide, and a maximum of four subunits bind to four nucleotides at any instant [22, 50]. Studies have shown that leading subunit accepts a nucleotide. Other subunits coordinate with each other for binding nucleotides and hydrolysing these or interact with DNA [132]. The subunits go through various NTP ligation states, including empty, NTP, NDP*Pi, and NDP. Each subunit’s affinity for DNA is affected by the state of NTP ligation and the type of nucleotide bound to it. These states control helicase affinity for DNA resulting in conformational changes that separate base pairs and drive helicase translocation. The cycle is completed when the last subunit releases DNA.

**Figure 2.10** Schematic representation of DNA unwinding cycle: helicase subunits go through three possible states (Empty, NDP+Pi, NTP) as shown
According to the uncoordinated model, each helicase subunit functions independently. It accepts a nucleotide, binds ssDNA, hydrolyzes NTP, liberates Pi and then ultimately releases the DNA strand. During each state the affinity of subunits for DNA changes. This causes DNA to be transferred along the enzyme [58]. Translocation proceeds at a rate of 130 nt/s along ssDNA, hydrolyzing one NTP per three-base movement. Estimated kinetic step size of unwinding by T7 helicase is around 10 bp, where it unwinds ~4 bp of AT-rich DNA or 1–2 bp of GC-rich DNA. Recent studies have revealed that DNA helicase and polymerase work in conjunction to unwinding and replicate DNA in single nucleotide steps [48]. This finding may suggest that helicase is capable of unwinding DNA in single nucleotide steps alone, and that all 6 subunits are actively engaged during this process. Hence ssDNA passes on from one subunit to the next in steps of single nucleotides.

2.5 Discussion

Evolution of DNA sequencing techniques has been towards direct, label free and real-time detection (Figure 2.16).

**Figure 2.11** Evolution of DNA sequencing techniques towards single molecule, real-time and label-free technologies

These offer huge implications in early diagnostics, preventative and personalized medicine, forensics, defense and bioterrorism, pharmaceutics, food and agriculture, and
environmental monitoring. First and second generations depended heavily on PCR, DNA amplification and fluorescence imaging. Third and fourth generations have allowed using individual DNA molecule as sequencing template through direct measurement and/or real-time imaging of primer extension. such as DNA nanopore and transistor mediated techniques have demonstrated the use of nucleotide specific charge and mass properties for electronic sensing. DNA sensing based on intrinsic molecular charge can allow direct real-time detection by eliminating the use of DNA probes as bioreceptors, and employing other biological and synthetic probes or nanoparticles. This has already been shown for fourth generation sequencing methods SMRT and DNA nanopore technology. Single-atom electronic sensitivity of nanoparticles such as graphene, carbon nanotubes and nanowires can overcome fourth generation sequencing problems such as limited spatial and temporal resolution and simplify readout. Conjugation of DNA related proteins with nanoparticles for electronic sensing can offer single-base resolution by exploiting speed processivity of a biological process. This can eliminate the complicated steps associated with classical sensing techniques such as denaturation, fragmentation, labelling, modification, hybridization and amplification of DNA. This can be exploited towards development of sensors that can quickly and accurately detect and read minute nucleic acid samples, requiring minimal training for use. Electronic transduction using nanoparticles has the potential to be the next-generation DNA biosensors and sequencing devices
3 SELECTION OF ATTACHMENT METHOD

This chapter discusses steps involved in selecting the optimal method for attaching helicase to CNTs, predicts possible interactions between the protein and CNT, and experimental design of nano-biosensor.

3.1 Rationale

Immobilization methods affects selectivity, sensitivity, responsiveness and stability of a biosensor. Different proteins may interact differently with CNTs based on their structure, amino acid composition and attachment method [28, 102, 103, 112]. It is important to select the optimal method for attaching helicase to CNT, without compromising protein function and CNT electronic structure.

3.2 Purpose

To develop an enzyme-CNT conjugate by attaching helicase to CNTs, for incorporating into a portable DNA detector that would sequence DNA using individual charges on nucleotide bases, by following steps outlined below:

![Figure 3.1 Schematic of Proposed Helicase-CNT DNA sensor](image-url)
a. To compare helicase structure with other protein structures for selecting appropriate immobilization method
b. To inspect Helicase-CNT attachment using physical and non-covalent methods
c. To analyze DNA binding and NTP hydrolysis to the Helicase-CNT conjugates
d. To determine DNA unwinding by Helicase-CNT conjugates
e. To investigate preservation of CNT electronic structure and properties for electronic sequencing

Figure 3.1 shows a schematic of the proposed conjugate.

3.3 Introduction

Different methods of bio-conjugation for proteins with CNTs were reviewed and three proteins lysozyme (LSZ), Bovine Serum Albumin (BSA) and glucose oxidase (GOx) were selected for comparison due to the following reasons:

1. LSZ has been extensively studied as a model protein for its interaction with CNTs [121]
2. GOx has been successfully employed in the fabrication of CNT-based glucose sensor [44-46]
3. BSA is similar in size to helicase, and has been attached to CNTs for various applications [28, 43]

Different types of interactions are possible between CNTs and proteins based on surface chemistry of the two aromatic structures. Protein composition and structure greatly affects type of interactions between the two surfaces [21]. Size of protein has a significant effect on protein adsorption by CNTs, where smaller molecules adsorb more readily than larger proteins [33, 117].

Table 3.3-1 shows possible interactions between CNTs and three different amino acids BSA, GOx and LSZ.
### Table 3.3-1 Type of interactions between CNTs and different amino acids for BSA, GOx and LSZ

<table>
<thead>
<tr>
<th>Type of interaction</th>
<th>Type of interacting residue (Protein)</th>
<th>Type of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vander Waals forces</td>
<td>Non-polar residues</td>
<td>BSA</td>
</tr>
<tr>
<td>Pi-pi stacking</td>
<td>Aromatic residues</td>
<td>BSA, LSZ, GOx</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>Hydrophobic surface residues, total hydrophobic residues</td>
<td>BSA</td>
</tr>
<tr>
<td>Electrostatic interaction</td>
<td>Positively charges residues</td>
<td>LSZ, BSA</td>
</tr>
<tr>
<td>Amphiphilicity</td>
<td>Amphiphilic residues</td>
<td>BSA, LSZ</td>
</tr>
</tbody>
</table>

The aliphatic index of a protein indicates the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). Aliphatic amino acids are different from aromatic residues. They do not form a stable ring of atoms, and are non-polar and hydrophobic. Knowledge of aliphatic index can help in predicting a protein’s interaction with other aromatic and aliphatic compounds. Increasing aliphatic index leads to greater retention of protein secondary structure.

Iso-electric point (pI) of a protein is defined as the pH at which a protein is neutral. Shifting the pH of the surrounding medium away from its pI can activate polar residues, facilitating electrostatic interactions between protein and other compounds.

The Grand average of Hydropathicity Value (GRAVY) of a protein determines how active the protein will be in aqueous solutions. It is calculated as the sum hydropathy values of all the amino acids, divided by the number of residues in the sequence. It ranges on a scale
of -2 to +2, where -2 is the most hydrophilic and +2 is the most hydrophobic. Percentages of hydrophobic residues greater than 35% can disperse CNTs [38].

3.4 Methods

3.4.1 Protein Analysis using ProtParam

ProtParam was used to calculate physical features mentioned in section 3.3 for LSZ, BSA and GOx using protein sequence (Tables 3.4.1-1).

Table 3.4.1-1 Comparison of protein parameters for Helicase, GOx, BSA and LSZ. Detailed information on helicase composition can be found in Appendix A.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Helicase</th>
<th>GOx</th>
<th>BSA</th>
<th>LSZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of amino acids</td>
<td>566</td>
<td>583</td>
<td>583</td>
<td>129</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>62655.45</td>
<td>63273.40</td>
<td>66432.96</td>
<td>14313.14</td>
</tr>
<tr>
<td>Theoretical pI</td>
<td>5.35</td>
<td>4.94</td>
<td>5.60</td>
<td>9.32</td>
</tr>
<tr>
<td>Total number of negatively charged residues (Asp + Glu)</td>
<td>84</td>
<td>66</td>
<td>99</td>
<td>9</td>
</tr>
<tr>
<td>Total number of positively charged residues (Arg + Lys)</td>
<td>65</td>
<td>37</td>
<td>82</td>
<td>17</td>
</tr>
<tr>
<td>Aromatic residues</td>
<td>Trp, 10, 1.8%</td>
<td>Trp, 10, 1.7%</td>
<td>Trp, 2, 0.3%</td>
<td>Trp, 6, 4.7%</td>
</tr>
<tr>
<td></td>
<td>Tyr, 15, 2.7%</td>
<td>Tyr, 27, 4.6%</td>
<td>Tyr, 20, 3.4%</td>
<td>Tyr, 3, 2.3%</td>
</tr>
<tr>
<td></td>
<td>Phe, 19, 3.4%</td>
<td>Phe, 18, 3.1%</td>
<td>Phe, 27, 4.6%</td>
<td>Phe, 3, 2.3%</td>
</tr>
<tr>
<td>Aliphatic index</td>
<td>74.93</td>
<td>81.51</td>
<td>76.14</td>
<td>65.12</td>
</tr>
<tr>
<td>Grand average of hydropathicity (GRAVY)</td>
<td>-0.44</td>
<td>-0.241</td>
<td>-0.475</td>
<td>-0.472</td>
</tr>
</tbody>
</table>

3.5 Results

Protein analysis results included:

1. BSA is the most closely related protein to helicase in terms of size, aliphatic index, isoelectric point, hydrophobicity and amino acid composition (Table 3.4.1-1)
2. Most attachment methods involving GOx to CNTs are based on covalent techniques, and hence not suitable to use for helicase. Dispersion limit of GOx for CNTs was also found to be considerably lower than BSA and LSZ [105].

3. LSZ was found to have the highest dispersion limit, however attachment methods involving LSZ included sonicating CNTs in protein buffer which results in a loss of enzyme structure.

Based on the comparison, non-covalent attachment was selected due to its potential for preserving enzyme structure and function, without damaging CNT optical and electronic structure.

3.6 Discussion: Possible interactions between T7 helicase and CNT

Attachment methods similar to BSA were employed for attaching helicase to CNTs, and possible interactions were predicted based on previously reported ones for BSA (Figure 3.4). T7 helicase has two main regions, C-terminal (helicase domain) that forms the core, and N-terminal (primase) that forms the outer ring [23, 137]. Helicase domain is responsible for DNA binding and unwinding [22, 35]. Primase domain is responsible for addition of nucleotide bases for DNA replication [18, 138]. Most proteins have
hydrophobic residues buried inside the protein. Helicase core mainly consists of basic residues lysine, arginine and histidine [43]. This is because DNA binding occurs at the center of the ring, and since DNA is acidic, the core needs to be basic for it to attract DNA. Asparagine, an aliphatic amino acid also plays a vital role in ssDNA binding in the core [32]. These residues form a central β-hairpin that binds ssDNA.

Figure 3.3 NTP binding site for T7 Helicase between two subunits. Adapted from “Promiscuous usage of nucleotides by the DNA helicase of bacteriophage T7: Determinants of nucleotide specificity”, Satapathy, Ajit K. Crampton, Donald J. Beauchamp, Benjamin B. Richardson, Charles C., Journal of Biological Chemistry, 2009, vol 284, no 21, pp 14286-14295.

NTP binding occurs at the subunit interface which is composed of several conserved residues (figure 3.5) [30]. These include Gly-317, Lys-318, Ser-319, Glu-343, Asp-424, His-465, and Tyr-522 and responsible for NTP hydrolysis. The arginine finger of the adjacent subunit also takes part in coordinating hydrolysis with subunit conformational change. A side chain of Tyr-535 plays an important role in NTP binding site. Replacement with other residues led to a loss of catalytic activity, except for when it was replaced by another aromatic residue. Due to the aromatic nature of this site, it can possibly interact with CNT, affecting NTP binding.

DNA unwinding and NTP hydrolysis are coupled by a Phe-523 located at the subunit interface, and is exposed on to the surface. Upon NTP hydrolysis, a conformational change
causes the residue to get buried within the interface [136]. Consisting of a hydrophobic side chain, this site might interact with CNT surface, resulting in loss of hydrolytic activity.

Figure 3.4 N-terminal polymerase domain includes a TRP-69 and may possibly interact with CNT sidewall using aromatic pi-pi stacking. The site is important for initiating DNA synthesis. Adapted from “Direct role for the RNA polymerase domain of T7 primase in primer delivery.”, Zhu, Bin Lee, Seung-Joo, Richardson, Charles C, Proceedings of the National Academy of Sciences of the United States of America, 2010, 107, 20, pp 9099-104

The primase domain can further be divided into C-terminal Zinc Binding Domain (ZBD) and N-terminal RNA polymerase binding domain (RPD) (Figure 3.6) [39]. The aromatic residue tryptophan mainly reside in primase domain, and has a key role in recognizing primase sites on DNA, for primer delivery and DNA synthesis by DNA polymerase [39]. Since tryptophan is one of the potential residues for aromatic interaction with CNTs and other aromatic molecules, the N-terminal domain might interact with CNTs. Tryptophan also plays a major role in stabilizing proteins due to its hydrophobic nature [137]. Trp-69 side chain in primase region of helicase may contribute to increase adsorption as reported earlier for designed peptides with larger Trp side chain [23]. Other tryptophan residues in
the primase domain include: Trp-42 in the ZBD and Trp-97, -147, and -255 in the RNA polymerase domain [137].

Figure 3.5. Helicase structure generated using PDB viewer showing helicase (left) and primase (right) regions. TRP-69 located in primase region may interact with CNT surface.

Figure 3.7 shows detailed picture of helicase and primase structure that was generated using PDB in section 3.4. Replacement of these tryptophan residues with other amino acids affected primer synthesis and NTP binding ability. However, since these tryptophan residues are located in the primase domain, loss of function may not have any effect on helicase processivity. Helicase may interact with carbon nanotube sidewalls using aromatic pi-pi stacking, Vander Waal’s forces and hydrophobic interactions. Since majority of Trp residues reside in the primase domain it may be possible that CNT will interact with the primase domain only through hydrophobic and pi-pi interactions.
3.7 Conclusion

Helicase is like BSA in terms of size, aliphatic index, isoelectric point, hydrophobicity and amino acid composition. These properties play an important role in directing protein attachment to CNT sidewalls. Hence, non-covalent attachment methods that have been reported in literature for attaching BSA to CNTs may be used for attaching helicase to CNTs as well.
4. PREPARATION OF CARBON NANOTUBE SUSPENSION FOR HELICASE ATTACHMENT

4.1 Rationale

Aromatic protein residues can non-covalently bind to CNT surface via pi-pi, Vander Waals, electrostatic and hydrophobic interactions [88, 97, 141]. Separation and solubilizing of CNTs is the first step for protein attachment. Type of attachment method was selected in chapter 3. Different solvents, organic and inorganic can be used for preparation of CNT suspension and can affect protein interaction with CNT surface. Therefore, it is important to investigate the best solvent for helicase attachment to CNTs.

4.2 Purpose

The purpose of this chapter was:

1. To prepare a CNT suspension in an organic solvent Dimethyl formamide (DMF), and non-organic solvent Milli-Q water in the presence of a surfactant Tween-20, using ultrasonication (bath sonication and tip sonication)
2. To investigate helicase attachment to CNT surface in the two solvents using non-covalent adsorption
3. To investigate the effect of enzyme concentration on CNT solubility
4. To compare tip sonication with bath sonication for helicase attachment

4.3 Introduction

Protein attachment to CNTs imparts solubility, biocompatibility and biosensing ability to CNTs, while CNTs stabilize protein upon attachment. This is generally carried out by suspending CNTs using ultrasonication in a solvent followed by protein incubation [29, 48, 109, 112, 116, 149, 150]. Direct sonication in protein solution is also sometimes employed [28, 47, 102, 103, 112]. This method is often assisted by addition of a surfactant for improved solubility [99, 105, 108]. A surfactant is an amphiphilic molecule that dissolves in both aqueous and non-aqueous solvents [98]. The hydrophobic head binds to the CNT sidewall, and the hydrophilic tail remains exposed to the solvent. This prevents carbon nanotubes from reaggregation after separation and non-specific binding of proteins.
by limiting CNT surface available to proteins for adsorption. Figure 4.1 outlines the steps followed for attaching a protein to CNT.

![Flowchart representation of steps involved in protein attachment to CNTs](image.png)

**Figure 4.1.** Flowchart representation of steps involved in protein attachment to CNTs

### 4.4 Methods

Single walled carbon nanotubes (CNTs) produced by laser evaporation were obtained from National Research Council Canada and used without further purification. T7 Helicase (1 mg/mL, purified from an E. coli strain that overexpresses the gp4A gene isolated from the T7 phage) was purchased from BioHelix Corporation (MA, USA), stored at – 20 °C (20mM TrisHCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 50% Glycerol) and used without further purification. All other chemicals were purchased from ThermoFisher Scientific or Sigma Aldrich and used as obtained. Two different sonication methods, bath sonication and tip sonication were used. Detailed methods can be found in Appendix D.

#### 4.4.1 Helicase attachment to CNTs using bath sonication in DMF and Milli-Q water with Tween 20

**Bath sonication**

Two samples of 0.106 g CNTs were carefully measured in a vial using an analytical balance and 80 mL of DMF and 80 mL of Milli-Q water (with Tween 20 1X) was added to each respectively. Samples were vorticed using Vortex Genie 2 for 1 min followed by bath sonication using Cole Palmer Ultrasonic 8891 (115 VAC, 42 kHz) for 60 min. Next, samples were centrifuged using Thermo Scientific Sorvall Legend X1R at 10000 rpm for 30 min. Supernatant was collected for each sample and analyzed visually.

**Enzyme incubation**
Helicase solution was prepared by adding 50 µg of T7 helicase to 400 uL Milli-Q water to yield 0.125 mg/mL of T7 Helicase. 200 uL of helicase solution was added to 2 mL of CNT dispersions for each solvent and stirred on shaking incubator for 2 h at 25 degrees and 250 rpm. The dispersions were then transferred to Eppendorf tubes; 1X PBS (Phosphate Buffered Saline) was added and micro-centrifuged at 14*g for 60 min. This helped to remove unbound enzyme from supernatant which was stored separately to measure final enzyme concentration.

4.4.2 Effect of increasing enzyme concentration on helicase attachment

Increasing concentrations of helicase solution (0.0, 0.2, 0.4, 0.6, 0.8 mg/mL) were added to 2 mL of CNT dispersions and incubated for 2 h at 25°C and 250 rpm. This was followed by micro-centrifugation for 60 min at 20°C and 14 *g, and supernatant was collected.

4.4.3 Bath sonication compared to tip sonication for helicase attachment

80 mg of CNTs was tip sonicated in 100 mL DMF and 100 mL Milli-Q water for 1 min at 85% amplitude using a tip sonicator. The resultant suspensions were centrifuged using Thermo Scientific Sorvall Legend X1R for 30 min at 10000 rpm. Supernatant was collected for each sample and analyzed visually before and after helicase incubation (0.71428 mg/mL).

4.4.4 Helicase attachment through bath sonication in enzyme solution

4 mL of each CNT dispersion was bath sonicated with 2 mL helicase solution (1 mg/mL) for 60 min using Branson Ultrasonic at 45 W followed by centrifugation using Thermo Scientific Sorvall Legend X1R for 30 min at 10000 rpm. Supernatant was collected for each sample and analyzed visually.

4.5 Results

4.5.1 Helicase attachment to CNTs using bath sonication in DMF and Milli-Q water with Tween 20

1. Bath sonication in DMF and Milli-Q water yielded colored solutions (figure 4.2).
2. DMF-CNT dispersion was darker in color than Milli-Q water-CNT dispersion.
3. Microcentrifugation after enzyme incubation allowed removal of all undissolved particles from DMF and Milli-Q water suspensions, yielding colored solutions (figure 4.3).

**Figure 4.2** CNTs in Milli-Q water (left) and DMF (right) after bath sonication

**Figure 4.3** CNTs in DMF and Milli-Q before and after helicase attachment (left to right)

### 4.5.2 Effect of increasing enzyme concentration on helicase attachment

1. CNT dispersions in DMF and Milli-Q water showed color variations with increasing enzyme concentration.
2. Dispersions in DMF, highest concentration of enzyme yielded darkest color dispersion, even darker than the control sample with no enzyme (figure 4.4 (a)).
3. Dispersions in Milli-Q water yielded lighter colored dispersions when compared with DMF dispersions at the same enzyme concentration (figure 4.4 (b)).
4. Sample B for DMF dispersion became unstable after a week, while the rest remained stable and samples A-D for Milli-Q water precipitated after a week (figure 4.5).

![Figure 4.4](image1.png) CNTs in (a) DMF and (b) Milli-Q water centrifuged after helicase incubation. Decreased enzyme concentrations from left to right (from 0 to 0.8 ug/mL).

![Figure 4.5](image2.png) CNTs in (a) DMF. Samples in DMF remained clear (b) Milli-Q water one week after enzyme incubation. Some samples in Milli-Q water precipitated out of solution (B, C and D). These can be seen as tiny black spots.

4.5.3 Bath sonication compared to tip sonication for helicase attachment
1. Tip sonication in DMF yielded a darker colored solution with small aggregates suspended throughout the solvent. Tip sonication in Milli-Q water yielded a lightly colored solution with small aggregates settled down at the bottom (Figure 4.6).
2. Tip sonication in Milli-Q yielded a clear, colored solution upon enzyme attachment and microcentrifugation. Tip sonication in DMF yielded a clear, darker colored solution (figure 4.7).

### 4.5.4 Helicase attachment through bath sonication in helicase solution

1. Bath sonication in helicase solution did not yield a homogenous dispersion, and large particles were still visible upon inspection.

![Figure 4.6](image)

**Figure 4.6** Tip sonicated CNTs in DMF and Milli-Q water before adding helicase. Samples in DMF retained higher concentration of CNTs, and the solution appeared darker in color.

![Figure 4.7](image)

**Figure 4.7** Tip sonicated CNTs in DMF and Milli-Q water and bath sonicated samples after helicase incubation and centrifugation
4.6 Discussion

Supernatant color can be used to visually determine amount of CNTs dissolved in a solvent [112, 142]. As amount of dissolved CNTs in a solution increases, the color gets darker. Colorless solutions indicate little or no dissolved CNTs. Colored solution indicates dissolved individual CNTs and/or dissolved CNT bundles. Bath sonication yields more bundled CNTs, while tip sonication produces less aggregates.

Table 6-1 Initial Concentrations of helicase and CNT

<table>
<thead>
<tr>
<th>Method</th>
<th>Initial Concentration of Helicase (mg/mL)</th>
<th>Initial Concentration of CNT* (mg/mL)</th>
<th>Number of Helicase molecules</th>
<th>Number of CNT atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bath sonication followed by enzyme incubation</td>
<td>0.125</td>
<td>1.325</td>
<td>1.19*10^{18}</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td>Effect of increasing enzyme concentration</td>
<td>0</td>
<td>1.325</td>
<td>0</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.325</td>
<td>1.91*10^{18}</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.325</td>
<td>3.82*10^{18}</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.325</td>
<td>5.73*10^{18}</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1.325</td>
<td>7.64*10^{18}</td>
<td>6.65*10^{22}</td>
</tr>
<tr>
<td>Bath sonication compared to Tip sonication</td>
<td>0.71428</td>
<td>0.8</td>
<td>6.83*10^{18}</td>
<td>4.01*10^{22}</td>
</tr>
</tbody>
</table>

Four different methods were employed to select the best attachment method for helicase to CNTs: Bath sonication, tip sonication, bath sonication in protein solution and effect of...
increasing protein concentration on CNT solubility. Two solvents DMF and Milli-Q water were used. Since Milli-Q water is inorganic, a surfactant Tween-20 was added to prevent reaggregation of CNTs. Bath sonication in DMF produced darker-colored suspensions than Milli-Q Water, indicating higher solubility of CNTs in DMF as compared to Milli-Q Water.

Effect of increasing enzyme concentration on CNT adsorption was investigated. Increase in enzyme concentration increased CNT adsorption. However, upon centrifugation after removal of excess enzyme, stability of samples in Milli-Q water decreased and tubes precipitated out of solution. Tip sonicated samples were compared to bath sonicated samples. Tip sonication yielded darker colored dispersion in DMF as compared to bath sonication, but lighter colored dispersion for Milli-Q water. It was concluded that tip sonication produced less bundled CNTs in both solvents, while bath sonication produced more bundled CNTs (Figure 4.7). Bath sonication in protein solution was unable to break down large aggregates. This could be due to protein denaturation upon sonication, or because the concentration of helicase used was not enough to disperse CNTs. This method is not suitable for helicase attachment to CNT.

Initial concentration of CNTs was very high. Concentration decreased greatly after centrifugation. It was assumed that all helicase adsorbed onto CNT surface, with no free enzyme remaining in solution. Amount of CNT remaining after centrifugation was estimated based on literature.

4.7 Conclusion

Tip sonication in DMF and Milli-Q water can be used to prepare stable CNT dispersions for helicase attachment. The concentration of helicase should be greater than 1 mg/mL for 0.8 mg/mL of CNTs.
5 TESTING HELICASE ATTACHMENT TO CARBON NANOTUBES USING ABSORBANCE SPECTROSCOPY

5.1 Rationale

Spectroscopic techniques such as UV-Vis-NIR spectroscopy, Raman spectroscopy and Fluorescence spectroscopy may be used to confirm non-specific protein attachment to CNT surface. These can also be used to study the effect of protein attachment on CNT solubility and optical properties [149-153].

5.2 Purpose

The purpose of this chapter was to confirm helicase attachment to CNT, investigate CNT solubility upon protein attachment and preservation of CNT electronic structure.

5.3 Introduction

5.3.1 UV-Vis-NIR Spectroscopy

UV-Vis-NIR spectroscopy can be used to determine presence of CNTs and proteins in a sample based on their absorbance in different regions of the electromagnetic spectrum [142, 148]. Protein functionalization alters optical properties of carbon nanotubes resulting in shifts in spectra [142]. This is used for characterization of CNTs samples in different solvents and surfactant solutions, and for analyzing protein-conjugated carbon nanotubes in NIR, where biological systems tend to exhibit zero absorbance (figure 5.1) [8, 17, 18, 21, 146].

Proteins can absorb in far-UV (180-230 nm) region due to the presence of peptide bonds, mainly tryptophan, tyrosine and phenylalanine, and in near-UV (240-300 nm) region due to aromatic residues which absorb in both these regions. Table 5.3.1-1 shows absorbance of aromatic amino acids and peptide bonds. Most proteins exhibit maximum absorbance within a defined range of 275-280nm [19, 155, 158]. These peaks are primarily attributed to absorbance of the two aromatic amino acids tryptophan and tyrosine. Phenylalanine absorbs very weakly around 257 nm (Figure. 5.2).
Figure 5.1 Proteins absorb in the Ultraviolet region, and CNTs absorb in the Near Infrared region of electromagnetic spectra.

Figure 5.2 (a) Absorption spectrum of a peptide (b) Absorption spectrum of T7 Helicase. The absorption band ~280 nm is due to aromatic residues. Absorption band in the far UV region arises due to peptide bond electronic transitions.
Table 5.3.1-1 Absorbance of aromatic amino acids and peptide bonds in UV region

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>218 and 280 (smaller side peak at 288 and shoulder at 271)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>222 and 275 (shoulders at 267 and 282)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>257, 258 (205, 242, 252, 263, 267)</td>
</tr>
<tr>
<td>Peptide bonds</td>
<td>228-230</td>
</tr>
</tbody>
</table>

CNTs exhibit high absorbance in NIR region because of electronic transitions called first or second van Hove singularities. These singularities are a result of rolling process of the graphene sheet during tube formation. Each transition is seen as a distinct absorption peak at a wavelength specific to a type of CNT and can provide information about sample composition in a mixture of carbon nanotube sample [28, 112, 149, 153]. Protein functionalization alters absorption spectra of a sample by either broadening an absorption peak, or causing a shift in maximum absorption peak (Figure 5.3).

Figure 5.3 UV-Vis-NIR spectra of raw (grey line) and LSZ functionalized (black line) CNTs. Metallic (M$_{11}$) and semiconducting (S$_{22}$ and S$_{11}$) carbon nanotubes each absorb within a define range of spectrum. Protein functionalization increases amount of individual tubes in a sample which is an increase in absorption maxima. Adapted from “PH-sensitive dispersion and debundling of single-walled carbon nanotubes: Lysozyme as a tool”, Nepal, Dhriti Geckeler, Kurt E., Small, 2006, vol 2, no 3, pp 406-412.
5.4 Methods

UV-Vis-NIR spectroscopy was used to characterize CNT dispersions in DMF and Milli-Q water before and after enzyme incubation, between 200 nm-1200 nm at a scan rate of 600 nm/s. Absorbance of Helicase in Milli-Q water and DMF was compared in the UV-Vis region. Absorbance in NIR region was used to determine type and amount of CNTs present in the samples before and after helicase attachment. Concentration of CNTs remaining was estimated using extinction coefficient reported in literature [145, 146].

5.4.1 Calculating enzyme loading on CNT surface

Beer’s law may be used to estimate concentration of a substance in each solute at a specific wavelength. However, for CNTs, application of Beer’s law is not straightforward. This is because total absorbance of a CNT sample is a sum of CNT absorbance, as well as background π plasmon transition of CNTs and impurities.

According to Beer’s law

\[ A = \epsilon \times C \times l, \]

Where \( \epsilon \) is the extinction coefficient (355 L mol\(^{-1}\) cm\(^{-1}\)), \( C \) is mole of CNTs per L, \( l \) is path length of the cuvette (1 cm), and \( A \) is absorbance of the sample.

Table 5.5.1-1 shows calculation of enzyme loading on CNTs for tip sonication in water and DMF.

**Table 5.5.1-1** Calculation of enzyme loading on CNTs for tip sonication in water and DMF

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Absorbance (800 nm)</th>
<th>Calculated concentration (µg/ml)</th>
<th>No of CNT molecules</th>
<th>No of helicase molecules</th>
<th>Enzyme loading (µg T7/µg CNT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.15</td>
<td>63.4</td>
<td>3.18 *10(^{18})</td>
<td>6.83*10(^{18})</td>
<td>0.79</td>
</tr>
<tr>
<td>DMF</td>
<td>0.25</td>
<td>105.6</td>
<td>5.3*10(^{20})</td>
<td>6.83*10(^{18})</td>
<td>0.47</td>
</tr>
</tbody>
</table>

5.5 Results

Absorbance measurements in UV-Vis region confirmed that helicase was successfully attached to CNT surface. Absorbance measurements in the NIR region confirmed the
presence of CNTs in the sample, and their solubility and dispersibility. The concentration of enzyme was estimated by using absorbance at 280 nm [147]. It was assumed that protein was in excess of CNTs, and no free protein was left after centrifugation [38]. Concentration of CNTs after centrifugation was estimated using absorbance at 800 nm [148]. The following results were obtained:

1. Control samples with Milli-Q water containing no enzyme showed absorbance maxima at 260 nm. Addition of enzyme resulted in two absorbance peaks in UV-Vis region at 228 and 260 nm (figure 5.4), and two absorbance peaks in NIR region, at 970 and 1190 nm (figure 5.5). Higher absorbance was observed at 260 nm upon addition of enzyme.

2. Control samples with CNTs in DMF containing no enzyme showed no absorbance peak in UV-Vis region. Addition of enzyme resulted in an absorbance peak at 260 nm (figure 5.7), increased absorbance between 700-1200 nm, and a peak around 1200 nm (figure 5.8).

3. Helicase-CNT samples in DMF demonstrated higher stability (up to 2 weeks or more), while helicase-CNT samples in Milli-Q water became unstable after 1 week, and tubes precipitated out of solution.

4. Enzyme loading was calculated to be 0.79 µg T7/µg CNT for samples in water, and 0.47 µg T7/µg CNT in DMF.

5. Enzyme concentration was calculated to be approximately 0.21 mg/ml and 0.1 ml/ml for water and DMF respectively.
Figure 5.4. UV Absorption spectra of CNT dispersion in Milli-Q water before and after helicase attachment. Addition of enzyme results in a shift from 260 nm to 255 nm. There is also a decrease in absorbance intensity from 0.8 to 0.3 at 250 nm due to removal of unfunctionalized CNTs upon centrifugation.

Figure 5.5. Vis-NIR Absorption spectra of CNT dispersion in Milli-Q water before and after helicase attachment and separation of some tubes and results in two absorption bands.

Figure 5.6 UV absorption spectra of CNT dispersions in DMF without helicase and with helicase. Interaction of helicase with CNTs results in a blue shift. Addition of enzyme results in another absorption band between 240 and 270 nm.
Figure 5.7 Vis-NIR absorption spectra of CNT dispersions in DMF without helicase and with helicase. Increase in absorbance can be seen in the Vis-NIR region after addition of helicase.

Figure 5.8 Comparison of CNT solubility in DMF and Milli-Q water for UV-Vis (top) and NIR regions (bottom). Samples in water had lower concentration of CNTs (0.2 Abs at 400 nm) as compared to samples in DMF (0.5 Abs at 400 nm).

Figure 5.8 shows a comparison of CNT solubility in DMF and Milli-Q water in the presence of enzyme. Both samples exhibited similar absorbance pattern in the UV region, with samples in water had a distinct peak at 260 nm, indicating better CNT dispersibility in the solvent. CNTs in DMF had higher solubility than in water, as can be seen by higher absorbance values at the same wavelength in both samples. Two absorption peaks in the
NIR region for samples in water further confirmed better dispersibility of CNTs in water. Enzyme concentration was calculated for both samples using an extinction coefficient of $76100 \, \text{M}^{-1}\text{cm}^{-1}$ at 280 nm, and was found to be 0.1 mg/ml and 0.21 mg/ml for DMF and water respectively. Higher concentration of enzyme for samples in water proved better attachment of helicase and CNTs, which resulted in better dispersibility of CNTs in water.

**Figure 5.9** A comparison of helicase-CNT samples in DMF (a) and Water (b) with a CNT mixture containing 12 different types of CNTs. A CNT sample Absorption peaks can be attributed to specific species in a mixture [149]. Samples contained different types of tubes.

### 5.6 Discussion

Absorption spectroscopy provides insight into many properties of carbon nanotubes including sample composition, separation and purity. A CNT sample contains a mixture of tubes with varying rolling angle and diameters which are produced when a graphene sheet
rolls to form a CNT. Many types of interactions are possible between protein and CNT [21]. Protein adsorption results in changes in optical properties of CNTs, which can be detected as variations in absorbance intensity (broadening or increase in absorption peak) or as wavelength shifts of pristine carbon nanotube samples [147, 152]. The broadening and/or shift of the peak may be affected by two factors: type of solvent used for CNT dispersion and type of adsorbed protein. Comparison of absorption maxima at different wavelengths for the two solvents suggested that CNT samples exhibited variation in protein unfolding.

Enzyme-conjugated samples for both solvents showed absorbance at 260 nm, which may be attributed to surface Plasmon resonance of CNTs as an indicative of good dispersibility [143, 146, 153]. A red shift was observed from 260 nm to 228 nm for helicase-CNT conjugates in Milli-Q water, accompanied with lower intensities than free CNTs. This is consistent with previously reported findings for other proteins adsorbed on CNTs [94, 145]. This may be attributed to $\pi \rightarrow \pi^*$ transitions of peptide bonds (aromatic C-C bonds), or to absorbance by aromatic side chain of histidine [153, 154]. This could be due to hydrogen bonding between helicase and CNTs which exposes the peptide bonds. For samples in DMF, interaction between CNTs and helicase was mainly attributed to hydrophobic interactions and Van der Waals forces. Increasing concentration of enzyme resulted in an increased absorbance, indicating that attachment to helicase increased solubility of CNTs in both solvents (Figure 5.5 and Figure 5.7).

The results were compared to absorption spectra of a mixture of CNTs [149]. It is predicted that helicase may be selective for different types of tubes as seen in figure 5.9. Helicase attachment resulted in separation of types of tubes which are visible as peaks on the figure, while the others remained bundled.

5.7 Conclusion

Helicase was successfully attached to CNTs using non-covalent adsorption which preserved CNT optical characteristics and improved CNT solubility.
6. HELICASE FUNCTIONALITY TEST USING FLUORESCENT Pi SENSOR

6.1 Rationale

Fluorescence methods can be used to investigate changes in protein function upon interaction with other materials [154]. Once protein attachment is confirmed, changes in intrinsic fluorescence of helicase can be used to investigate NTP hydrolysis.

6.2 Purpose

The purpose of this chapter was:

1. To study changes in NTPase (Nucleotide Triphosphatase) activity of helicase
2. To compare enzymatic activity in DMF and Milli-Q water of helicase-CNT conjugates with that to free helicase for selecting the best solvent for sensor fabrication

6.3 Single Stranded DNA binding and Nucleotide Triphosphatase activity determination using Fluorescence Intensity Measurement

When a molecule absorbs electromagnetic radiation, excitation of atoms results in the emission of light. This process is termed as fluorescence. Fluorescence is a three-stage process characterized by absorption of a photon, creation of an excited state, and followed by the emission of another photon (Figure 6.1). Fluorescent probes (a fluorophore or a fluorescent dye) can detect products of a biological reaction [155]. A fluorescence detector system can be used to capture fluorescence. It is composed of a light source, fluorophore and a wavelength filter to distinguish between excitation and emission wavelengths. Four types of such detectors are available: plate readers, microscopes, flow cytometers and scanners.

Intrinsic fluorescence of a protein arises from three aromatic amino acids: Tryptophan (Trp), Tyrosine (Tyr) and Phenylalanine (Phe) [156]. The spectrum is mainly dominated by Trp and Tyr. An excitation wavelength of 280 nm can excite both, and an excitation wavelength of 295 nm can be used to selectively excite Trp only [156]. The fluorescence spectrum varies with changes in the protein’s surrounding environment, and hence may be used to investigate conformational changes in protein structure due to interactions between
protein and other molecules [156, 157]. The native protein state is usually hydrophobic. These residues are buried in the core, resulting in a high quantum fluorescence yield. Exposure to hydrophilic solvent causes the protein to unfold, exposing these residues, and thus decreasing fluorescence spectrum. Slight perturbations to the protein because of hydrogen bonding, electrostatic interactions or hydrophobic interactions may cause partial unfolding, affecting fluorescence spectrum [95]. However, these will not have a significant effect on the protein’s function unless protein is permanently denatured physically or chemically.

**Figure 6.1** Three stage process of fluorescence: absorption, excited state and emission [157]

DNA helicase is a motor protein that translocates along ssDNA in steps of single nucleotide base, by using energy from ATP hydrolysis. Presence of ssDNA greatly enhances ATPase activity of helicase [44]. Because of this translocation, dsDNA is converted to ssDNA, and an inorganic phosphate is released at each step. Fluorescent, reagentless sensors in solution can provide rapid and sensitive response in a single step for measuring function of ATP-coupled motor proteins such as myosin, kinesins and helicases [50, 158, 159]. This can be done by capturing products of ATP hydrolysis reaction, such as ADP or inorganic phosphates- Pi. This allows measurement of step size, or ATP hydrolysis rate in real-time under varying conditions, providing insight into protein conformational changes and molecular interactions.
Figure 6.2 (a) Schematic representation of mechanism of MDCC-PBP binding with organic phosphate (b) flowchart showing how helicase activity is measured using MDCC-PBP

For measuring NTPase activity, the protocol used here employs a special phosphate sensor (Pi). A coumarin fluorophore is covalently attached to a single cysteine on the surface of phosphate binding protein (MDCC-PBP). As Pi binds to the sensor, there is a change in fluorescent intensity that can be detected using a plate reader. This method has been used for measuring ATPase activity of T7 helicase and other helicases [49, 130].

The rate of Pi binding to PBP-MDCC is very fast, around $1.4\times10^8 \text{ M}^{-1} \text{ s}^{-1}$ which allows capturing of Pi in milliseconds[44]. Structure of helicase was discussed in chapter 3. Helicase consists of 10 Trp, 15 Tyr, and 19 Phe which can contribute to the protein's
intrinsic fluorescence. Fluorescence intensity measurements were made using both the solvents, DMF and Milli-Q water with Tween 20 for two reasons:

1. to study how CNT attachment affects helicase function and
2. how subsequent DNA binding affects fluorescence spectrum of native helicase [160]

Results were compared to fluorescence measurements of free enzyme in the same solvents, in the presence and absence of ssDNA and dsDNA.

6.4 Methods

Reagents and buffers
CNTs produced by laser vaporization were obtained from National Research Council of Canada and used without further purification. T7 Helicase (1 mg/mL, purified from an E.coli strain that overexpresses the gp4A gene isolated from the T7 phage) was purchased from BioHelix Corporation (MA, USA), stored at -20 °C (20mM TrisHCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 50% Glycerol) and used without further purification. EDTA was used as a quenching solution. dTTP was used for hydrolysis due to helicase preference over other NTPs. Helicase reaction buffer consisted of 35 mM Tris–acetate, pH 7.5, 11 mM magnesium acetate, 5 mM DTT, 0.01% Triton X-100. Phosphate mop was prepared using 200 uM 7-methyl guanosine and 0.1-1.0 U/mL purine nucleoside phosphorylase to eliminate potential inorganic phosphate contamination for ATPase assay. Refer to Appendix E for detailed methods.

Oligonucleotides
Previously purchased DNA from GenScript™ available at OHRI (Ottawa Heart Research Institute) was used for investigating ssDNA binding to helicase-functionalized CNTs. Table 6.1 shows the length and sequence of oligonucleotides used.

NTPase activity was measured by linking NTP hydrolysis to the change in fluorescence observed in the Pi sensor MDCC-PBP. Reactions were carried out in a 96 well plate (75-200 uL working volume per well). 10 µL of helicase-CNT dispersion (serially diluted with DMF) was added to each well in column 2, 10 µL of helicase-CNT dispersion (serially diluted in Milli-Q water) was added to each well in column 3. Column 1 and 5-8 were
controls as follows: empty, helicase in DMF, Helicase in Tween20-Milli Q, Tween20-MilliQ, DMF, Milli-Q and empty.

**Table 6.4-1** Length and sequence of oligonucleotides from GenScriptTM used for investigating ssDNA binding and NTP hydrolysis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Length</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24</td>
<td>5'-ACTTCGACTAGCTAGCTCTATTCG</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>5'-GCACCGCGCGCGCGCGATTGCGATTG</td>
</tr>
</tbody>
</table>

This was followed by addition of 20 µL of reaction buffer to each well, followed by addition of 20 µL of DNA (1 mM ssDNA). The plate was read 2 min before adding dTTP. Reactions were initiated by adding of 10 µL of dTTP (2 mM). The plate was incubated at 37 degrees for 30 minutes and EDTA was added to stop the reactions. Next, 40 µL of 2X Phosphate Sensor was added and the read plate immediately using BMG Labtech Fluostar Omega microplate reader (BMG Labtech, ON, Canada) for microplate fluorescence readings experiments. The cycle time was set at 5-20 s at 37 degrees at a gain of 85%. Two set of excitation and emission wavelengths were used: 355/460 nm and 485/460 nm, due to unavailability of the setting 436/455 nm on the instrument, which are the required excitation emission wavelengths for Phosphate sensor. The experiments were repeated 3 times and normalized with a control reaction in the absence of substrate. Background fluorescence was subtracted.

Helicase-attached CNTs were incubated with 0.1 umol ssDNA and dsDNA and fluorescence intensity measurements were made using BMG Labtech Polarstar Omega plate reader. Excitation wavelength of 310 nm was used to study changes in helicase structure for helicase-attached CNTs before and after DNA incubation and compared to that of free helicase.

### 6.5 Results

Fluorescence measurements were used to determine ssDNA binding and NTP hydrolysis by helicase-CNT conjugates in DMF and Milli-Q water (figure 6.3) and compared to that of free enzyme (figure 6.4) in the same solvents. The following results were obtained:
1. Helicase-CNT conjugates in both solvents exhibited an increase in fluorescence with time, indicating interaction between ssDNA and NTP hydrolysis (Figure 6.3).

2. Samples in DMF and Milli-Q water exhibited similar NTPase activity. However, conjugates in DMF showed higher fluorescence intensity reaching a maximum of 30000 a.u, than those in Milli-Q water (Figure 6.3). Samples in Milli-Q water reached a maximum of 26000 a.u. There was also a delay of 2 seconds, where samples in CNT reached maximum fluorescence faster than samples in Milli-Q water.

3. Figure 6.4 compares activity of free helicase in the two solvents. Fluorescence intensity of free enzyme in water was observed to be lower than that for DMF. Higher fluorescence in DMF was attributed to intrinsic fluorescence of DMF. Helicase-CNT conjugates exhibited fluorescence quenching than free enzyme for both solvents. (Figure 6.3 and 6.4).

4. Effect of increasing enzyme concentration on activity of conjugates was compared (Figure 6.5). Increase in fluorescence was observed for both samples with increasing enzyme concentration up to 0.4 mg/ml of helicase. After 0.4 mg/ml, both samples exhibited variation in fluorescence activity. For samples in Milli-Q water, increase in helicase concentration led to higher fluorescence intensity. However, samples in DMF exhibited a slight decrease in fluorescence, with fluorescence become steady at 0.65 mg/ml of helicase. Both samples exhibited maximum absorbance at around 0.3 mg/ml of helicase, 25000 a.u for DMF and 22000 for water. 0.45 mg/ml was the maximum enzyme saturation of CNTs for both samples.
Figure 6.3 Comparison of fluorescence intensity of Helicase-CNT conjugates in DMF and Milli-Q water

Figure 6.4 Comparison of Fluorescence intensity of free helicase in DMF and Milli-Q Water
Figure 6.5 Change in dTTPase Activity with increasing Helicase concentration for Helicase-CNT conjugates in DMF and Milli-Q water

6.6 Discussion

Changes in fluorescence intensity were compared for helicase-CNT samples in DMF and Milli-Q water. As dTTP is added, helicase unwinds DNA by hydrolyzing dTTP, releasing an inorganic phosphate. This inorganic phosphate binds with PBP to produce fluorescence. Figure 6.3 shows fluorescence of samples exhibited a biphasic fluorescence intensity, similar to results reported earlier[44]. There was a rapid increase in fluorescence intensity over time, upon initiation of reaction, followed by a steady dTTPase rate. Helicase-CNT samples in DMF demonstrated higher fluorescence than helicase-CNT conjugates in Milli-Q water. This was attributed to intrinsic DMF fluorescence, and not due to release of inorganic phosphate. To confirm this, activity of free helicase was compared for the two solvents. Similar results were observed where samples in DMF showed higher fluorescence than samples in Milli-Q water.

Effect of increasing enzyme concentration was investigated for both helicase-CNT conjugates in DMF and Milli-Q water. Increasing enzyme concentration resulted in an increase in fluorescence for helicase-CNT conjugates in Milli-Q water. This continued
increase in fluorescence for samples in Milli-Q water were attributed to increase of free enzyme in solution with addition of more enzyme. On the other hand, increasing enzyme concentration had no significant increase in fluorescence for helicase-CNT conjugates in DMF. Thus, DMF samples were not used for DNA unwinding analysis.

![Figure 5.6](image)

**Figure 5.6** Stern-Vermon plot for binding between tau protein and carbon nanotubes

Figure 6.6 shows changes in fluorescence of helicase upon interaction with CNTs. Stern-Volmer equation was used to compare the enzyme activity in DMF and Milli-Q water[161].

\[
\frac{F_0}{F} = K_{sv} [\text{CNTs}] + 1
\]

Where \(F_0\) and \(F\) are the steady state fluorescent intensities of protein before and after attachment to CNTs, \(K_{sv}\) is the Stern-Volmer quenching constant, [CNTs] is the concentration of CNTs. Higher \(K_{sv}\) indicates stronger binding between protein and CNT. \(K_{sv}\) of sample in Milli-Q water and DMF was compared to those reported earlier [161]. It was calculated to be 7.8 ml/ug and 4.54 ml/ug, for water and DMF respectively, indicating stronger binding of CNTs to helicase in water as compared to that in DMF. This was similar
to $K_{sv}$ for tau protein (7 ml/ug) which indicated strong binding between protein and CNT (figure 6.6).

**Figure 6.6** Comparison of fluorescence intensity of free helicase with CNT attached helicase. Attachment to CNT increased fluorescence activity of helicase.

Intrinsic fluorescence of a protein is affected by placement of fluorescent residues within a protein. Changes in protein structure can lead to increased or decreased fluorescence upon binding. Fluorescence quenching of helicase upon attachment to CNT can be attributed to changes in protein structure upon binding. To further establish retention of enzymatic activity and to determine whether immobilized helicase is active, DNA unwinding tests are described in the next section. Changes in fluorescence of helicase-CNT conjugates may also be indicative of helicase binding to ssDNA, since a protein’s fluorescence spectrum may change upon binding to DNA. This was further confirmed by studying interaction between helicase and dsDNA.
6.7 Conclusion

Helicase retained ssDNA binding ability and NTP hydrolysis function after attachment to CNT.
7. TESTING DNA UNWINDING BY HELICASE-CNT CONJUGATES USING FLUORESCENCE RESONANT ENERGY TRANSFER (FRET)

7.1 Rationale

Once DNA binding and NTP hydrolysis by helicase-CNT conjugates is confirmed, further assessment is needed to investigate helicase ability to unwind DNA.

7.2 Purpose

The purpose of this chapter was:

1. To investigate DNA hybridization and helicase-DNA interactions using fluorescence intensity measurements
2. To investigate DNA unwinding by helicase-CNT conjugates

7.3 Monitoring DNA unwinding using Fluorescence Intensity and FRET (Fluorescence Resonant Energy Transfer)

7.3.1 Fluorescence Intensity

Fluorophore labelled oligonucleotides can be used to investigate DNA hybridization, unwinding and protein-DNA interactions using fluorescence intensity measurements [161, 162]. Upon interaction, changes in fluorescence intensity takes place. For DNA unwinding, two complementary oligonucleotides are used. One is called a lagging strand and is slightly longer than the other and is partially complementary. The shorter strand is called the leading strand. One of the strands is labelled with a fluorophore. Hybridization between the two strands quenches fluorescence.

7.3.2 FRET

FRET is a spectroscopic technique that has been used to investigate biomolecular interactions for nucleic acid analysis (Figure 7.1). It makes use of energy transfer between a donor, usually a fluorophore, to an acceptor. FRET makes use of several acceptor-donor pairs. The acceptor must absorb energy at the emission wavelength of the donor. FRET has
been used for studying DNA unwinding mechanisms and kinetics of T7 helicase and other helicases [16, 53, 54, 161].


Nucleic acids such as DNA may be labelled with many different fluorescent molecules. The most common of these are the fluorescent dye pairs. In this research, fluorescein-hexachlorofluorescein dye pair is employed for labeling complementary DNA oligonucleotides at 3’ and 5’ ends respectively [60]. Fluorescein may be excited at 480 nm and emits at 520 nm [164].

7.4 Materials and Methods

Reagents and Buffers
The oligonucleotides were suspended in TE buffer (10 mM Tris, pH 7.5-8.0, 1 mM EDTA). Annealing buffer was prepared using 10 mM Tris, 1 mM EDTA and 50 mM NaCl, pH 8.0. All unwinding reactions were performed in helicase buffer which consisted of 35 mM Tris–acetate, pH 7.5, 11 mM magnesium acetate, 5 mM DTT and 0.01% Triton X-100.

**Oligonucleotides**

**Table 7.4-1** Length and sequence of oligonucleotides from Sigma Aldrich used for investigating DNA unwinding using Fluorescence Intensity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Duplex Length</th>
<th>Composition and Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>14</td>
<td>5’-AATCCGTCGAGCAGagttagggttaggttaggttag (t25) 3’-TTAGGCAGCTCGTC</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>5’-ATCCGTCGAGCAGagttagggttaggttaggttag (t25) 3’-TTAGGCAGCTCGTC</td>
</tr>
<tr>
<td>C</td>
<td>14</td>
<td>5’-AATCCGTCGAGCAGagttagggttaggttaggttag(t24)T-F 3’-TTAGGCAGCTCGTC</td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>5’-AATCCGTCGAGCAGAGttagggttaggttaggttagctctagcagt (t18) 3’-TTAGGCAGCTCGTC</td>
</tr>
<tr>
<td>E</td>
<td>16</td>
<td>5’-aatccgtagageagagttag(GTGTAGCTCTAGCAGT CCAATCGAGATCGTCA-F-5’</td>
</tr>
<tr>
<td>F</td>
<td>22</td>
<td>5’-AATCCGTCGAGCAGAGTTAGGtaggttagggttag (t25) 3’-TTAGGCAGCTCGTCTCAATCCC</td>
</tr>
<tr>
<td>G</td>
<td>36</td>
<td>5’-AATCCGTCGAGCAGAGTTAGGGTTAGGGTTAGGGTTAGGTTag (t25) 3’-TTAGGCAGCTCGTCTGAATCCCAATCCCAATCCCAATCCCA</td>
</tr>
<tr>
<td>H</td>
<td>40</td>
<td>5’- CAGCAGCGGGAATGTAACCATCGTGGTGGCAGCAGGGA(t25) 3’-GTCGTCGCCCCCTACATTGGTAGCAACCAGCGTCGTCGCGG-5’</td>
</tr>
</tbody>
</table>

All oligos were stored at – 20 °C as 100 uMolar stock solution with foil covered tubes to prevent photobleaching. Equal volumes of equimolar oligonucleotides were annealed using heat block method to yield double stranded DNA (dsDNA) in annealing buffer. This was
done by heating complementary oligonucleotides at 95 °C for 5 minutes followed by slow cooling at room temperature.

Table 7.4-1. shows size, composition and sequence of oligonucleotides labelled with fluorescein at 3’ end [59]. Table 2. shows size, composition and sequence of oligonucleotides labelled at 3’ and 5’ ends [59].

Table 7.4-2 Length and sequence of oligonucleotides from Sigma Aldrich used for investigating DNA unwinding using FRET

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Duplex Length</th>
<th>Composition and Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>14</td>
<td>5’HEX-AATCCGTCGAGCAGttagggttagggttagggttag(t24)T-F 3’F-TTAGGCAGCTCGTC</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>5’HEX-AATCCGTCGAGCAGttagggttagggttagggttagtctctagcagt (t18) 3’F-TTAGGCAGCTCGTCTC</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>5’HEX-AATCCGTCGAGCAGttagggttagggttagggttagtctctagcagt (t25) 3’F-TTAGGCAGCTCGTCTCAATCCC</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>5’HEX-AATCCGTCGAGCAGttagggttagggttagggttagggttagggttagggtag (t25) 3’F-TTAGGCAGCTCGTCTCAATCCC</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>5’HEX-CAGCAGCGGGGAAATGAAATCCATCGTGTTGGCTGCGCAGCGGCCGTTCGCGTCCCGG (t25) 3’F-GTCGTCGCCCTTACATTGGTAGAACCAGCCGCTGTCGCCG-5’</td>
</tr>
</tbody>
</table>

Methods

DNA binding and T7 helicase catalyzed DNA unwinding was simultaneously investigated using FRET [52, 53, 163]. UV-Vis absorption and fluorescence spectra of buffer was recorded. Fluorescence spectra of fluorescein labelled ssDNA (FLC-ssDNA) was measured at an excitation wavelength of 485/520 nm, and hexachlorofluorescein labelled DNA (HEX-ssDNA) at 544/590 nm before hybridization with unlabelled complementary oligonucleotides[163]. ssDNA substrates with fluorescein labelled 3’ end over hang and
hexachlorofluorescein labelled 5’ end was added to helicase unwinding buffer at 25°C (150 ul total volume) using Nunc™ 96-Well Polypropylene MicroWell™ Plates. The helicase-functionalized CNTs were then added and emission spectrum was measured after 5 min. The unwinding reaction was initiated by the rapid addition of 2mM dTTP serially diluted at various concentrations. Fluorescence spectra were measured at 485 and 520 nm, absorption and emission after 5 minutes. Spectra was corrected by subtraction of the solvent spectra measured under the same conditions at 25 °C.

7.5 Results

Fluorescence intensity measurements were used to compare structural changes in helicase structure before and after DNA binding.

1. Free helicase: Bath sonicated samples in DMF showed highest fluorescence, followed by tip sonicated samples in DMF and bath sonicated samples in Milli-Q water. Tip sonicated samples in Milli-Q water showed the least fluorescence.
2. Helicase-attached CNTs: fluorescence intensity of protein is reduced to one-third of that of free protein upon attachment (F₀/F).
3. Addition of DNA to helicase-attached CNTs: Decrease in fluorescence of fluorescein-labelled DNA was observed suggesting possible interaction between helicase and DNA, or between DNA and CNT. Decrease in fluorescence was higher for ssDNA binding than for dsDNA binding.

As DNA strands hybridize, fluorescence quenching occurs. Fluorescence spectra of FLC-ssDNA and HEX-ssDNA before annealing, after annealing and upon DNA unwinding was compared and was found to be similar to ones reported previously (Figure 7.3)[60].
Figure 7.2. Fluorescence intensity analysis of DNA unwinding by helicase-CNT conjugates in Milli-Q water at 485/520 nm. Fluorescence intensity of FLC-ssDNA decreases upon DNA hybridization. Upon DNA unwinding by helicase-CNT conjugates FRET increases again.

Figure 7.3. Fluorescence intensity analysis of DNA unwinding by helicase-CNT conjugates in Milli-Q water at 544/590 nm. As DNA unwinding by helicase-CNT conjugates progresses, FRET decreases again and fluorescence intensity of HEX-ssDNA increases.

The important results included:
1. Initial fluorescence of FLC-ssDNA and HEX-ssDNA was higher than dsDNA at both 485/520 nm and 544/590 nm
2. After addition of dTTP, enhancement of FLC emission for same excitation wavelength (492 nm) due to DNA unwinding was observed
3. After addition of dTTP, decrease in HEX emission for same excitation wavelength (544 nm) upon DNA unwinding was observed
4. Variations in length of DNA sequences did not affect fluorescence intensity measurements
5. Changes in fluorescence measurements, and hence DNA unwinding were in the order of milliseconds (ms)

7.6 Discussion

DNA helicase is a motor protein that translocates along DNA, deriving energy from phosphate hydrolysis. Studies have shown that this movement is unidirectional and helicase unwinds 2 to 3 base pairs of DNA per second. Many studies have employed FRET for studying DNA unwinding by helicase and to investigate the step size (number of base pairs unwind) of helicase [44, 164]. For each 2-3 base pair unwound, one molecule of dTTP is hydrolyzed.

DNA labelled with fluorophores can allow monitoring of helicase-catalyzed DNA unwinding using FRET. Each of the DNA strands can be labelled with a donor and an acceptor molecule, such as fluorescein and hexachlorofluorescein. The fluorescence emission spectrum of dsDNA is composed of individual fluorescence spectrums of fluorescein and hexachlorofluorescein labelled ssDNA strands [60]. Emission spectrum from 500-540 nm is dominated by fluorescein labelled ssDNA, and emission spectrum from 540-600 nm is dominated by hexachlorofluorescein labelled ssDNA, with a spectral overlap at 540 nm for the two dyes.

To determine whether helicase-attached CNTs can unwind DNA, fluorescence measurements were performed before and after DNA unwinding using dTTP hydrolysis. Two different excitation wavelengths were used for FLC-ssDNA and HEX-ssDNA. Fluorescence intensity measurements were recorded only for samples in Milli-Q water.
DMF samples showed very high fluorescence intensity readings and were not used because of possible helicase and DNA denaturation. Figure 7.2 shows fluorescence intensity analysis of DNA unwinding by helicase-CNT conjugates in Milli-Q water at 485/520 nm. Fluorescence intensity of FLC-ssDNA was recorded and compared to intensity measurements after hybridization. As DNA hybridizes to dsDNA, fluorescence intensity measurements decreased to about 1/3 of the initial measurements (figure 7.2).

As DNA unwinding was initiated by helicase-CNT conjugates, fluorescence measurements increased again. The final fluorescence measurements were lower than that of initial FLC-ssDNA, possibly due to fluorescence quenching over time.

Helicase-CNT conjugates were able to hydrolyze dTTP and unwind DNA, as determined by FRET [52, 53]. No fluorescence was observed in samples without dTTP added. Upon addition of dTTP, DNA unwinding was initiated. Fluorescence intensity of fluorescein decreased as the reaction progressed, and that of hexachlorofluorescein increased, confirming progression of DNA unwinding (figure 7.3).

7.7 Conclusion

Helicase retained its ability to unwind DNA after attachment to CNT.
8. SUMMARY OF RESULTS, CONCLUSIONS AND FUTURE DIRECTIONS

This chapter summarizes results, discusses the significance and contribution made towards DNA sequencing and provides future directions.

8.1 Key Results

In this research, helicase was non-covalently attached to CNT for development of a novel DNA sensor. The conjugation method was derived from previously reported methods of attaching proteins to carbon nanotubes, using BSA as a model protein. The conjugate was tested for preservation of helicase structure, and its ability for detecting and unwinding DNA. Helicase non-specifically adsorbed onto CNT surface as was confirmed by taking absorbance measurements in UV-Vis-NIR regions before and after helicase addition to CNT samples. UV-Vis-NIR spectra of helicase-CNT samples in the DMF and Milli-Q water also showed variation in protein adsorption, suggesting different type of interaction between helicase and CNT for the two solvents. Increasing concentration of enzyme resulted in increased UV-Vis-NIR absorbance, indicating that helicase increased solubility of carbon nanotubes in both solvents. Upon comparison with attachment results of other enzymes it was proposed that helicase may be specific in its attachment to a specific type of tube.

Structural analysis of helicase was conducted using intrinsic fluorescence measurements of helicase. The measurements decreased upon attachment to CNTs, suggesting changes in tertiary structure of the protein. Helicase-CNT conjugates retained their ability to bind to DNA. Samples with helicase and dsDNA together, showed higher fluorescence than free helicase at the same concentration due to helicase-DNA binding.

Functional analysis of DNA unwinding showed that helicase retained its dTTP hydrolysis and DNA unwinding function upon attachment. Helicase activity was reduced after attachment to CNTs, when compared to free helicase, as was discovered using fluorescence measurements. Fluorescence measurements, and hence DNA unwinding by helicase was recorded in milliseconds.
8.2 Conclusion

In this research, DNA unwinding by helicase was investigated by attaching it to CNTs for DNA sequencing. Helicase was successfully attached to CNTs using non-covalent adsorption methods, with preservation of protein function and CNT electronic structure. The interaction is likely dominated by pi-pi and hydrophobic interactions similar to other proteins that are comparable to helicase in terms of structure and physical properties and have been attached to CNTs previously. These helicase-CNT conjugates have the potential to be incorporated into a DNA device that unwinds DNA and sequences it simultaneously.

8.3 Comparison with other methods

This research was directed at overcoming drawbacks of currently available DNA sequencing techniques. Current DNA sensing techniques are complex, time consuming and require specialized training. The study proves that the helicase-attached CNTs can eliminate several steps from existing sequencing methods (DNA extraction, purification, amplification).

8.4 Significance/Contribution

This research demonstrated a novel concept where helicase catalyzed unwinding can be reproduced in vitro for facilitating DNA sequencing. Successful conjugation of helicase and CNT for sensing of low amounts of DNA samples, and rapid DNA unwinding. Results from this study can help in the development of an electronic, portable and compact DNA sensor. The research also verified separation and isolation of semi-conducting CNTs using a protein (helicase) and a surfactant (Tween-20). Using a similar procedure, solubilisation and separation of CNTs can be extended beyond biosensing applications, such as tissue engineering, biocatalysis and drug delivery.

8.5 Future work

Further work is needed to investigate electronic detection by helicase-attached CNTs and to facilitate the formation of this device. Non-specific binding of helicase molecules to CNT can be prevented to ensure a single helicase molecule binds to a single CNT. Primase region of helicase can be chemically attached to CNT to ensure that helicase activity is not
affected upon attachment. DNA has a very high affinity for CNTs. It might be useful to insulate CNT surface using polymers to ensure DNA strand does not bind to CNT and affect DNA unwinding. Insulation may also facilitate electronic signal transduction through CNTs. Helicase-attached CNTs can be deposited on silicon electrodes for measurement of electronic signals based on charges on individual nucleotide bases.
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## Appendices

### Appendix A - Color coding scheme for amino acids in Protein Data Bank (PDB)

<table>
<thead>
<tr>
<th>Amino Acid (Residue)</th>
<th>Color</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP, GLU</td>
<td>Bright red</td>
<td>230, 10, 10</td>
</tr>
<tr>
<td>LYS, ARG</td>
<td>Blue</td>
<td>20, 90, 255</td>
</tr>
<tr>
<td>PHE, TYR</td>
<td>Mid blue</td>
<td>50, 50, 170</td>
</tr>
<tr>
<td>GLY</td>
<td>Light grey</td>
<td>235, 235, 235</td>
</tr>
<tr>
<td>ALA</td>
<td>Dark grey</td>
<td>200, 200, 200</td>
</tr>
<tr>
<td>CYS, MET</td>
<td>Yellow</td>
<td>230, 230, 0</td>
</tr>
<tr>
<td>SER, THR</td>
<td>Orange</td>
<td>250, 150, 0</td>
</tr>
<tr>
<td>ASN, GLN</td>
<td>Cyan</td>
<td>0, 220, 220</td>
</tr>
<tr>
<td>LEU, VAL, ILE</td>
<td>Green</td>
<td>15, 130, 15</td>
</tr>
<tr>
<td>TRP</td>
<td>Pink</td>
<td>180, 90, 180</td>
</tr>
</tbody>
</table>
Appendix B- Amino acid composition of Helicase-Primase

Amino acid composition of a protein affects how it will interact with other materials. Amino acids can be charged, polar or hydrophobic. Hydrophobic residues are buried inside the core, while polar and charged residues tend to accumulate on the surface. Attachment to CNT sidewalls varies with the number and type of amino acids. Solvent medium affects which residues will take part in the interaction. For instance, at a pH that is above or below protein’s isoelectric point activates charges residues, facilitating interaction through electrostatic forces. At the protein’s isoelectric point, hydrogen bonds through polar residues dominate. Hydrophobic interactions take place when a solvent denatures protein, exposing the buried hydrophobic residues to the surface.

<table>
<thead>
<tr>
<th>Amino Acid (Residue)</th>
<th>Number of residues</th>
<th>Percentage of residues (%)</th>
<th>Type of residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALANINE (ALA)</td>
<td>37</td>
<td>6.5</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>ARGinine (ARG)</td>
<td>27</td>
<td>4.8</td>
<td>Polar</td>
</tr>
<tr>
<td>ASPARAGINE (ASN)</td>
<td>25</td>
<td>4.4</td>
<td>Polar</td>
</tr>
<tr>
<td>ASPARTIC ACID (ASP)</td>
<td>41</td>
<td>7.2</td>
<td>Polar</td>
</tr>
<tr>
<td>CYSTINE (CYS)</td>
<td>13</td>
<td>2.3</td>
<td>Polar</td>
</tr>
<tr>
<td>GLNUTAMINE (GLN)</td>
<td>17</td>
<td>3.0</td>
<td>Polar</td>
</tr>
<tr>
<td>GLUTAMIC ACID (GLU)</td>
<td>43</td>
<td>7.6</td>
<td>Polar</td>
</tr>
<tr>
<td>GLYCINE (GLY)</td>
<td>54</td>
<td>9.5</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>HISTIDINE (HIS)</td>
<td>15</td>
<td>2.7</td>
<td>Polar</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Number</td>
<td>Charge</td>
<td>Physical Property</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------</td>
<td>--------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Isoleucine (ILE)</td>
<td>27</td>
<td>4.8</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Leucine (LEU)</td>
<td>44</td>
<td>7.8</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Lysine (LYS)</td>
<td>38</td>
<td>6.7</td>
<td>Polar</td>
</tr>
<tr>
<td>Methionine (MET)</td>
<td>17</td>
<td>3.0</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Phenylalanine (PHE)</td>
<td>19</td>
<td>3.4</td>
<td>Hydrophobic</td>
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<td>Proline (PRO)</td>
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<td>2.1</td>
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</tr>
<tr>
<td>Serine (SER)</td>
<td>46</td>
<td>8.1</td>
<td>Polar</td>
</tr>
<tr>
<td>Threonine (THR)</td>
<td>28</td>
<td>4.9</td>
<td>Polar</td>
</tr>
<tr>
<td>Tryptophan (TRP)</td>
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<td>1.8</td>
<td>Polar</td>
</tr>
<tr>
<td>Tyrosine (TYR)</td>
<td>15</td>
<td>2.7</td>
<td>Polar</td>
</tr>
<tr>
<td>Valanine (VAL)</td>
<td>38</td>
<td>6.7</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>
Appendix C- Ultrasonication: Tip sonication and Bath sonication

Ultrasonication is an effective method to solubilize CNTs in different solvents. Ultrasonication can be carried out using a bath sonicator or a probe sonicator. Both methods make use of ultrasound waves to break down large CNT bundles into individual CNT particles. Bath sonication uses low energy and uneven distribution of ultrasound. This prevents damage to CNT structure but is ineffective in separating individual CNT particles. The resulting dispersion mainly consists of CNT bundles. Tip sonication uses higher energy which is more localized. As a result, it is more successful in solubilizing tubes in a medium.

![Figure D.1 Principle of ultrasonication: Bath sonicator and probe sonicator](image)

**Bath Sonicator**
- unfocused transducer
- non-contact

**Probe Sonicator**
- focused waveguide
- sample contact
Appendix D-Carbon nanotubes and chirality

Chirality is defined as the direction of vector, chiral vector, along which a graphene sheet folds to form a carbon nanotube. The vector determines whether a carbon nanotube will be metallic or semi-conducting.
Figure E.1 photograph showing Nunc 96 well polysterene plate used for fluorescence experiments
Figure E.2 Layout for the Nunc 96 well polystyrene plate used for fluorescence experiments

Phosphate Mop

Phosphate is commonly found in biological and experimental materials, buffers and reagents, in the form of ribose-1-phosphate. In order to use phosphate sensor, it is important to make sure that plastic and glass labware is free from phosphate contamination, Phosphate mop is used for this purpose.