AN ABSTRACT OF THE DISSERTATION OF

Adeniyi A. Adenuga for the degree of Doctor of Philosophy in Chemistry
presented on July 8, 2013

Title: Functionalization of Carbon Nanotubes for Effective Biosensing and Potential Biomedical Applications

Abstract approved:

______________________________________________
Vincent T. Remcho

Since their discovery in the 1990s, the great potential of carbon nanotubes (CNTs) has made them a focus of many research endeavors, including their application as components of biosensors. The inherent chemical “inertness” of CNTs makes their application to biosensing a challenge. It is necessary to “decorate” their surfaces to endow them with molecular recognition capability along with a signal transduction function. Many research challenges still remain, most importantly in the area of immobilization of bioreceptors, in a controlled orientation, onto the sidewalls of CNTs with sufficient stability and without compromising either the activity of the biomolecule or the electrical properties of the nanotubes. On the other hand, it is also necessary to assess potential environmental toxicity effects in advance of widespread application of CNTs in nanomedicine and clinical
devices. Effective functionalization of CNTs could render them very hydrophilic and ensure their stability in suspension and in physiological environments, though it also might alter their potential toxicity. The work described in this dissertation focused on the development of novel techniques for functionalization of carbon nanotubes to enable their use as components of biosensors for use in physiological conditions. The goal was to develop cost effective detection techniques with novel mechanisms of detection. Also presented is a new method of functionalization of CNTs to render them highly hydrophilic and stable in aqueous suspension. CNTs modified using this approach were studied to ascertain their toxicity using a zebrafish model.

For biosensor development, carbon nanotubes were functionalized with 4-carboxybenzenediazonium tetrafluoroborate salt, which covalently grafted a carboxyphenyl moiety on the CNT sidewall without excessively compromising the electronic integrity of the nanotube. The carboxyphenyl moieties were then used to chemically attach biorecognition probes to the nanotubes in a controlled orientation, so as to preserve the activity of the probes. Initially, potentiometric biosensors were constructed by covalently coupling the carboxyphenyl moieties on the CNTs via an amine-modified anti-thrombin oligonucleotide (aptamer) or antibody. We explored the performance of the biosensor under physiological conditions in an effort to ensure the sensors would be suitable for biological analyses. The biosensors showed high sensitivity with a limit of detection for thrombin in the picomolar range, and exhibited reusability and reproducibility.

A second approach focused mainly on antibodies as bioreceptors on CNT transducers. Because of the fragile nature of antibodies, it was necessary to apply “gentle”
immobilization methods that would not compromise the activity of the antibody. This study, therefore, exploited the sulfhydryl group of the intrinsic disulfide groups in the hinge region of the antibody as a point of attachment to the functionalized CNT using \(3-(2\text{-pyridyldithio})\)propionyl hydrazide (PDPH) as a crosslinker. Because the number of disulfide bonds is quite small, a self-assembled monolayer of antibodies on CNTs was envisiaged. This approach was intended to ensure the correct orientation of the antibodies and to preserve their activity. To demonstrate this principle, C-reactive protein was used as the model target using anti-C-reactive protein antibody as biorecognition molecule. This biosensor demonstrated high sensitivity over physiologically relevant concentrations of CRP with limits of detection in the picomolar range. We also demonstrated in these studies the dependence of the sensitivity of potentiometric biosensors on the pH of the electrolyte buffer and proposed a novel mechanism for protein detection by the biosensor.

Lastly, we demonstrated effective functionalization of CNTs to render them highly hydrophilic - which led to enhanced suspension stability in physiological solutions. The prepared functionalized nanotubes were well characterized to evaluate their physical morphology and elemental composition. We also evaluated the functionalized CNTs for possible toxicological effects using the zebrafish model. The results showed that none of the CNTs studied caused significant adverse developmental effects. These results support the potential safe use of CNTs as components of indwelling medical devices such as tissue growth scaffolds, monitoring devices, and drug delivery.
Functionalization of Carbon Nanotubes for Effective Biosensing and Potential Biomedical Applications

by
Adeniyi A. Adenuga

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APPROVED:

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Major Professor, representing Chemistry

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Chair of the Department of Chemistry

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

__________________________
Adeniyi A. Adenuga, Author
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CONTRIBUTION OF AUTHORS

My research advisor, Dr. Vincent T. Remcho edited and assisted in writing of all chapters in this dissertation. His name appears on all published and submitted work contained herein. Dr. Neal Sleszynski was involved with the design and editing of chapters 1, 2 and 3. Dr. Ethan D. Minot was involved with the design of chapter 2. Sumate Pengpumkriat assisted with data collection in chapter 2. Dr. Robert Tanguay and Dr. Lisa Truong were involved in data collection and writing of chapter 4.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Carbon Nanotubes</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Structure and properties of carbon nanotubes</td>
<td>3</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Production and characterization of carbon nanotubes</td>
<td>6</td>
</tr>
<tr>
<td>1.1.3</td>
<td>Applications and challenges of carbon nanotubes as components of biomedical devices and biosensors</td>
<td>7</td>
</tr>
<tr>
<td>1.1.4</td>
<td>Functionalization of CNTs</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>Biosensors</td>
<td>16</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Transduction methods in Biosensors</td>
<td>17</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Biorecognition Elements in Biosensors</td>
<td>21</td>
</tr>
<tr>
<td>1.3</td>
<td>Carbon nanotubes in electrochemical biosensors</td>
<td>25</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Potentiometric sensors based on carbon nanotubes as ion-to-electron transducers</td>
<td>28</td>
</tr>
<tr>
<td>1.4</td>
<td>Objectives</td>
<td>33</td>
</tr>
<tr>
<td>1.5</td>
<td>References</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>DEVELOPMENT AND APPLICATION OF A SOLID-STATE CARBON NANOTUBE BASED POTENTIOMETRIC BIOSENSOR FOR DETECTION OF PROTEINS UNDER PHYSIOLOGICALLY RELEVANT HIGH IONIC STRENGTH</td>
<td>55</td>
</tr>
<tr>
<td>2.1</td>
<td>Abstract</td>
<td>56</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
<td>57</td>
</tr>
<tr>
<td>2.3</td>
<td>Experimental section</td>
<td>60</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Materials and reagents</td>
<td>60</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Synthesis and characterization of 4-Carboxybenzene diazonium tetrafluoroborate salt</td>
<td>61</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Covalent functionalization of GCE-CNT with 4-carboxybenzene diazonium salt and sensor preparation</td>
<td>62</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Characterisation of CNT modified glassy carbon electrode (CNT-GCE) and aptamer functionalized CNT-GCE</td>
<td>64</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.5. Electrochemical measurements</td>
<td>65</td>
</tr>
<tr>
<td>2.4. Results and Discussion</td>
<td>66</td>
</tr>
<tr>
<td>2.4.1. Characterization of the biosensor electrode surface</td>
<td>66</td>
</tr>
<tr>
<td>2.4.2. Optima pH condition for the biosensors</td>
<td>68</td>
</tr>
<tr>
<td>Figure 2.4 (a) Aptamer biosensor responses in buffer solutions at different pH, (b) effect of pH on sensor response at thrombin concentration of $1 \times 10^{-8}$ M</td>
<td>70</td>
</tr>
<tr>
<td>2.4.3. Performance of the biosensor based on sensitivity and detection limits</td>
<td>71</td>
</tr>
<tr>
<td>2.4.4. Repeatability and reproducibility</td>
<td>74</td>
</tr>
<tr>
<td>2.4.5. Selectivity</td>
<td>76</td>
</tr>
<tr>
<td>2.4.6. Antibody biosensor</td>
<td>80</td>
</tr>
<tr>
<td>2.4.7. Control experiments</td>
<td>82</td>
</tr>
<tr>
<td>2.5. Proposed mechanisms of detection</td>
<td>84</td>
</tr>
<tr>
<td>2.6. Conclusions</td>
<td>85</td>
</tr>
<tr>
<td>2.7. References</td>
<td>86</td>
</tr>
</tbody>
</table>

CHAPTER 3 ............................................................................................................. 90

IMMOBILIZATION OF ANTIBODIES AS SELF ASSEMBLED MONOLAYERS ON CARBON NANOTUBE-BASED POTENTIOMETRIC BIOSENSORS AND THE APPLICATION OF THESE DEVICES FOR DETECTION OF PROTEINS .................................................................................. 90

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1. Abstract</td>
<td>91</td>
</tr>
<tr>
<td>3.2. Introduction</td>
<td>92</td>
</tr>
<tr>
<td>3.3. Experimental</td>
<td>94</td>
</tr>
<tr>
<td>3.3.1. Materials and reagents</td>
<td>94</td>
</tr>
<tr>
<td>3.3.2. Fragmentation and reduction of anti-CRP (mouse IgG1) antibody</td>
<td>95</td>
</tr>
<tr>
<td>3.3.3. Covalent functionalization of GCE-CNT</td>
<td>96</td>
</tr>
<tr>
<td>3.3.4. Characterization of functionalized CNTs for assessment of surface morphology and elemental composition</td>
<td>99</td>
</tr>
<tr>
<td>3.3.5. Electrochemical measurements</td>
<td>100</td>
</tr>
<tr>
<td>3.4. Results and Discussion</td>
<td>100</td>
</tr>
<tr>
<td>3.4.1. Characterization of diazonium functionalized CNTs</td>
<td>100</td>
</tr>
</tbody>
</table>
### TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.2.</td>
<td>Identification of optimal pH conditions for CRP biosensors</td>
<td>105</td>
</tr>
<tr>
<td>3.4.3.</td>
<td>Performance of the biosensor based on sensitivity and detection limits</td>
<td>106</td>
</tr>
<tr>
<td>3.4.4.</td>
<td>Repeatability and reproducibility</td>
<td>109</td>
</tr>
<tr>
<td>3.4.5.</td>
<td>Selectivity</td>
<td>109</td>
</tr>
<tr>
<td>3.4.6.</td>
<td>Control experiments</td>
<td>112</td>
</tr>
<tr>
<td>3.4.7.</td>
<td>Assessment of the new technique of antibody sensor fabrication with the commonly used whole antibody functionalized sensors</td>
<td>114</td>
</tr>
<tr>
<td>3.5.</td>
<td>Conclusions</td>
<td>116</td>
</tr>
<tr>
<td>3.6.</td>
<td>References</td>
<td>117</td>
</tr>
<tr>
<td>3.7.</td>
<td>Control experiments</td>
<td>112</td>
</tr>
<tr>
<td>3.8.</td>
<td>Assessment of the new technique of antibody sensor fabrication with the commonly used whole antibody functionalized sensors</td>
<td>114</td>
</tr>
<tr>
<td>3.9.</td>
<td>Conclusions</td>
<td>116</td>
</tr>
<tr>
<td>3.10.</td>
<td>References</td>
<td>117</td>
</tr>
<tr>
<td>4.1.</td>
<td>Abstract</td>
<td>121</td>
</tr>
<tr>
<td>4.2.</td>
<td>Introduction</td>
<td>122</td>
</tr>
<tr>
<td>4.3.</td>
<td>Experimentals</td>
<td>124</td>
</tr>
<tr>
<td>4.3.1.</td>
<td>Materials and reagents</td>
<td>124</td>
</tr>
<tr>
<td>4.3.2.</td>
<td>Preparation procedure for soluble carbon nanotubes</td>
<td>125</td>
</tr>
<tr>
<td>4.3.3.</td>
<td>Characterization</td>
<td>125</td>
</tr>
<tr>
<td>4.3.4.</td>
<td>Toxicity testing</td>
<td>126</td>
</tr>
<tr>
<td>4.4.</td>
<td>Results and Discussions</td>
<td>127</td>
</tr>
<tr>
<td>4.4.1.</td>
<td>Characterization</td>
<td>127</td>
</tr>
<tr>
<td>4.4.2.</td>
<td>Toxicity</td>
<td>135</td>
</tr>
<tr>
<td>4.5.</td>
<td>Conclusions</td>
<td>139</td>
</tr>
<tr>
<td>4.6.</td>
<td>References</td>
<td>140</td>
</tr>
<tr>
<td>5.1</td>
<td>Summary and conclusions</td>
<td>145</td>
</tr>
<tr>
<td>5.2</td>
<td>Future work</td>
<td>148</td>
</tr>
<tr>
<td>6.1</td>
<td>BIBILOGRAPHY</td>
<td>149</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure of graphene and carbon nanotubes</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic honeycomb structure of a graphene sheet and different structures of carbon nanotubes</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>The chemical oxidation of SWNTs and the covalent binding of receptor molecules. EDC: 1 - Ethyl - 3 - [3 -dimethylaminopropyl]carbodiimide hydrochloride ; NHS :N - hydroxysuccinimide.</td>
<td>14</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic diagram showing the main components of a biosensor.</td>
<td>16</td>
</tr>
<tr>
<td>1.5</td>
<td>Schematic diagrams of antibody and fragments.</td>
<td>22</td>
</tr>
<tr>
<td>1.6</td>
<td>A potentiometric cell composed of a reference electrode and an indicator electrode (ion-selective electrode).</td>
<td>30</td>
</tr>
<tr>
<td>1.7</td>
<td>Schematic representaion of the ion-to-electron transduction process of the GCE/SWCNT/electrolyte system. C(^+) = cation, A(^-) = anion, e(^-) = electron.</td>
<td>32</td>
</tr>
<tr>
<td>2.1</td>
<td>Functionalization and Immobilization scheme</td>
<td>64</td>
</tr>
<tr>
<td>2.2</td>
<td>(a) Surface morphology of the developed sensor electrode examined with SEM showing successful attachment of large amount of CNTs entangled together in a spaghetti form (b) EDAX spectra of the CNTs on developed sensor electrode after functionalization with the diazonium salt showing the elemental compositions of the surface. The inset table displayed the percent elemental contents.</td>
<td>67</td>
</tr>
<tr>
<td>2.3</td>
<td>(a) Fluorescence microscope image of GCE-CNT surface (no aptamer) after exposure to Dylight-labeled thrombin. (b) Fluorescence microscope image of Dylight-labeled thrombin bound to the developed sensor.</td>
<td>68</td>
</tr>
<tr>
<td>2.4</td>
<td>Aptamer biosensor response in buffer solutions at different pH.</td>
<td>70</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 Calibration curve for thrombin detection in the concentration range of $1 \times 10^{-10} - 1.8 \times 10^{-7}$ M. (a) Time response showing change in potential as analyte’s concentration increases. (b) Calibration curve obtained from three different sensors with the error bars representing the standard deviation of the potentiometric measurements.</td>
<td>73</td>
</tr>
<tr>
<td>2.6 Demonstration of repeatability and reproducibility of the biosensors</td>
<td>75</td>
</tr>
<tr>
<td>2.7 Specificity of the sensor in response to (a) non-specific protein targets (elastase and HSA) compared with the specific binding of thrombin. (b) Specific binding of thrombin in the presence of $3.5 \times 10^{-9}$ M elastase and $5 \times 10^{-7}$ M HSA compared to response without elastase and HSA.</td>
<td>79</td>
</tr>
<tr>
<td>2.8 (a) Sensitivity of the antibody sensor to thrombin in different pH buffer electrolytes (b) calibration curve for the response of antibody sensor to thrombin compared to that of aptamer sensor.</td>
<td>81</td>
</tr>
<tr>
<td>2.9 Comparison of the responses for the developed sensors with those of the control sensors.</td>
<td>84</td>
</tr>
<tr>
<td>3.1. Fragmentation of anti-CRP antibody using Fab &amp; F(ab’)$_2$ fragmentation kit for mouse IgG$_1$ obtained from G-Biosciences.</td>
<td>96</td>
</tr>
<tr>
<td>3.2. Reduction of the hinge disulfide bond of antibody using TCEP.</td>
<td>96</td>
</tr>
<tr>
<td>3.3. Functionalization and Immobilization scheme for covalent attachment of reduced F(ab’)$_2$ antibody.</td>
<td>98</td>
</tr>
<tr>
<td>3.4. Thermogravimetric analysis curves of (a) pristine SWCNT, and (b) diazonium functionalized SWCNT, showing the percent weight loss as a function of temperature and mass change per °C temperature (dMass (mg)/dTemperature (°C)).</td>
<td>102</td>
</tr>
<tr>
<td>3.5 (a) SEM image of diazonium functionalized CNTs shown the surface morphology, (b) TEM image revealing the tubular structure of the functionalized CNTs, (c1 &amp; c2) HAADF STEM image showing the relative abundance of carbon atoms and oxygen atoms, respectively, in the diazonium functionalized CNTs, and (d1 &amp; d2) HAADF STEM image showing the relative abundance of carbon atoms and oxygen atoms, respectively, in the pristine CNTs.</td>
<td>104</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6 Calibration curve for CRP detection in the concentration range of $6.25 \times 10^{-12} - 8.33 \times 10^{-9}$ M. (a) Time response showing change in potential as a function of analyte’s concentration. (b) Calibration curve obtained from three different sensors with the error bars representing the standard deviation of the potentiometric measurements.</td>
<td>108</td>
</tr>
<tr>
<td>3.7 CRP sensor response to (a) non-specific protein targets (thrombin and HSA) compared with the specific binding to CRP. (b) Specific binding of CRP in the presence of $2.5 \times 10^{-6}$ M HSA compared with the sensor response to CRP in buffer only.</td>
<td>111</td>
</tr>
<tr>
<td>3.8 Comparison of CRP sensor’s responses with those of the control sensors.</td>
<td>113</td>
</tr>
<tr>
<td>3.9 Comparison of the performance of whole antibody and reduced F(ab)2 CRP sensors under the same experimental conditions.</td>
<td>115</td>
</tr>
<tr>
<td>4.1 (a) Solution of the prepared CNT after washing, drying and re-suspension in water. (b) An SEM image of the prepared SWCNT taken with 15KV accelerating voltage and 200,000 magnifications, and (c) TEM image the prepared SWCNT taken with operating voltage of 120KV and magnification of 200,000; demonstrating retention of structural integrity of the nanotubes.</td>
<td>129</td>
</tr>
<tr>
<td>4.2 Thermogravimetric analysis curves of pristine and treated SWCNT (after washing and drying) showing (a) percent weight loss as a function of temperature and (b), mass change per °C temperature ($dMass (mg)/dTTemperature (°C)$).</td>
<td>131</td>
</tr>
<tr>
<td>4.3 X-ray photoelectron spectroscopy (XPS) data demonstrates the elemental composition of SWCNTs before and after the acid treatment, indicating that only minor changes (attributable to surface fictionalization with carboxylic groups) arise following treatment.</td>
<td>132</td>
</tr>
<tr>
<td>4.4 FTIR spectra of untreated and acids treated CNTs (after washing and drying)</td>
<td>135</td>
</tr>
</tbody>
</table>
4.5  Mortality and adverse effects induced by seven different types of water soluble CNTs. Dechorionated embryos were exposed to the CNTs from 6-120 hpf and 4 endpoints were evaluated at 24 hpf, and 18 at 120 hpf. Endpoints evaluated are defined as follows: MO24 = mortality observed at 24 hpf; DP24 = developmental progression at 24 hpf; SM24 = spontaneous movement at 24 hpf; NC24 = notochord malformation at 24 hpf. Endpoints evaluated at 120 hpf were: MORT = cumulative mortality; YSE = yolk sac edema; AXIS = axis defects; EYE = eye defects; SNOU = snout defect; JAW = jaw defect, OTIC = otic (ear) defect; PE = pericardial edema; BRAI = brain defect; SOMI = somite defect; PFIN and CFIN = pectoral and cadual fin defect; PIG = pigmentation abnormalities; CIRC = circulation defects; TRUN = trunk defect; SWIM = swim bladder abnormalities; NC = notochord defect at 120 hpf and TR = touch response abnormality.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Comparison of the performance of the biosensor electrodes evaluate the repeatability and reproducibility using a thrombin concentration $5 \times 10^{-9}$ M.</td>
<td>76</td>
</tr>
<tr>
<td>3.1 Change of potential as a function of concentration of CRP in buffer of different pH values.</td>
<td>106</td>
</tr>
<tr>
<td>4.1 Description of the nanotubes and their zeta potential in Milli-Q water and zebrafish embryo medium.</td>
<td>128</td>
</tr>
</tbody>
</table>
To the glory of God,

this dissertation is dedicated to my wife, Oyebola Folasade Adenuga, who was there for me all the time and to my children, Ifeoluwa, Fiyinfoluwa and David.

I love you all
FUNCTIONALIZATION OF CARBON NANOTUBES FOR EFFECTIVE BIOSENSING AND POTENTIAL BIOMEDICAL APPLICATIONS

CHAPTER 1

INTRODUCTION
The main aim of the present Doctoral Thesis is to study and develop new approaches for functionalization of carbon nanotubes for: (1) effective biosensing and (2) potential clinical applications. In order to achieve the first goal, biosensors were developed with single-walled carbon nanotubes incorporated as transducers on glassy carbon electrodes, with bioreceptors covalently attached to the sidewall of the nanotubes in a controlled orientation to ensure that their activities are preserved. For the second goal, a new approach was used to functionalize different varieties of carbon nanotubes which rendered them hydrophilic. The stable homogenized suspension of nanotubes obtained was subjected to toxicity studies to determine their safety in biomedical applications.

In this chapter, a four-part general overview to carbon nanotubes is provided and their use as components of biosensors and in biomedical applications are reviewed. A particular emphasis is placed on the challenges encountered in the use of nanotubes for different applications, and the need for new approaches to enhance their effective application. The first section of this chapter provides an overview of the useful properties of carbon nanotubes and a review of functionalization and characterization techniques. The second section is an overview of the concepts of biosensors. The third section introduces the concept of carbon nanotubes as transducers for electrochemical biosensing with special remarks on their use as transducers in all-solid-state potentiometric biosensors. The fourth and last section presents the specific objectives of this Doctoral Thesis.
1.1. Carbon Nanotubes

1.1.1. Structure and properties of carbon nanotubes

Carbon nanotubes (CNTs) were discovered in 1991 by Iijima\(^1\) and represent an important class of nanoscale materials that have received great attention in recent years due to their enormous potential in many different fields. They possess one of the simplest chemical compositions and atomic bonding configurations, but show the most extreme diversity and potential among nanomaterials based on their structure and associated properties\(^2\). Carbon nanotubes are built from a well-ordered hexagonal arrangement of sp\(^2\)-hybridized carbon atoms in nanoscale dimensions and can be visualized as graphene sheets rolled up into seamless molecular cylinders\(^2\)–\(^5\). Depending on the arrangement, they can be classified as single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) with varying intrinsic bandgaps and helicities\(^6\)–\(^8\). SWCNT are comprised of a single cylinder, while MWCNT consist of multiple concentric cylinders that share a common longitudinal axis (Figure 1.1)\(^4\)–\(^9\),\(^10\). Carbon nanotubes usually have a high aspect ratio (breadth-to-length ratio), typically 1000 or greater, which is why nanotubes are often modeled as one dimensional structures\(^11\)–\(^13\). In this approach, every nanotube is considered a network solid, which behaves as a single molecule consisting of millions of atoms, with lengths that can vary from a few micrometers to millimeters. However, their diameters are a function of the type; SWNTs are 0.4–2 nm in diameter and MWNTs are 2–100 nm in diameter\(^2\),\(^5\),\(^11\)–\(^16\).
By folding a graphene sheet into a cylinder so that the beginning and end of an \((m; n)\) lattice vector in the graphene plane join together, an \((m; n)\) nanotube is obtained, where \(n\) and \(m\) are integers corresponding to the number of hexagons traversed in the two unit-vector directions, \(a_1\) and \(a_2\), of the graphene lattice\(^{19}\). The \((m; n)\) indices determine the diameter and chirality of the tube, which are crucial parameters of a nanotube and dictate numerous relevant properties. Nanotubes where \(m=n\) are ‘arm-chair’ tubes because the atoms around the circumference are in an arm-chair pattern. Nanotubes where \(n=0\) are termed ‘zigzag’ based on the atomic configuration along the circumference, while chiral nanotubes have \((m\neq n)\) with the rows of hexagons spiraling along the nanotube axes\(^{19}\). As an example, in Figure 1.2, the two basis vectors \(a_1\) and \(a_2\) are shown. Folding of the \((8,8)\), \((8,0)\), and \((10,-2)\) vectors lead to armchair, zigzag and chiral nanotubes, respectively. Depending on the chirality, CNTs have divergent properties such as optical activity, mechanical strength and electrical conductivity.

CNTs possess unique physicochemical properties different from those of other allotropes of carbon, such as graphite, diamond, or fullerenes. In terms of mechanical properties,
nanotubes are among the strongest and most resilient materials known to exist in nature, with Young’s modulus values greater than 1.0 TPa (approximately 500% higher than that of steel), densities as low as 1.3 g.m$^{-3}$ (less than that of Al), and exhibit impressive thermal stability, surviving heating to 1400°C in a vacuum$^{20,21}$. Theoretical and experimental reports have shown that some carbon nanotubes can display metallic behavior, reaching conductivities of 1000 times greater than that of copper, while exhibiting high thermal and chemical stability$^{22,23}$. CNTs are also extremely elastic and have tensile strength of about a hundred times higher than steel$^1$ and can tolerate large strains before mechanical failure$^{2,11,24}$.

Figure 1.2. Schematic honeycomb structure of a graphene sheet and different structures of carbon nanotubes$^{19}$.

The electrical properties of nanotubes depend on their $(m; n)$ indices and therefore on the diameter and chirality. A single walled carbon nanotube can be either metallic if $(m-n)$ equals zero or $(m-n)/3$ is an integer; otherwise, it will be semiconducting, with a bandgap inversely proportional to the diameter and ranging from approximately 1800 meV, for
nanotubes with very small diameters, to 180 meV for the largest diameter SWCNT\textsuperscript{13,25}. Thus, armchair nanotubes are metallic while zigzag and chiral tubes are either metallic or semiconducting\textsuperscript{26}. The differences in conductivity can easily be derived from the properties of graphene sheets\textsuperscript{27}. The electrical resistance of nanotubes is determined by quantum mechanics and has been ascertained to be independent of the nanotube length\textsuperscript{28}. Thus, there are many possibilities in the type of carbon nanotube molecules, and each nanotube could exhibit distinct physical properties.

1.1.2. Production and characterization of carbon nanotubes

Carbon nanotubes are generally produced by one of three major methods: electric arc discharge\textsuperscript{29}, laser ablation\textsuperscript{30,31} or chemical vapor deposition (CVD)\textsuperscript{32}. In these methods, CNTs are produced from the vaporization of graphite targets (arc discharge, laser ablation) or by passing a carbon containing vapor (e.g., CO) over a supported metal catalyst in a furnace at a temperature above 700 °C (CVD)\textsuperscript{33,34}. CVD is the most widely used commercial method of producing carbon nanotubes. Most of these synthesis procedures yield CNT aggregates with a narrow distribution of diameters, lengths and defect concentrations, although their variability of length and diameter may be tuned by controlling the growth conditions\textsuperscript{35}. Another widely used method for SWCNT growth is high pressure carbon monoxide (HiPCO) disproportionation, which has the advantage of producing SWCNTs on a large scale and with a high degree of purity, >90\%\textsuperscript{36}. Characterization of CNT samples can be a challenging task. It is difficult to obtain
unambiguous knowledge of their physical properties based on the data from only one characterization technique. Usually complementary techniques are employed including transmission electron microscopy (TEM), scanning electron microscopy (SEM), thermogravimetric analysis (TGA), Raman spectroscopy and XPS (X-ray photoelectron spectroscopy) among others.

1.1.3. Applications and challenges of carbon nanotubes as components of biomedical devices and biosensors

Inspired by the various properties of CNTs, research towards applying carbon nanotubes for biomedical applications has been progressing rapidly, and CNTs have become one of the most studied materials over the last two decades. Their applications include energy and gas storage, molecular electronic devices, composite reinforcements, biomedical and tissue engineering, nanoinjectors, neuroengineering, drug-delivery carriers, photo-thermal therapy, gene delivery systems, nanoprobes and biosensor technology. Some consumer products on the market now incorporate carbon nanotubes, such as battery electrode additives, racquets, surfboards, golf clubs, ice hockey sticks, mass transportation fuel system components, and plastics additives.

In biomedicine, CNTs have been used as substrates for directed cell growth and tissue regeneration, supporting materials for the adhesion of liposaccharides to mimic the cell membrane, transfection, and controlled drug release. Researchers have revealed the ability of SWNTs to cross cell membranes which enhances delivery of
peptides, proteins, and nucleic acids into cells\textsuperscript{56,62,63}. Because of this, CNTs could serve as an excellent vehicle to administer therapeutic agents, providing effective dosages of drugs and less elimination by macrophages. Additionally, utilization of their distinctive electrical, optical, thermal, and spectroscopic properties in a biological context has been exploited to yield advances in the detection, monitoring, and therapy of diseases\textsuperscript{64}.

In biosensing the application of CNTs as transducers provides a platform for electrical monitoring of biorecognition events\textsuperscript{65-67}. Based on their small size, high electrical conductivity, high surface-to-volume ratio, and the essentially one-dimensional structure, CNTs allow signals to be transported in a confined surface, making them extremely sensitive to electrical and chemical changes in their immediate environment\textsuperscript{68}. A number of CNT based biosensing techniques with fast response, high sensitivity, and easy miniaturization have been developed for selective sensing of DNA\textsuperscript{69-71}, antigens or antibodies\textsuperscript{72-76}, cells\textsuperscript{77-79}, and a variety of biological molecules\textsuperscript{80-87}. In general, the two major configurations of CNT-based biosensors are CNT field-effect transistors (CNT-FETs)\textsuperscript{88,89} and CNT electrochemical sensors\textsuperscript{90-92}.

In spite of the great potential of CNTs in biomedical and biotechnology applications, their manipulation is hampered by several problems. First, because of strong Van der Waals interactions, CNTs form bundles composed of hundreds of single nanotubes entwined together leading to formation of large aggregates\textsuperscript{93,94}. Second, pristine CNTs lack solubility in process-friendly solvents, which presents difficulties in processing and has imposed great limitations on the practical use of CNTs. Third, regardless of the production method,
metal and carbonaceous impurities remain in the samples and affect CNT properties, behaviors and interactions with biomolecules. Finally, fears of potential CNTs toxicity have severely hindered their widespread use in the areas of nanomedicine and nanobiotechnology\textsuperscript{94}. Despite the aforementioned difficulties, continuous efforts have been made towards solubilizing and functionalizing CNTs to improve their biocompatibility, and allow for their applications in biotechnology. Recent developments in the chemical functionalization of CNTs have greatly improved the stability, biocompatibility, solubility and dispersion of CNTs in water, subsequently opening the path for their handling and processing in physiological environments\textsuperscript{95}.

1.1.4. Functionalization of CNTs

1.1.4.1. Functionalization for solubilization

Solubility and biocompatibility are crucial factors for effective use of CNTs in biomedical applications\textsuperscript{96}. It is, therefore, important to have uniform and stable dispersions of CNTs in physiologically relevant conditions to enable biological applications. For biomedical applications, chemical modification or functionalization of the surface chemistry is required to make the nanotubes more hydrophilic and, as such, more water soluble. This makes them biocompatible, and transforms them into manageable materials as well as reducing or eliminating toxicity associated with CNTs\textsuperscript{97}. Surface functionalization of CNTs can be achieved by two main strategies – (1) by covalent interactions, and (2) non-covalent interactions or “wrapping”. Chemical reactions forming bonds with the nanotube
end or sidewall are performed in the covalent functionalization event, while non-covalent functionalization make use of favorable interactions between the hydrophobic domain of an amphiphilic molecule and the CNT surface, producing aqueous nanotubes “wrapped” by surfactant.

Several different covalent functionalization methods have been developed to render CNTs biocompatible and soluble, with sulfuric and nitric acid treatments being the most common\(^9\). During this process, functional groups including carboxylic acids and other oxygen containing groups are formed at the ends of tubes as well as at defect points on the sidewalls\(^10\). Zeng et al. observed sp\(^3\) carbon atoms on SWNTs after oxidation and further covalent conjugation with amino acids\(^10\). However, while oxidized CNTs are soluble in water, they aggregate in the presence of salts due to charge screening effects, and thus cannot readily be directly used for biological applications in physiological environments. Recently, we have prepared CNTs with moderate to good stability (with zeta potential ranging from \(-31\) to \(-61\) mV) in physiological solution by treating the nanotubes with mixtures of deuterated sulfuric and nitric acids\(^9\). As reported by other researchers, further modification of oxidized CNTs has been achieved by attaching hydrophilic polymers such as poly(ethylene glycol) (PEG), yielding CNT-polymer conjugates stable in biological environments\(^102-104\). Cycloaddition reactions are another commonly used type of covalent reaction to functionalize CNTs, which occurs on the aromatic sidewalls, instead of nanotube ends and defects as in the oxidation case. [2+1]-Cycloadditions have been
conducted by photochemical reaction of CNTs with azides\textsuperscript{105,106} or carbene generating compounds via the Bingel reaction\textsuperscript{107,108}.

Non-covalent or wrapping functionalization of CNTs has been performed with molecules such as amphiphilic surfactants, polymers, biomolecules, and polyaromatic compounds\textsuperscript{109}. The non-covalent interaction occurs primarily by weak interactions such as surface adsorption onto the side walls of CNTs, electrostatic interactions, hydrogen bonding, van der Waals or by $\pi$-stacking forces\textsuperscript{110,111}. One common approach to increase the solubility of CNTs in water is to treat them with detergents or surfactants such as sodium dodecyl sulfate (SDS) and Triton-X 100\textsuperscript{112}. Polymers that have been used in the formation of supramolecular complexes of CNTs include, among others, poly(ethylene glycol), polyvinyl pyrrolidone, polystyrene sulfonate, poly(1-vinyl pyrrolidone-co-vinyl acetate), dextran, dextran sulfate, poly(1-vinylpyrrolidone-coacrylic acid), poly(1-vinylpyrrolidone-codimethylaminoethyl methacrylate), and biologically derived materials such as bovine serum albumin, starch and chitosan\textsuperscript{23,96,113}. Taking advantage of the $\pi$-$\pi$ interaction between pyrene and the nanotube surface, researchers have used pyrene derivatives to noncovalently functionalize carbon nanotubes\textsuperscript{110,114}. Chen et al. showed that proteins can be immobilized on SWNTs functionalized by an amine-reactive pyrene derivative\textsuperscript{110}. Wu et al., recently demonstrated the use of pyrene conjugated glycodendrimers to solublize carbon nanotubes\textsuperscript{114}. Beside pyrene derivatives, single-stranded DNA molecules have been widely used to solubilize SWNTs by virtue of the $\pi$-$\pi$ stacking between aromatic DNA base units and the nanotube surface\textsuperscript{115-117}. 
1.1.4.2. Functionalization of CNTs for biosensing

CNTs cannot be directly used to detect biomolecules because they lack specific recognition elements. Introduction of a molecular recognition element onto the CNTs is therefore required to make a biosensor using CNTs, which will enable them to selectively recognize and transduce a signal in proportion to biomolecule concentration. A number of techniques have been established to modify CNTs with this molecular recognition capability through different functionalization processes. The basic idea for CNT functionalization for biosensing is to immobilize biomolecule probes, such as proteins, peptides, enzymes, aptamers, antibodies, or antigens onto the end, inner, or outer sidewalls of the CNTs. In biosensor development, useful functionalization should preserve the activity of the molecular recognition element being attached as well as the integrity of the CNT’s relevant electrical, mechanical, and chemical properties. It is, therefore, important to design techniques for immobilization of the biomolecular probes in a controllable and reproducible style. To conjugate bioreceptors to CNTs, both covalent bonding and non-covalent binding approaches have been employed.

In order to covalently attach biomolecules to CNTs, functional groups/desirable reactive species must first be created on CNTs (often at the tube ends or at defect sites), with the biorecognition element then attached at these sites. Creation of reactive sites on CNTs follows two general approaches: (1) oxidation at the nanotube open ends and defect sites or (2) nonselective attack of nanotube sidewalls by highly reactive species such as nitrenes and aryl diazonium salts. Oxidation processes can be performed through
wet chemical reactions with strong acids or dry plasma routes\textsuperscript{137} amongst others. These treatments can create several types of functional groups such as carbonyl, carboxyl and hydroxyl at the open ends or defect sites of CNTs. Using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as a cross-linker, biomolecules such as peptides\textsuperscript{138}, mononucleotides\textsuperscript{139} and proteins\textsuperscript{140,141} can be covalently conjugated onto carboxylated CNTs to act as nanotube-mediated biosensing devices (Figure 1.3).

A variety of biological species have been covalently bonded to CNTs\textsuperscript{131,142,143}. For example, in order to detect DNA hybridization, Wang et al. introduced carboxylic acid groups onto the surfaces or ends of nanotubes\textsuperscript{144}. Subsequently, amino-terminated probe oligonucleotides were immobilized by the formation of covalent amide bonds between these carboxyl groups and the amino groups at the ends of the DNA oligonucleotides. The occurrence of hybridization between the probe and the target DNA oligonucleotides was confirmed by monitoring changes in the voltammetric peak arising from the methylene blue label on the aptamer probe. Zelada-Guillén et al. showed that aptamer-based sensors can be used to detect living microorganisms, by linking aptamers to carboxylated SWNTs\textsuperscript{145}. The aptamers used in these studies were modified with an amine group at the 3’ end. This method can easily be applied to the attachment of CNTs to any useful entity including amine groups, such as nucleic acids, polymers, dendrimers, and even inorganic nanoparticles\textsuperscript{146,147}. 
Figure 1.3. The chemical oxidation of SWNTs and the covalent binding of receptor molecules. EDC: 1 - Ethyl - 3 - [3 -dimethylaminopropyl]carbodiimide hydrochloride; NHS :N - hydroxysuccinimide.

One of the advantages of covalent functionalization is good stability since it involves a direct attachment of the molecular recognition element to the CNT through formation of a stable chemical bond. Additionally, it offers better binding selectivity due to its ability to directly control the location and the number of the bioreceptors on the CNTs. However, this also often alters the intrinsic structure and properties of CNTs as well as the properties of the attached biomolecules\textsuperscript{148}. For biosensor applications, it is crucial to design techniques for covalent conjugation of bioreceptors to CNTs without compromising the electrical properties of the CNTs and the activity of the bioreceptors.

The noncovalent approach via electrostatic interaction, \( \pi-\pi \) stacking, or Van der Waals forces is also an efficient immobilization method for biomolecules. This functionalization
approach preserves the sp² nanotube structure, and thus its important electronic characteristics. Typically a bifunctional molecule, such as 1-pyrenebutanoic acid, succinimidyl ester, is irreversibly absorbed to SWCNTs as the pyrenyl group interacts with the hydrophobic surfaces of the SWCNTs due to π–π stacking. The succinimidyl ester groups are highly reactive to nucleophilic substitution with the amines on the surface of most proteins, resulting in the formation of an amide bond.

The first demonstration of SWCNT functionalization with proteins via π–π stacking was reported by Dai’s group at Stanford University. Proteins were immobilized through the nucleophilic substitution of N-hydroxysuccinimide by a protein amino group to form an amide bond. So et al used the same principle to develop a CNT field effect transistor (FET) for detection of thrombin. Later, Guldi’s group designed several new hybrid materials from CNTs and electron donors via two step reactions. They first prepared SWCNT/pyrene⁺ hybrids via π–π stacking and then immobilized negative charged electron donors onto SWCNT/pyrene⁺ via electrostatic interaction to yield the electron donor–acceptor nanohybrids. Alternatively, a direct assembly of water-insoluble porphyrin on SWCNT was designed based on the π–π noncovalent interaction between porphyrins and CNTs, resulting in a novel sensor for trichloroacetic acid.
1.2. Biosensors

Biosensors were first reported in the 1960s\textsuperscript{152}, and since then there have been many advances made in the field, including improved sensitivity and portability. In general, a biosensor consists of an active biorecognition element which is responsible for the selective detection of the target analyte(s), an interface architecture where a specific biological event takes place and gives rise to a signal picked up by a transducer that recognizes the biological event on the sensing element, converting it into an appropriate signal output; electrical, optical, thermal or magnetic\textsuperscript{152,153} (Figure 1.4). The selectivity of a biosensor is a function of the biorecognition element, while the sensitivity is a function of the transducer, the number of biorecognition elements present, and their viability and accessibility. The selection of the molecular recognition elements and method of transduction used in biosensors is determined by the analyte of interest and the physical property to be measured. Typical recognition elements used in biosensors are: antibodies, aptamers, nucleic acids, chemoreceptors, enzymes and larger entities such as whole cells, cell organelles and plant or animal tissue sections\textsuperscript{154}.

![Figure 1.4. Schematic diagram showing the main components of a biosensor](image-url)
The primary goals of miniaturized analytical devices include enhanced sensitivity, rapid response, low fabrication costs, selectivity and robustness. Biosensors are often able to meet these criteria. They are versatile types of analytical devices that can often be miniaturized easily and are ideally capable of selective identification of analytes at ultra-trace levels in real, untreated samples with little or no influence of the sample matrix. Biosensors are applicable in many different fields including; environmental monitoring of pollutants, clinical diagnosis of disease biomarkers, food processing, and detection of chemical and bio-warfare agents\textsuperscript{155,156}. In many cases the ability to obtain rapid and accurate results is important for appropriate judgments and control of situations, and to take punctual remediation to avert avoidable damages.

1.2.1. Transduction methods in Biosensors

A molecular recognition element can be interfaced to a number of different signal transducers which convert the molecular signal into an electrical, optical or digital signal that can be quantified, displayed, and analyzed. Transducers can be put into four general groups: optical (fluorescent, colorimetric, luminescent, and interferometric), mass based (piezoelectric and resonant/acoustic wave), electrochemical (amperometric and potentiometric), and calorimetric or thermal (temperature based)\textsuperscript{157}. 
1.2.1.1. **Optical detection biosensors**

Optical biosensors are based on various optical phenomena and measure properties including luminescence, absorption, fluorescence, phosphorescence, chemiluminescence, colorimetric, reflectance, and other optical signals generated as a result of the interaction of the analytes with the biorecognition element. Optical transducers convert changes in the optical properties, as a result of the analyte interaction with the recognition element, into an electrical/digital readout\textsuperscript{158}. Photonic crystal biosensors are a newly emerging class of biosensors that use a photonic crystal sensor to capture light from very small areas or volumes, which leads to greater measurement sensitivity. This method can detect when and where cells or molecules bind to or are removed from the crystal surface by measuring the light reflected by the crystal\textsuperscript{159}.

1.2.1.2. **Mass-based biosensors**

Resonant (or acoustic wave) and piezoelectric biosensors are in this category of biosensors. In resonant biosensors, an acoustic wave transducer is coupled with a molecular element, and a change in mass is observed as a result of the analyte interaction with the molecular probe. This leads to changes in the resonant frequency of the transducer, and the resulting frequency change is measured. Piezoelectric sensors are based on changes in the mass of quartz crystals when analytes are captured. Sorption of the analyte leads to a change in the mass of the crystal, generating a change in the frequency of oscillation\textsuperscript{160}. Immunosensors
and microcantilever sensors that use piezoelectric technology fall into this category, and have proven useful in the identification of cancer biomarkers\(^1\!
\)

### 1.2.1.3. Calorimetric biosensors

Calorimetric biosensors exploit one of the fundamental properties of biological reactions, namely heat change (absorption or production), which in turn changes the temperature of the medium in which the reaction takes place. They are constructed by coupling immobilized enzyme molecules with temperature sensors. Since many enzymatic reactions generate heat, the amount of heat generated can be used to measure analyte concentration\(^1\!
\). Medley et al., demonstrated the use of an aptamer-based gold nanoparticle calorimetric biosensor for the detection of cancer\(^1\!
\). The researchers used gold nanoparticles, and were able to differentiate between acute leukemia cells and Burkitt’s lymphoma cells. This report demonstrates the feasibility of combining aptamer based recognition elements with a calorimetric transducer to detect cancer cells and potentially discriminate between normal and cancer cells\(^1\!
\).

### 1.2.1.4. Electrochemical biosensors

Electrochemical biosensors are the most commonly used type of biosensor due to their cost effectiveness, sensitivity, portability, and ease of use\(^1\!
\). Some types of these biosensors can be used at home or in the doctor’s office as point-of-care (POC) devices. In these
devices the bioelectrochemical component serves as the main transduction element. The diabetic glucose monitor has revolutionized the way that diabetics measure blood glucose and is an electrochemical biosensor\textsuperscript{158}. The two most common types of electrochemical biosensors are potentiometric and amperometric. Potentiometric biosensors are almost always ion-selective electrodes which detect a change in measured potential based on the molecular recognition element\textsuperscript{154,159,162,163}. Amperometric sensors are operated at a fixed applied potential between the working electrode and the reference electrode, and the current produced is related to the concentration of target analytes. Amperometric transducers measure the current resulting from the oxidation or reduction of an electro-active species on the surface of a working electrode, and the signal produced is correlated with the concentration of target analytes\textsuperscript{154,164,165}. Typically, in amperometry the current is measured at a constant potential while in voltammetry the current is measured during controlled variations of the potential\textsuperscript{160}.

Other types in this class of transducers are; conductometric, which measure the change in the conductive properties of a medium due to the production or consumption of ions during the metabolic activity of microorganisms\textsuperscript{162}, impedimetric, which measures impedance (both resistance and reactance)\textsuperscript{166,167}, and field-effect, which uses transistor technology to measure current which is controlled by a potentiometric effect at a gate electrode\textsuperscript{160,168}.  

1.2.2. Biorecognition Elements in Biosensors

1.2.2.1. Antibodies

Antibodies are proteins found in the serum and other body fluids of vertebrates that react specifically with the antigens that induced their formation. Antibodies, also referred as immunoglobulins (IgGs), are immune-system protein complexes exhibiting a molecular weight of ca. 150 kDa. IgGs consist of four polypeptide chains, two identical heavy chains connected to two identical light chains, arranged in a Y-shape complex. Each IgG displays two antigen binding pockets at the “Fab” (fragment, antigen binding) region, which corresponds to an epitope (a site on an antigen at which an antibody can bind) for antigen binding, and the Fc region, which refers to the stem of the Y-shaped antibody; this region is known for its high degree of structural similarity among broad classes of antibodies (Fig. 1.5). Each arm of an antibody chain is connected by inter-chain disulfide bonds. An antigen binds to the paratope (the antigen-binding site of an antibody) of an antibody that is located at the top of the Y in a region called the antibody’s idiotype. An antibody binds to a specific antigen to form an immune complex. This is the basis for antibody-based immunoassays and immunosensor design. The IgG class of antibody is typically used in immunoassays because they bind antigens with greater affinity and specificity than other classes of antibodies. Recently antibody fragments, Fab, and F(ab’)2 are emerging as credible alternatives to whole antibodies. They provide the same specificity as whole antibodies but are smaller, which can be an advantage for biosensor applications. In their
main application field, biosensors and therapeutics, these small, highly specific reagents are often used as bi- or trimers\textsuperscript{172}. 

![Schematic diagrams of antibody and fragments](image)

**Figure 1.5 Schematic diagrams of antibody and fragments\textsuperscript{173}**

### 1.2.2.2. **Aptamers**

Aptamers are synthetic, functional single strand DNA or RNA oligonucleotide receptors engineered with a high affinity and specificity towards a specific target. Aptamers are oligonucleotides that can adopt complex secondary and tertiary structures, which facilitate specific interactions with other molecules and can be mass produced at a cost that is much less than that of antibodies, once their sequence is known. They are generated by an in vitro evolutionary selection process, referred to as Systematic Evolution of Ligands by Exponential Enrichment (SELEX)\textsuperscript{173,174}. Using SELEX, aptamers with distinguishing sequences and functional structures can be selected against a wide variety of targets
including proteins, cells, small molecules, small ions, organic molecules, large
glycoproteins and mucins.\textsuperscript{175,176}

They can be easily modified with different functional groups and tags, making them adaptable to various immobilization strategies for assay applications.\textsuperscript{175,177} Compared to antibodies, aptamers have a comparable affinity as capture molecules.\textsuperscript{178} The major advantage of aptamers over antibodies in electronic sensing is that they are smaller ~ 3nm; compared with antibodies with an average length of ~ 10 nm. Thus, aptamer – target binding can occur much closer to the sensor surface, which is advantageous for all surface - based sensors.\textsuperscript{147} In comparison to antibodies, aptamers can be prepared to a higher degree of purity, which reduces the batch-to-batch variation found in antibodies. Aptamers have higher temperature stability (they are stable at room temperature) and because of their small size, denser receptor layers can be generated on biosensor substrates. The animal-free production of aptamers is especially advantageous in cases where the immune response can fail when the target molecule (e.g. a protein) has a structure similar to endogenous proteins or when the antigen consists of toxic or non-immunogenic compounds.\textsuperscript{179}

\subsection{1.2.2.3. Enzymes}

Enzymes are proteins that act as powerful catalysts to convert specific substrates into products.\textsuperscript{180} The substrate is recognized by a binding pocket of the enzyme, similar to the antibody-antigen interaction.\textsuperscript{171} Redox enzymes catalyze reactions that produce or consume electrons. Enzymes with such specific binding pockets are used in
electrochemical enzymatic biosensing - where electrons are detected\textsuperscript{171,181,182}. The most widely used enzymes for amperobiosensors are glucose oxidase (GOx)\textsuperscript{183} and horseradish peroxidase (HRP)\textsuperscript{184}. The scheme of the reactions involving these enzymes are as shown in equations 1 & 2. Electron donors for the reaction with HRP are molecules such as phenols, aromatic amines, thioaminoles or iodide\textsuperscript{184}.

\begin{equation}
O_2 + \text{glucose} \xrightarrow{\text{GOx}} H_2O_2 + \text{gluconic acid} \quad (1)
\end{equation}

\begin{equation}
H_2O_2 + \text{donor} \xrightarrow{\text{HRP}} 2H_2O + \text{oxidized donor} \quad (2)
\end{equation}

Among the major problems when working with enzymes is their stability, which limits shelf life as well as operational stability. Because enzymes are very sensitive to their environment; deactivation, inhibition or unfolding upon adsorption and chemical or thermal inactivation are common if precautions are not applied\textsuperscript{185,186}.

\subsection{1.2.2.4. Other receptor types}

Other bioreceptors that have been used in biosensing include biomolecules like DNA, and various proteins. Larger biomaterials including whole cells and tissues have been used as receptors\textsuperscript{147}. For example, human olfactory receptors were employed as a recognition element in SWNT - FET sensors. The human olfactory receptors (hOR2AG1) were immobilized on a SWNT - FET, and the electronic responses to various odorant molecules measured\textsuperscript{187}. The sensor was found to exhibit very high sensitivity (\textasciitilde 100 fM), as well as
superior selectivity. An olfactory receptor normally bears ionizable cysteine residues, and once a specific odorant molecule has bound to a receptor molecule, the latter shifts to a negatively charged state. Negatively charged molecules most likely affect the work function of the metal contact electrodes on the SWNT-FETs, which is then translated into a conductance change. The sensor exhibited a clear decrease in conductance in the presence of amyl butyrate, whereas no change in conductance was observed for butyl butyrate.147

1.3. Carbon nanotubes in electrochemical biosensors

The International Union of Pure and Applied Chemistry (IUPAC) defined an electrochemical biosensor as “a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element”188. The sensitivity of electrochemical biosensors is combined with the inherent bioselectivity of the biological component to produce better detection. The biological component in the sensor recognizes its analyte resulting in a catalytic or binding event that ultimately produces an electrical signal, monitored by a transducer, which is proportional to the log of analyte concentration189. Electrochemical detection techniques have often been proven as inexpensive and rapid response methods with remarkable detection sensitivity, reproducibility, and ease of miniaturization.
The use of nanomaterials as a platform to achieve miniaturization of analytical instrumentation can enhance the development of low cost analytical devices requiring smaller sample volumes, decreased power and reagents consumption, with improved performance. Hence intensive ongoing research activities around the world have focused on developing new miniaturized materials and technologies for detection of analytes of interest are ongoing. Synergies in the advantageous properties of traditional carbon-based electrodes and those of nanomaterials have led to CNT electrode applications in electrochemical detection of various molecules and chemicals of interest in many fields, including clinical diagnosis, environmental monitoring, and food processing among others. Electrochemical biosensors that utilized CNTs as electrode materials have been shown to exhibit low limit of detection (LOD) and fast response due to the signal enhancement provided by high surface area and rapid electrode kinetics. The high thermal and electrical conductivity, mechanical strength and chemically stability of CNTs is also very appealing for sensing applications.

Compared with “conventionally scaled” materials and other types of nanomaterials, the following special nanostructural properties give CNTs some overwhelming advantages in fabricating electrochemical sensors;

(i) the large specific area produces high sensitivity;

(ii) the tubular nanostructure and the chemical stability allows the fabrication of ultrasensitive sensors consisting of as few as one nanotube;
(iii) good biocompatibility that is suitable for constructing electrochemical biosensors, especially for facilitating the electron transfer in enzymes, and other redox proteins;

(iv) the modifiable ends and sidewalls provide a chance for fabricating multifunctioned electrochemical sensors via the construction of functional nanostructures;

(v) the possibility of achieving miniaturization;

(vi) the possibility of constructing ultrasensitive nanoarrays\(^\text{199}\).

SWCNTs have been proven to have efficient electron transport through individual nanotubes and high current density, making them ideal materials for electronics and electrochemical applications\(^\text{200}\). It has been demonstrated that SWCNTs can carry up to 10\(^9\) A\(\cdot\)cm\(^{-2}\) current densities, more than any metal can carry\(^\text{201}\). The combination of high conductivity (1.0 x 10\(^6\) - 3.0 x 10\(^6\) S\(\cdot\)m\(^{-1}\))\(^\text{202,203}\) with their nanoscale size makes SWCNTs a good material for molecular wires in electrochemical applications and electronics. Furthermore, the small diameter of the SWCNTs makes them an ideal material for the construction of a nanoelectrode array, which can enhance the surface area of the electrode (compared with planar surfaces) for the covalent attachment and detection of biomolecules. The advances in SWCNT functionalization with single molecules open the possibilities to use SWCNTs as individual electrodes or nanoelectrodes\(^\text{204}\).

The use of CNTs for electrochemical biosensing was pioneered by the Wang group, who developed a CNT-based electrode to detect the reversible oxidation of dopamine\(^\text{205}\).
Intensive work has since been done to take advantage of CNTs as electrochemical biosensor transducers to realize the potential benefits of enhanced reactivity and sensitivity, higher currents, lower overvoltages, and enhanced electron transfer rates, even in proteins where the redox center is embedded deep within the glycoprotein shell\textsuperscript{206,207}. Different techniques have been used to incorporate CNTs onto electrochemical transducers, such as direct growth on the electrode surface, adsorption onto existing electrodes, incorporation in polymer coatings, or combining CNTs and a binder to make a paste electrode\textsuperscript{83,208-213}. Among the most frequently used electrochemical methods with CNT sensors are voltammetry, potentiometry, amperometry, electrical impedance spectroscopy, and measurement of resistance or conductance in field effect transistor (FET) sensors, where CNTs are used as the gate.

1.3.1. Potentiometric sensors based on carbon nanotubes as ion-to-electron transducers

Potentiometric sensors have become progressively more useful in many areas of biomedical applications, such as pharmaceutical development, disease screening and monitoring, and remain one of the workhorses in clinical laboratories. Potentiometry is one of the simplest electrochemical techniques with extraordinary analytical capabilities. Over the years, potentiometry has been a compelling option for numerous analyses owing to the low cost, simplicity and ease of use of the instruments employed, short response times, high selectivity, and very low detection limits. Potentiometry is based on the passive
measurement of the electric potential between two electrodes, a reference electrode and an indicator electrode, while the solution composition is unaffected. The reference electrode consists on an electrode with a well-known and stable electrode potential\textsuperscript{154,214,215}. For potentiometric sensors, the relationship between the analyte concentration and the potential is typically governed by the Nernst equation:

\[ E_{\text{cell}} = E_{\text{cell}}^\circ - \frac{RT}{nF} \ln Q \]

Where \( E_{\text{cell}} \) is the observed cell potential at zero current, which is also referred to as electromotive force (EMF), \( E_{\text{cell}}^\circ \) is a constant potential for the system, \( R \) is the universal gas constant, \( T \) is the absolute temperature in degrees Kelvin, \( n \) is the charge number of the electrode reaction, \( F \) is the Faraday constant and \( Q \) is the ratio of the oxidized ion to the reduced ion\textsuperscript{216}.

The indicator electrode can be made selective towards a specific target (ion or other charged species) by incorporating a selective recognition element at the transducer/solution interface. When the recognition element, which consists of an ionophore that is able to recognize a specific ion, is embedded in a permeable membrane between the transducer and the solution (as in the ion-selective membranes), the potentiometric electrode is termed as an ion-selective electrode (ISE) (Figure 1.6). However, other recognition elements such as biomolecules, aptamers and antibodies, can be included in the construction of an indicator electrode as an alternative to using selective membranes. In a circumstance as
this, the electrode can be custom-made to detect diverse targets as long as the transducer part is able to detect the electrochemical variations occurring during the recognition process. Among potentiometric techniques, EMF measurements based on solid state electrodes, referred to as solid-contact ion-selective electrode (SC-ISE), represent an attractive tool for chemical/biological analysis in liquid samples. SC-ISE is a promising alternative to conventional liquid-contact ISE, drawing tremendous attention from both the experimental and theoretical scientific communities ever since its discovery in 1971217-220.

![Figure 1.6 Potentiometric cell composed of a reference electrode and an indicator electrode (ion-selective electrode)](image)

In potentiometric analysis, the electric potential (EMF) measured is related to the analyte concentration when a thermodynamic equilibrium is reached between the free analyte in the solution and the analyte bound to the recognition element on the indicator electrode. In
the steady state, the decrease of free energy produced when the analyte diffused from the solution to the recognition layer is compensated by an increase of free energy at the recognition layer/transducer interface by the repulsion of charges of the same charge at the transducer. This change of free energy is thus measured as a change in voltage using a high impedance voltmeter. In a rapid-kinetic stabilization process, this equilibrium can be reached almost in real-time, and thus, the measurement of voltage between the reference electrode and the indicator electrode gives a signal related to analyte concentration\textsuperscript{221-224}. Solid-state potentiometric sensors present advantages over their counterparts with an internal solution, especially in terms of their ability to be miniaturized. Moreover, in the case of ion selective electrodes with internal solutions, leakage of the ions from the internal solution may occur in long-term measurements, which does not occur in a solid state ISE\textsuperscript{220}.

On the other hand, carbon nanotubes with an extremely high surface-to-volume ratio, outstanding electrical properties and the high charge transfer capacities are candidates of choice for construction of miniaturize sensors. Incorporation of CNTs as part of the transducing components in potentiometric biosensors adds advantages of enhanced electron transfer and catalytic behavior, rapid electrode kinetics, and increased accumulation of biomolecules. The combination of the two fields of potentiometry and nanomaterials is therefore a promising way to develop highly sensitive biosensors. Researchers have found the approach of using nanostructured materials as the solid-contact ion-to-electron transducers to be greatly promising and effective\textsuperscript{220}. Gold nanoparticles\textsuperscript{225}
and carbon-based nanomaterials like fullerene\textsuperscript{226}, single-walled, and multiwalled carbon nanotubes (CNTs)\textsuperscript{227-229}, have been employed to fabricate SC-ISEs. Charge transfer processes in carbon nanotubes are driven by the high double layer capacitance resulting from the large interface between the nanotubes and the solution\textsuperscript{230,231} (Figure 1.6). This large double layer capacitance has been shown to remarkably minimize the potential drift (less than 20 μV h\textsuperscript{-1}) of the SC-ISEs\textsuperscript{220}.

Figure 1.7 Schematic representation of the ion-to-electron transduction process of the GC/SWCNT/electrolyte system\textsuperscript{230,231}. C\textsuperscript{+} = cation, A\textsuperscript{-} = anion, e\textsuperscript{-} = electron.

Many successful applications of potentiometric biosensors have been reported in the literature. Düzgün et al.\textsuperscript{232} covalently linked a thrombin aptamer to a layer of carboxylated SWCNTs, thus developing aptasensor which is able to record a potentiometric signal that is directly proportional to the log concentration of the target protein with a limit of detection of 80 nM. Zelada-Guillén et al. demonstrated the feasibility of quantitatively determining the presence of very low levels of different bacteria in different experiments, in a rapid and
selective way using the hybrid material carbon nanotube–aptamer and also for detection of protein in blood samples\textsuperscript{233-236}.

1.4. Objectives

The main objective of this Doctoral Thesis work was the design, development, characterization and application of solid-contact indicator electrodes based on single-walled carbon nanotubes for the detection of clinically important proteins. In addition, the work sought to assess the toxicity of functionalized carbon nanotubes in aqueous media to determine their safety for use in biomedical applications.

This general objective is achieved through a series of specific objectives:

- Development of new approaches to functionalize single walled carbon nanotubes, as transducers on all solid state indicator electrodes, with aptamers and antibodies for biosensors using potentiometric detection, which demonstrated high sensitivity and selective recognition properties.

- Demonstration of the successful applications of the developed label free biosensors under clinically relevant high ionic strength without compromising their sensitivities, which is fundamental to the ability to apply the sensors for detections in unprocessed biological samples.
- Optimization of the pH buffer conditions for the successful use of the biosensors and demonstration of a new detection mechanism for the biosensors.
- Development and application of a new functionalization approach for production of highly hydrophilic carbon nanotubes.
- Demonstration of the stability of the prepared hydrophilic carbon nanotubes in physiological solutions and successful assessment of the developmental toxicity effect of the nanotubes using a zebrafish model.

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53


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CHAPTER 2

DEVELOPMENT AND APPLICATION OF A SOLID-STATE CARBON NANOTUBE BASED POTENTIOMETRIC BIOSENSOR FOR DETECTION OF PROTEINS UNDER PHYSIOLOGICALLY RELEVANT HIGH IONIC STRENGTH

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To be submitted
2.1. Abstract

The ability to obtain rapid and accurate analytical results is very important for appropriate judgments and control of situations, and punctual treatment to avert avoidable damages. Biosensors are versatile devices that are able to provide the necessary results because they possess the potential to monitor the target analytes in real-time, with high sensitivity and selectivity. We report here the development of a highly sensitive and very selective solid-state potentiometric biosensor capable of detecting proteins at picomolar concentrations. The potentiometric biosensors were constructed using carbon nanotubes (CNTs) deposited on a glassy carbon electrode, covalently functionalized by reacting with 4-Carboxybenzene diazonium tetrafluoroborate salt, which grafted a carboxyphenyl moiety on the CNT sidewalls. The carboxyphenyl moieties on the CNTs were then covalently coupled to an amine modified oligonucleotide (aptamer) or antibody. In this study, thrombin was used as a model protein (analyte) and we compared the performance of both anti-thrombin aptamer and antibody functionalized biosensor electrodes. We explored the performance of the biosensor under physiologically relevant ionic strength conditions to ensure it was suitable for real biological samples analyses. Linearity was obtained for log thrombin concentrations from $5 \times 10^{-10} \text{ M}$ to $7 \times 10^{-8} \text{ M}$ with a sensitivity of $-31.01 \pm 1.41 \text{ mV/decade}$ and regression coefficient $\geq 0.99$. The limit of detection (determined according to classical potentiometric sensors as the intersection of the two extrapolated straight lines) was $4.1 \times 10^{-10} \text{ M}$. 
2.2. Introduction

Unambiguous recognition and quantification of clinically important biomolecules is critical in many areas of clinical sciences, security and biotechnology, from diagnosis of life-threatening diseases to detection of biological agents in warfare or terrorist attacks. In many cases the ability to obtain rapid and accurate results is important for appropriate judgments and control of situations, as well as providing necessary treatment to avert avoidable damage. Biosensors possess the ability to monitor their targets in real samples, in a real-time, with high sensitivity and selectivity and with little or no influence of the sample matrix. The field of sensor research is progressing so rapidly that new achievements appear monthly, especially those focused on electrochemical methods of detection. Potentiometric sensors are one of the simplest types of electrochemical sensors, with extraordinary analytical capabilities. They have become progressively more useful in many biomedical applications, such as new drug development, disease screening and monitoring. Over the years, potentiometry has been a compelling option for numerous analyses owing to the low cost, compatibility with novel microfabrication technologies, simplicity, instrument ease of use, low power requirements, short response times, high selectivity, very low detection limits, and independence of sample turbidity.

The incorporation of nanomaterials in biosensors is increasing due to their ability to enhance sensitivity, which is of major importance for clinical diagnostics since the concentration of targets can be very low in biological samples. Hence intensive research activities around the world focused on developing new miniaturized materials and
technologies for sensing and detection of analytes of interest are ongoing. Electroc
chemical sensing using CNTs as electrode materials have been shown to exhibit low limit of detection (LOD) and fast response due to the signal enhancement provided by high surface area, low overvoltage, and rapid electron transfer kinetics. The high thermal and electrical conductivity, mechanical strength, and chemical stability of CNTs is very appealing for sensing applications. Their high surface-to-volume ratio, functional surfaces, excellent biocompatibility, and modifiable sidewall make CNTs ideal candidates for constructing high performances sensors, and make them a definite candidate for the development of biosensing platforms for single-molecule detection. Combining the advantageous properties of traditional carbon-based electrodes with those of nanomaterials, CNTs electrodes have been applied in electrochemical detection of compounds of interest in different fields including: environmental monitoring of pollutants, clinical diagnosis, food processing, and even in defense and homeland security systems. The combination of the two fields of potentiometry and nanomaterials is therefore a promising way to develop highly sensitive biosensors.

Different potentiometric biosensors have been reported for detection of various clinically important biomolecules. However, most of these sensors operate under low ionic strength (ca 1mM or lower) to avoid ionic screening effects. Therefore, for practical applications such sensors will almost always require the time-consuming and often uncontrollable biological sample manipulation steps such as desalting processes or dilution by several order of magnitude. Based on the fact that physiologically ionic strength
of most relevant biological samples is greater than 100 mM, developing sensors that are capable of functioning under such high ionic strength solutions is crucial to achieve real time detection in unprocessed biological samples.

Thus, in this study we demonstrate the successful fabrication of a simple and effective all-solid-state biosensors operating under clinically relevant high ionic strength solution using potentiometric detection techniques. We fabricated biosensors with either anti-thrombin aptamer or antibody, and characterized them using thrombin as the target protein. Thrombin is a multifunctional serine protease that plays an important role in procoagulant and anticoagulant functions\textsuperscript{1,19}. However, it is also associated with some pathological processes, including cardiovascular diseases, angiogenesis, metastasis, inflammation, and has also been shown to contribute to tumor progression in manners both coagulation-dependent and coagulation-independent\textsuperscript{19-22}. The successful application of biosensors developed with either aptamer or antibody as bioreceptors, under the same condition of high ionic strength, is an indication that the mechanism of detection in this study is not a function of the nature of the bioreceptors but on the charge formation and accumulation of the target proteins that binds to the biosensor and the response of the electrode transducer to such interactions.
2.3. Experimental section

2.3.1. Materials and reagents

The nanotubes used were 90% semiconducting single walled carbon nanotubes in solution, a high performance semiconducting nanotube ink (‘IsoNanotubes-S 90%’ by NanoIntegris, Inc.), with 1.2nm -1.7nm diameter and length ranged from 300 nm to 5 micron. Anti-thrombin aptamer with sequence: 5’-(GGT TGG TGT GGT TGG)-(CH2)3-NH2-3’ was purchased from Biosythesis Inc, mouse monoclonal anti-thrombin antibody(IgG1 isotype) was obtained from Abcam, and human α-thrombin was supplied by Enzyme Research Laboratories. The reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS) were purchased from Thermo Scientific Pierce, sodium phosphate monobasic and diabasic (NaH2PO4 and Na2HPO4), sodium chloride (NaCl), potassium chloride (KCl), chitosan and tris base were from Sigma, N-morpholinoethane sulfonic acid (MES) sodium salt and human serum albumin (HSA) were purchased from CALIBIOCHEM - EMD biosciences, and elastase from USB corporation. The water used was purified with Milli-Q Advantage A10 system (EMD Millipore Corporation) equipped with BioPak catridge to produce pyrogem free, DNase free and RNase free ultrapure water.
2.3.2. Synthesis and characterization of 4-Carboxybenzene diazonium tetrafluoroborate salt

Covalent functionalization of CNT using 4-Carboxybenzene diazonium tetrafluoroborate salt to graft carboxyphenyl moieties on CNT sidewalls has been described in the literature.\textsuperscript{19,20} However, the diazonium salt is not commercially available hence it had to be synthesized. 4-Carboxybenzene diazonium tetrafluoroborate salt was synthesized from a 4-aminobenzoic acid precursor as described in the literature.\textsuperscript{21,22} 4-Aminobenzoic acid, obtained from Alfa Aesar, (6.85 g, 50 mmol) was dissolved in 36.25 ml of 48% fluoroboric acid, obtained from BDH, (200 mmol) and 50 mL water. The solution was heated until all the solids dissolved completely and was then cooled in an ice water bath. Sodium nitrite, from Alfa Aesar, (3.65 g, 53 mmol) dissolved in 10 mL water was added dropwise to the reaction mixture with stirring. The solution was allowed to warm to room temperature and was concentrated by rotary evaporation to approximately half the original volume. The concentrated solution was cooled in an ice bath, and the resultant white solid was filtered and washed with cold ether and cold water, and dried in a vacuum oven. The synthesis product of diazonium salt was confirmed with proton nuclear magnetic resonance (\textsuperscript{1}H NMR) spectroscopy (Bruker Avance III 700 MHz NMR, CD\textsubscript{3}CN) and IR (Spectrum Two IR spectrometer by PerkinElmer). The NMR data showed δ (ppm) values, two doublets at 8.439-8.461 and 8.616-8.637 and the diazonium function by IR spectroscopy at about 2306 cm\textsuperscript{-1}. These data are in agreement with the literature data by Saby et al.\textsuperscript{26}
2.3.3. Covalent functionalization of GCE-CNT with 4-carboxybenzene diazonium salt and sensor preparation

The solid contact sensor was prepared using a Glassy Carbon electrode (BAS, Inc.) with OD of 6 mm and ID of 3 mm. The tip of the electrode was sequentially polished using 1 µm, 0.3 µm and finally 0.05 µm grain size alumina powder slurry to obtain a smooth surface which was observed under optical microscope before modification. Residual alumina were removed from the electrode surface by sonicating the electrode tip in Milli-Q water for 5 min. The tip of the electrode was then soaked for 4 hrs in 1% chitosan solution prepared with 1% acetic acid solution. The electrode was then rinsed briefly with Milli-Q water and dried with N₂ gas. 20 µL of IsoNanotubes-S 90% semiconducting SWCNTs solution (0.08 mg/mL) was deposited on the electrode’s surface and allowed to dry for 45 min at 70 °C in an oven. The deposition process was repeated eight times, with DI rinsing by dipping the surface in Milli-Q water and stirring for 1 min at every third interval. CNTs on the GCE surface were then reacted with 20 mM 4-Carboxybenzene diazonium tetrafluoroborate salt solution for 90 min at 45 °C to modify nanotube sidewalls with carboxyphenyl moieties. The electrode surface was then washed sequentially with acetone, isopropy alcohol and Milli-Q water, and dried with gentle stream of N₂. The bioreceptors (Anti-thrombin aptamer or antibody) were then immobilized onto the electrode using EDC/NHS bioconjugation technique, forming amide bonds (Figure 2.1).

The carboxylic acid of the 4-carboxybenzene moieties on the nanotube sidewalls were activated by placing electrodes at room temperature for 30 min in an aqueous solution of
100 mM N-(3-dimethylaminopropyl)-N’-ethylenediamine hydrochloride (EDC) and 50 mM sulfo-N-Hydroxysulfosuccinimide (sulfo-NHS), prepared with activation buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid sodium salt (MES), 0.5 M NaCl which was pH adjusted to 5.6 with HCl). Immediately afterwards electrodes were briefly dunked in the activation buffer, and then placed in a solution of anti-thrombin DNA aptamer (1 µM) or antibody (100 µg/mL) prepared in 50 mM phosphate buffered saline (PBS) of pH 7.4 (containing 140 mM NaCl and 5 mM KCl) for 5 hr at room temperature. The antibody had previously been fragmented F(ab’)_2 to reduce the distance between the electrode surface and the capture part of the antibody, reducing the antibody size to a size relatively similar to that of the aptamer. Fragmentation was done using Fab & F(ab’)_2 fragmentation kit for mouse IgG1 obtained from G-Biosciences and following the manufacturer’s procedures. Upon completion, electrodes were washed sequentially with PBS and Milli-Q water before drying under a gentle stream of N₂. The sensors were store in a fridge at 4 °C when not in use.
2.3.4. Characterisation of CNT modified glassy carbon electrode (CNT-GCE) and aptamer functionalized CNT-GCE

CNT modified electrodes were characterized using a scanning electron microscope (FEI QUANTA 600F environmental SEM) to assess the surface morphology and elemental composition of CNT-GCE after functionalization with the diazonium salt. Fluorescence microscopy (FM) was used to assess the binding of thrombin to the anti-thrombin aptamer on CNT-GCE (biosensor) using a Zeiss Axio Imager M1m microscope (Thornwood, NY, USA). To achieve this, thrombin was fluorescently labeled with a photostable protein.
fluorescent labeling dye, DyLight 633 (Thermo Scientific) using the labeling kits provided by the company and following the instruction protocol. Briefly, 2 mg/mL of thrombin was prepared in 0.05 M borate (pH 8.5) buffer and 0.5mL was added to the vial of DyLight reagent, vortexed gently, pipette up and down to mix and centrifuged briefly. Then the reaction mixture was incubated for 60 minutes at room temperature protected from light. To remove the excess dye, after incubation the reaction mixture was added to a spin column (part of the kit) and mixed with the resin inside by vortexing. It was then centrifuged for about 1 minute at ~1000 × g to collect the purified proteins. The biosensor was exposed to 1μM dylight labeled thrombin and allowed to react for 30 min after which it was rinsed with milli-Q water and dried with nitrogen gas before taken the fluorescence images. As a control, CNT-GCE was subjected to the same process described above but without aptamer modification.

2.3.5. Electrochemical measurements

Electrochemical measurements were performed in a 5 mL buffer solution under continuous stirring using a digital Ionalyzer (Orion Research Inc, 701A). The two electrode system was made up of the functionalized CNT-GCE as the indicator electrode and a silver-silver chloride (Ag/AgCl) electrode as reference. All potentiometric measurements were performed at room temperature (23.3 ± 0.8 °C) and sample was added to the electrochemical cell with stepwise increase in concentration from 1 x 10^{-10} up to 1.4x 10^{-6} M. After use, the aptamer sensors were regenerated by soaking the electrode tip in a 6 M
guanidine hydrochloride solution for 20 min, then in 2 M NaCl solution for 10 min before rinsing thoroughly with Milli-Q water.

2.4. Results and Discussion

2.4.1. Characterization of the biosensor electrode surface

The successful use of chitosan as an adhesive molecule for the immobilization of CNTs onto electrodes for electrochemical detection has been reported in the literature.27,28 In this study, the surface morphology of the CNT modified GCE examined with SEM showed large amount of CNTs entangled together in a spaghetti form (Figure 2.2a). This confirmed the successful attachment of CNTs to the electrode with the aid of chitosan. The elemental composition of the diazonium CNTs on GCE as shown in the Energy Dispersive Analysis X-Ray (EDAX) spectra (Figure 2.2b) revealed the presence of oxygen (O) and carbon (C) atoms and their relative abundances shown in the inset table. A weight percent of about 96% and 4% were obtained for C and O respectively. The oxygen content is attributed to the carboxyphenyl moieties grafted onto the sidewall of SWCNTs.
Figure 2.2 (a) Surface morphology of the developed sensor electrode examined with SEM showing successful attachment of large amount of CNTs entangled together in a spaghetti form (b) EDAX spectra of the sensor, including glassy carbon on developed sensor electrode after functionalization with the diazonium salt showing the elemental compositions of the surface. The inset table displayed the percent elemental contents.

The successful immobilization of anti-thrombin DNA aptamer to the CNT functionalized electrode surface, and the formation of aptamer-antigen complex was assessed by fluorescent microscopic analyses. The biosensor electrode was exposed to 1 µM solution of fluorescently labelled thrombin solution, and incubated for 30 min followed by thorough washing with Milli-Q water and dried under a stream of N₂ gas. As control a CNT-functionalized electrode, without the aptamer, was subjected to the same process. Figure 2.3 (a & b) shows the fluorescent image of the control sensor and the aptamer-antigen complex on the biosensor electrode. The labeled thrombin bound to the anti-thrombin
aptamer was observed randomly throughout the sensor electrode surface. These results clearly confirmed the successful immobilization of aptamers to the electrode surface through the attached CNTs and their complexes with thrombin.

![Fluorescence microscope images](image)

Figure 2.3 (a) Fluorescence microscope image of GCE-CNT surface (no aptamer) after exposure to Dylight-labeled thrombin. (b) Fluorescence microscope image of Dylight-labeled thrombin bound to the developed sensor.

### 2.4.2. Optima pH condition for the biosensors

In order to optimize the buffer pH for the sensitive operation of the biosensor we examined the sensor’s response in buffers at different pH; from pH 5.4 to pH 8.3. Each buffer solution was 50 mM and also contained 140 mM NaCl and 5 mM KCl. This was to ensure that high salt concentration would not be an issue if the biosensor were used with undiluted
biological samples. The concentration of thrombin was varied from $1 \times 10^{-9}$ to $1.4 \times 10^{-6}$ M using stepwise addition. As shown in Figure 2.4a, the response in the moderately acidic buffer (pH 5.4) showed the best sensitivity, the sensitivity deceased as we moved across the pH scale to slightly basic. This is attributed to the rate of charge formation and accumulation as thrombin was bound by the aptamers immobilized on the biosensor electrode. It is worth noting that we ran the experiment with a more acidic buffer (pH 3.8), an example is shown in Figure 2.4b for $1 \times 10^{-8}$ M thrombin concentration. Though higher sensitivity was observed, we could not obtain an equilibrium signal at a single concentration for the reaction. This could be due to degradation of thrombin in the acidic medium or to destabilization of the aptamer/thrombin complex. Based on the revelation of these results further studies were done in the pH 5.4 buffer. From the plot for the responses of the biosensor electrodes in buffer of pH 5.4 (Fig 2.4a), two linear regions were identified; one at lower concentrations ($< 4 \times 10^{-7}$ M) and the other at higher concentrations ($\geq 4 \times 10^{-7}$ M). Since our goal was to produce biosensors that can detect very low concentrations of protein we focused our attention on the lower range of concentrations.
Figure 2.4  (a) Aptamer biosensor responses in buffer solutions at different pH, (b) effect of pH on sensor response at thrombin concentration of $1 \times 10^{-8}$ M.
2.4.3. Performance of the biosensor based on sensitivity and detection limits

The characteristic time response for detection of thrombin with the developed all solid state potentiometric sensor in 50 mM acetate buffer of pH 5.4 (containing 140 mM NaCl and 5 mM KCl) is as shown in Figure 2.5a. The biosensor’s responses were examined with thrombin concentrations from $1 \times 10^{-10} - 1.8 \times 10^{-7}$ M. The total analysis time for the thrombin samples, as a function of how long it takes the signal to reach equilibrium (with a change less than 0.1 mV per minute) after thrombin introduction, varied from 1 min at higher concentrations of thrombin to 6 min at very low concentrations ($\leq 2.5$ nM), but an immediate change was observed once thrombin was added at every concentration level. For this study three independent aptamer sensors were prepared and each sensor was independently tested across the entire concentration change four times. After use, the aptamer sensors were regenerated by soaking the electrode tip in a 6 M guanidine hydrochloride solution for 20 min, then in 2 M NaCl solution for 10 min before rinsing thoroughly with milli-Q water. As shown in Figure 2.5b, the response was linear from $5 \times 10^{-10}$ M to $7 \times 10^{-8}$ M with a slope of $-31.01 \pm 1.41$ mV/decade and regression coefficient $\geq 0.99$. A calculated limit of detection was determined according to the classical potentiometric sensor criteria of the intersection of the two straight lines and has a value of $4.1 \times 10^{-10}$ M. The physiologically relevant concentrations of thrombin in plasma are 5–500 NIH units/mL (1 NIH unit = 0.324 ± 0.073 µg), which is equivalent to $\sim4.3 \times 10^{-8} – 4.3 \times 10^{-6}$ M$^{29-31}$. It is therefore crucial to be able to detect the thrombin content of plasma samples at these low concentrations. The very low limit of detection we obtained in this
study will enable the application of the sensor for biological samples even after dilutions of up to 100:1. In general for potentiometric sensors, dilution will reduce the effects of common interferences, and allow better control of ionic strength and pH.
Figure 2.5 Calibration curve for thrombin detection in the concentration range of $1 \times 10^{-10}$ – $1.8 \times 10^{-7}$ M. (a) Time response showing change in potential as analyte’s concentration increases. (b) Calibration curve obtained from three different sensors with the error bars representing the standard deviation of the change in potentiometric measurements.
2.4.4. Repeatability and reproducibility

Figure 2.6 showed the average responses of three independent biosensors, each used repeatedly four times for detection of thrombin concentrations from $1 \times 10^{-10}$ M to $1.8 \times 10^{-7}$ M. Repeatability of each sensor’s response was demonstrated by the small standard deviations and the reproducibility of sensors was revealed by the closely packed data. To further examine the repeatability of the sensor responses, we compared the responses of a single biosensor electrode for three repeated potential readings for a $5 \times 10^{-9}$ M thrombin concentration. As shown in Table 2.1, the results were very reproducible, with a relative standard deviation of 2.7%. We also demonstrate the repeatability of the sensor over the useful linear range, after regeneration of the sensor electrode surface as described in the experimental details, by examining the slopes of four repeated experiments. Our results showed that the slopes had a relative standard deviation of less than 4%, confirming the regeneration of the biosensor electrode. The reproducibility of multiple sensors was used to evaluate the consistency of the fabrication method. For this purpose, we functionalized three independent aptamer immobilized carbon nanotube based biosensor electrodes and compared the responses of the three electrodes for three repeated potential readings with a $5 \times 10^{-9}$ M thrombin concentration. Results shown in Table 2.1, demonstrated the high reproducibility of the sensors, with relative standard deviations ranging from 2.7% - 5.2% with an overall standard deviation of 3.6%. Also considering the reproducibility of the sensors over the useful linear range, after regeneration of the biosensor electrode surface, each of the biosensors was used repeatedly four times over the concentration range of $1 \times 10^{-10}$ – $1.8 \times 10^{-7}$ M and an overall relative standard deviation of 4.6% was obtained from
the slopes of the linear calibration curves. It is worth mentioning that sensor 2 was stored at room temperature (22 °C) while the other two were stored in a refrigerator (4 °C) when not in use and each developed sensor was used over a period of three weeks. No significant difference was observed in the performance of the sensors, though sensor 2 gave the largest standard deviations. Without further experimentation we cannot link this observation to the storage conditions. However, we recommend storage of such sensor in a refrigerator or at a very low temperature to ensure the stability and durability of the sensor.

Figure 2.6 Demonstration of repeatability and reproducibility of the biosensors
Table 2.1 Comparison of the performance of the biosensor electrodes evaluate the repeatability and reproducibility using a thrombin concentration $5 \times 10^{-9} \text{ M}$ and the slopes of the calibration curves.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Sensor response (mV)</th>
<th>%Relative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-33.2</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>-33.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-32.1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>Average sensor response (mV) ± standard deviation (n=3 for each sensor)</th>
<th>%Relative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor 1</td>
<td>-33.1 ± 0.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Sensor 2</td>
<td>-32.6 ± 1.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Sensor 3</td>
<td>-34.1 ± 1.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensors</th>
<th>Slope (mV/log[thrombin]) ± standard deviation (n=4 for each sensor)</th>
<th>% Relative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensor 1</td>
<td>-32.23 ± 1.15</td>
<td>3.38</td>
</tr>
<tr>
<td>Sensor 2</td>
<td>-29.47 ± 1.16</td>
<td>3.94</td>
</tr>
<tr>
<td>Sensor 3</td>
<td>-31.33 ± 1.16</td>
<td>3.70</td>
</tr>
<tr>
<td>Overall average (N=12)</td>
<td>-31.01 ± 1.41</td>
<td>4.56</td>
</tr>
</tbody>
</table>

### 2.4.5. Selectivity

The ability of a sensor to respond to the target analyte with high affinity in the presence of other analytes of like nature is the definition of selectivity, and it is a crucial parameter in the assessment of the performance of any sensor. Two different proteins present in human plasma were used to measure the selectivity of the developed sensor; human serum albumin (HSA) - a higher molecular weight protein that is common in human plasma with high
abundance, and elastase – a serine protease enzyme with an isoelectric point and molecular weight similar to that of thrombin and has a normal concentration of about 3.2 nM in the blood.\textsuperscript{32}

The biosensor response to each of these proteins was examined separately in the concentration range between $5 \times 10^{-10}$ – $5 \times 10^{-6}$ M and also the response of the sensor to thrombin in the presence of these proteins was examined. Figure 2.7a shows the high specificity of the sensor for the target analyte (thrombin) with very low effect of non-specific binding of non-target analytes at much higher concentration. As shown in the figure, elastase did not show any significant response until concentrations above $1.5 \times 10^{-6}$ M, while for HSA, no significant change in signal was observed below $1 \times 10^{-6}$ M. In our quest to understand how the presence of other proteins will affect the sensitivity of the developed sensors we carried out experiments to detect thrombin in the presence of 3.5 nM elastase and 0.5 µM HSA, and compared the sensor response to thrombin with the results obtained in the absence of those other proteins. As shown in Figure 2.7b, the presence of these two non-specific proteins only reduced the sensitivities of the sensors by about 15 % but without any effect on the detection limits of the biosensors. Owing to the fact that the human blood contains higher concentrations of HSA than other proteins, we carried out another experiment to detect thrombin in the presence of higher concentrations of HSA (up to 25 µM). It was observed that the sensitivities reduced by up to 33 % (results not shown) with a slight increase in the detection limits (ca $2.5 \times 10^{-9}$ M). This could be due to reduction in the rate of diffusion of thrombin to the sensor electrode.
surface. However, because of the very low detection limit achieved in this study, it will be easy to dilute biological sample to a level that will significantly minimized the screening effects of higher concentrations of other proteins.
Figure 2.7 Specificity of the sensor in response to (a) non-specific protein targets (elastase and HSA) compared with the specific binding of thrombin done. (b) specific binding of thrombin in the presence of $3.5 \times 10^{-9}$ M elastase and $5 \times 10^{-7}$ M HSA compared to response without elastase and HSA.
2.4.6. Antibody biosensor

In this study, we compared the effectiveness of an antibody functionalized sensor with an aptamer biosensor for the same target molecule (thrombin). In order to eliminate or at least reduce the negative effects of the larger size of the antibody (ca 12 nm), which moves the binding site farther away from the sensor electrode surface compared to aptamer (ca 3 nm), we fragmented the antibody to obtain a smaller size antibody (fab’)2 fragment as described in the experimental section. The resulting biosensor was used for detection of thrombin in the same concentration range as for the aptamer biosensor. As shown in Figure 2.8a, the response and sensitivity of the antibody sensor is similar to that of the aptamer sensor with best performance obtained in a moderately acidic buffer (pH 5.5), with thrombin concentration from $1 \times 10^{-9}$ to $1.4 \times 10^{-6}$ M. At around neutral pH, no significant signal was observed at concentrations below $1 \times 10^{-7}$ M. Comparing the sensitivities of both biosensor types with thrombin concentrations of $1 \times 10^{-10}$ to $2.0 \times 10^{-7}$ M, the two biosensor types show high sensitivity for detection of thrombin with the aptamer sensor displaying about 30% more sensitivity than the antibody biosensor (Figure 2.8b). However, the detection limits and the linear range are similar for both sensors.
Figure 2.8 (a) Sensitivity of the antibody sensor to thrombin in different pH buffer electrolytes (b) calibration curve for the response of antibody sensor to thrombin compared to that of aptamer sensor.
2.4.7. Control experiments

Control experiments were performed to evaluate the potency of the immobilization technique and the importance of the presence of aptamer on the sensor electrodes. The first control experiment immobilized aptamers to the CNTs on the electrode without prior functionalization of the CNTs with diazonium salt. This confirmed the importance of creating reactive species on the CNTs to facilitate covalent attachment of the aptamers. An experiment was also conducted using an electrode that was functionalized with CNTs only, to determine whether the presence of CNTs alone might generate enough signal for detection of thrombin. Lastly, a modified GCE with the aptamer alone was subjected to the same treatment as was used for the preparation of the fully developed sensor, but without CNTs and the subsequent diazonium salt solution treatment. This enabled us to determine whether adsorbed aptamers or aptamer coupled to the GCE surface by covalent attachment through any reactive species (COOH, OH, CO) on the GCE itself might play a role in the success obtained with the sensor electrodes.

The control sensors were evaluated by measuring their response to thrombin using the same experimental conditions for the developed sensor in the concentration range of 1 x 10^{-10} to 1.8 x 10^{-7} M. As shown in Figure 2.9, the presence of CNTs alone on the GCE surface resulted in a very slight potential increase at much higher concentrations of thrombin (above 5 x 10^{-8} M) with a sensitivity of approximately -5.6 mV/decade. This result is similar to what was obtained for the response of the developed sensor to elastase concentrations (Figure 2.7a) and may be attributed to the non-specific binding of the
protein onto the surface of the non-functionalized carbon nanotubes. Control sensors with CNTs and aptamer but without diazonium treatment, and the GCE with aptamers but no CNTs and no diazonium treatment did respond to thrombin concentration increases; this did not happen until concentrations above $2.0 \times 10^{-8}$ M were reached, and yielded modest sensitivities of -14 mV/decade and -11 mV/decade, respectively. The increased signals may be attributed to the presence of small amounts of aptamer adsorbed to the surface or covalently linked to the surface through a few reactive species, especially carboxylated groups, present on the electrode surfaces$^{33,34}$ as well as non-specific binding of proteins to the electrode surfaces. For the control sensor modified with CNTs, higher signals were judged to be a result of increased surface area for adsorption/attachment of aptamers as well as non-specific binding of proteins to the surface. These results indicated that the aptamer functionalized sensors had a much higher specific interaction with thrombin molecules than the non-functionalized sensors, and also showed that diazonium treatment of CNTs on the electrode surface enhanced the attachment of aptamers to the sensor surface.
Figure 2.9 Comparison of the responses for the various sensors with those of the control sensors

2.5. Proposed mechanisms of detection

Despite superficial similarities with Ion Selective Electrodes, we believe that an entirely different response mechanism, one that is not governed by the Nernst Equation, is responsible. At pHs below 7 positively charged thrombin molecules interact with the anti-thrombin aptamers or antibodies on the biosensor electrodes. The increasing positive charge on the solution side of the electrode interface forces accumulation of negative charge on the electrode surface, forming an electrical double layer capacitor. The biosensing mechanism would then be based on accumulation of positively charged
thrombin molecules interacting with the anti-thrombin aptamers to yield corresponding accumulation of negative charge on the sensor electrode surface resulting in formation of a double layer capacitor. Owing to the high isoelectric point (pI) values for thrombin, ranging near pH 8.35, it is positively charged under the experimental conditions for this study. As shown in Figures 2.4 and 2.7a for the aptamer sensor and the antibody sensor, respectively, as the pH decreases, the rate of formation of positively charged thrombin molecules increases and the sensitivities increase. The higher the concentration of (positively charged) captured analyte, the more negative the electrode side of the interface becomes, supplying the other side of the capacitor. Hence the decrease in potential as the concentration of positively thrombin is increased.

2.6. Conclusions

We have successfully demonstrated the fabrication and application of a label free solid-state potentiometric biosensor usable at high ionic strength that is capable of detecting thrombin across a broad, physiologically relevant range of concentrations. The high salt concentration in the electrolyte ensured compatibility with the usual high ionic strength of any unprocessed biological sample, thereby making room for the consequences of such high salt concentration and making the sensor relevant in analysis of real biological samples. The prepared biosensor was adjudged to exhibit satisfying reusability and reproducibility. A high linearity was obtained for thrombin concentrations from $1 \times 10^{-10}$ M to $1.8 \times 10^{-7}$ M for both the aptamer and the antibody modified biosensors with a limit
of detection in the pico molar range. We also demonstrated here the dependence of the sensitivity of potentiometry biosensor on the pH of the electrolyte buffer and proposed a novel mechanism for protein detection by the biosensor. The successful application of the developed biosensors with both aptamer and antibody bioreceptors, under the same condition of high ionic strength, is an indication that the mechanism of detection in this study was not as a result of folding of the aptamer as it binds to the antigen, as widely reported in the literature, or a result of the nature of the bioreceptors, but rather is dependent on charge formation and accumulation of the protein target that binds to the biosensor and the response of the electrode transducer to such interactions.

2.7. References


Screening Limitations of Nanowire-Based FET Devices *Nano Letters* **2012** 12 (10), 5245-5254.


CHAPTER 3

IMMOBILIZATION OF ANTIBODIES AS SELF ASSEMBLED MONOLAYERS ON CARBON NANOTUBE-BASED POTENTIOMETRIC BIOSENSORS AND THE APPLICATION OF THESE DEVICES FOR DETECTION OF PROTEINS

Adeniyi A. Adenuga, Neal Sleszynski and Vincent T. Remcho

To be submitted
3.1. Abstract

Controllable, reproducible immobilization of biomolecular probes is crucial to the performance of biosensors. Antibodies are generally fragile in nature, and it is a considerable challenge to immobilize them in a controlled orientation when using the intrinsic amine group in their structure to attach them to a substrate without compromising their activities. This study proposed a novel approach for the immobilization of antibodies onto carbon nanotubes for sensor construction by exploiting the sulfhydryl group of the intrinsic disulfide groups in the hinge region of the antibody as a point of attachment to CNT using 3-(2-pyridyldithio)propionyl hydrazide (PDPH) as a crosslinker. C-reactive protein (CRP) was used as the model target using anti-C-reactive protein antibody as biorecognition molecule. The biosensor was built by first covalently attaching PDPH, through its amine group, to the carboxyl group of the carboxyphenyl moieties, grafted to CNT sidewalls through diazonium salt treatment of the CNT. Fragmented antibodies [F(ab)2] were then reduced to gain access to the sulfhydryl group of the disulfide groups in their hinge region and thus allowed us to react the sulfhydryl group with the pyridyl disulfide group on PDPH forming a disulfide bond. The performance of this potentiometric biosensor under physiologically relevant ionic strength conditions demonstrated high sensitivity over physiologically relevant concentrations of CRP with limits of detection at 4.96 x 10^{-11} M. A useful linear interval from 5 x 10^{-11} M to 4.17 x 10^{-9} M was obtained and the average sensitivity, in terms of slope, was -14.65 ± 0.83 mV per log concentration of CRP with regression coefficient ≥ 0.99.
3.2. Introduction

The incorporation of carbon nanotubes (CNTs) into sensor transducers is gaining great attention in the field of electroanalytical chemistry. This is as a result of the important roles that CNTs are playing in the enhancement of the sensitivities of sensors; such as increasing surface area for biomolecule attachment, enabling fast heterogeneous electron transfer, and facilitating long range electron transfer\textsuperscript{1,2}. Despite the great potentials of CNTs in the construction of biosensors, the challenges in building a very versatile CNT- based biosensor still remain, most importantly in the area of immobilization of antibodies as molecular probe onto the sidewalls of CNT in a controlled orientation with sufficient stability without compromising the activity of the biomolecule and the electrical properties of the nanotube. The attachment of antibodies in a correct orientation and at high-density to nanotube sidewalls is therefore a critical step in CNT- antibody based biosensor development.

The use of antibodies as probes in biosensors has garnered great attention recently. However, a considerable fraction of these antibody based biosensors fail to function properly due to denaturation, unpredictable conformational changes on surfaces, or unwanted reactivities mediated by their Fc component\textsuperscript{3,4}. This may have been one of the reasons for comparatively modest use of antibodies in biosensor development relative to aptamer based biosensors. Additionally, the random orientation of antibodies that can arise upon their immobilization on sensor surfaces leads in turn to variability in antigen binding, which is often not the case in solution based immunoassays\textsuperscript{3,5,6}. Studies have shown that
random-coupling of antibodies lowers their antigen binding capacities by 2–3-fold compared to the capacities of well-oriented antibodies\(^5,7,8\). Various scientific reports have revealed that proper orientation of antibodies, with their antigen binding sites well exposed to the solution phase lead to exhibition of higher antigen binding capacities compared to randomly oriented antibodies\(^5,9,10\).

C-reactive protein (CRP) is synthesized in the liver and it is referred to as an acute-phase protein because its plasma concentration changes (increase or decrease) in response to inflammation and are \(^{11-14}\). Several clinical studies have evaluated the use of CRP levels for diagnostic purposes in cardiovascular diseases\(^{15,16}\) or as a prognostic indicator in gastroesophageal cancer\(^{17-19}\). CRP is made up of five identical subunits of \(\sim 23\) kDa each and the whole protein is \(\sim 115\) kDa. An elevated CRP level in the blood is a reliable indicator for cardiovascular disease (CVD)\(^{20,21}\), and chronic inflammatory processes\(^{22}\). CRP can stimulate phagocytosis and is considered a significant keystone of the human immune response\(^{20}\). As classified by the American Heart Association and the United States Center for Disease Control; a CRP concentration less than 1.0 mg/L represents low risk, a 1.0–3.0 mg/L range represents moderate risk, and levels above 3.0 mg/L are an indicator of high risk\(^{20,23,24}\). Early and precise quantification of CRP is a step towards of improving the outcome of cardiovascular or inflammatory disease through suitable intervention or treatment\(^{25}\).

The successful application of the developed CNT- antibody based biosensors under high ionic strength demonstrated high sensitivity over physiologically relevant concentrations
of CRP with limits of detection in the low picomolar range. We also demonstrated in these studies the dependence of the sensitivity of potentiometric biosensors on the pH of the electrolyte buffer and proposed a novel mechanism for protein detection by the biosensor.

3.3. Experimental

3.3.1. Materials and reagents

The nanotubes used were 90% semiconducting single walled carbon nanotubes in solution, purchased from “IsoNanotubes-S 90%” by NanoIntegris, Inc. with diameters from 1.2 nm -1.7 nm and lengths ranging from 300 nm to 5 micron. Monoclonal anti-C-Reactive Protein clone CRP-8 (mouse IgG1 isotype) and human plasma C-reactive protein (CRP) were purchased from Sigma Aldrich. Human α-thrombin was supplied by Enzyme Research Laboratories, and human serum albumin (HSA) were purchased from CALIBIOCHEM - EMD biosciences. The crosslinker, 3-(2-Pyridyldithio)propionyl hydrazide (PDPH) was purchased from Proteochem. The reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), and crystalline Tris(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl) were purchased from Thermo Scientific Pierce, sodium phosphate monobasic and diabasic (NaH$_2$PO$_4$ and Na$_2$HPO$_4$), sodium chloride (NaCl), potassium chloride (KCl), ethanolamine, chitosan and tris base were from Sigma, and N-morpholinoethane sulfonic acid (MES) sodium salt was from USB corporation. Zeba Micro Spin Desalting Columns, 7K MWCO, were obtained from Thermo Scientific. The water used was purified with Milli-Q Advantage A10 system.
(EMD Millipore Corporation) equipped with BioPak cartridge to produce pyrogem free, DNase free and RNase free ultrapure water.

### 3.3.2. Fragmentation and reduction of anti-CRP (mouse IgG1) antibody

The anti-CRP antibody was fragmented F(ab\textsprime)_2 to reduce the distance between the electrode surface and the capture part of the antibody. Fragmentation was done using a Fab & F(ab\textsprime)_2 fragmentation kit for mouse IgG\textsubscript{1} obtained from G-Biosciences and following the manufacturer’s procedures (Figure 3.1). The fragmented antibody F(ab\textsprime)_2 was reacted with TCEP-HCl, to reduce disulfide bonds in the hinge region of the antibody without also reducing those connecting heavy and light chains. This was performed according to procedure reported by Hermanson\textsuperscript{26} (Figure 3.2) with little modification. Briefly, an 8 molar excess of TCEP-HCl in 10 mM PBS buffer solution was added to 1 mg/ml solution of anti-CRP antibody [F(ab\textsprime)_2] prepared in 10 mM PBS solution and incubated at room temperature for 90 min while mixing with an end to end mixer. Then the reduced antibody was desalted with a Zeba Micro Spin Desalting Column to remove excess TCEP-HCl. The resulting antibody fragment was used immediately.
3.3.3. Covalent functionalization of GCE-CNT

The 4-Carboxybenzene diazonium tetrafluoroborate salt used to functionalize the CNTs by grafting carboxyphenyl moieties on the sidewall was synthesized and characterized as described in the previous chapter. The solid contact sensor was prepared using a Glassy Carbon electrode (BAS Inc.) with a diameter of 3 mm. The tip of the electrode was first
polished using 1 µm, 0.3 µm and finally 0.05 µm grain size alumina powder slurry to obtain a smooth surface, which was observed under an optical microscope before modification. The tip of the electrode was then soaked for 2 hrs in 1% chitosan solution prepared with 1% acetic acid solution. The electrode was then rinsed with Milli-Q water and dried with N₂ gas. 25 µL of an IsoNanotubes-S 90% semiconducting SWCNT suspension (0.04 mg/mL) was deposited on the electrode’s surface and allowed to dry for 45 min at 60 °C in an oven. The deposition and drying processes were repeated five times on each individual electrode prior to further use or treatment. To remove the surfactant from the CNTs on the electrode surface the electrode tip was soaked in Milli-Q water for 1 min, removed and dried in an oven at 60 °C. This was repeated twice before the electrode tip was finally soaked in Milli-Q water and stirred for 10 min, then dried with a steam of nitrogen gas. CNTs on the GCE surface were reacted with 20 mM 4-Carboxybenzene diazonium tetrafluoroborate salt solution for 90 min at 45°C to modify the nanotube sidewalls with carboxyphenyl moieties²⁸,²⁹. The electrode surface was then washed sequentially with acetone, isopropyl alcohol and Milli-Q water, and dried with a gentle stream of N₂. To deactivate the excess reactive groups on CNTs, the electrode surface was soaked in 20 mM solution of ethanolamine solution for 30 min, rinsed thoroughly with Milli-Q water and dried with N₂. Anti-CRP antibodies were immobilized onto the CNTs covalently through a disulfide linkage with the aid of an heterobifunctional crosslinker, 3-(2-Pyridyldithio)propionyl hydrazide (PDPH) (Figure 3.3).
The carboxylic acids of the 4-carboxybenzene moieties on the nanotube sidewalls were activated by placing electrodes in an aqueous solution of 100 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 50 mM sulfo-N-Hydroxysulfosuccinimide (sulfo-NHS), prepared with activation buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid sodium salt (MES), 0.5 M NaCl, and pH adjusted to 5.8 with HCl) at room temperature for 30 min. Immediately afterwards, electrodes were briefly dunked in the activation buffer and placed in a solution of PDPH (20 mM to be prepared in 0.1M PBS, pH 7.3) for 3 hr at room temperature. Upon completion, the electrode was washed with PBS and water and dried under a gentle stream of N₂.
The sulfhydryl-reactive pyridyl disulfide group on PDPH was then reacted with the sulfhydryl group on the antibody [reduced F(ab')$_2$] to form a disulfide linkage. This was achieved by placing the PDPH-functionalized CNT electrode in 200 µg/ml solution of anti-CRP antibody fragment [reduced F(ab')$_2$] prepared in 10 mM phosphate buffered saline (PBS) of pH 7.4 (containing 137 mM NaCl and 5 mM KCl) for 3 hr at room temperature and left in fridge at 4 °C overnight. Electrodes were then washed sequentially with PBS solution and Milli-Q water before drying under a gentle stream of N$_2$. The biosensor electrodes were stored in a fridge at 4 °C when not in use.

3.3.4. Characterization of functionalized CNTs for assessment of surface morphology and elemental composition

To effectively assess the effect of diazonium salt treatment on carbon nanotubes, bulk IsoNanotubes-S 90% semiconducting SWCNT in solution was functionalized with diazonium salt as described for the CNT modified GCE. The nanotubes were treated with 20 mM 4-Carboxybenzene diazonium tetrafluoroborate salt solution for 3 hrs at 45° C. The functionalized CNTs were then washed with acetone and filtered. It was further washed with isopropanol and thoroughly washed with Milli-Q water, then dried in a vacuum oven. As control a portion of the nanotubes solution was filtered and thoroughly washed with Milli-Q water and dried in a vacuum oven overnight. These nanotubes samples were subjected to thermal gravimetric analysis (Shimadzu TGA-50 thermogravimetric analyzer) to study the mass loss behavior of the CNTs. This was done under flowing Argon gas (20
ml/min) at a heating rate of 5°C/min from ambient temperature to 1000°C. Scanning electron microscope (FEI QUANTA 600F environmental SEM) transmission electron microscopy (FEI Titan 80-200) were used to assess the surface and structural morphology as well as the abundance of the elemental components of the nanotubes.

3.3.5. Electrochemical measurements

Electrochemical measurements were performed in a 5 mL buffer solution under continuous stirring using a digital Ionalyzer (Orion Research Inc, 701A). The two electrode system was made up of the functionalized CNT-GCE as the indicator electrode and a silver-silver chloride (Ag/AgCl) electrode as reference. All potentiometric measurements were performed at room temperature (23.3 ± 0.8 °C) and the target analyte was added to the electrochemical cell with stepwise increase in concentration from $6.25 \times 10^{-12}$ up to $8.3 \times 10^{-9}$ M.

3.4. Results and Discussion

3.4.1. Characterization of diazonium functionalized CNTs

The results of TGA (Figure 3.4) show that pristine SWCNT have maximum oxidation temperature at 702 °C (between 600 °C and 850 °C) with a residual percent of 8 % and no weight loss at temperature less than 450 °C. The diazonium functionalized SWCNT show a weight loss of 5 % in the temperature range between 116 °C and 350 °C with midpoint
at 220°C. This is attributed to pyrolysis of the carboxyphenyl moieties on the tube sidewalls. It also shows a maximum oxidation temperature at 748 °C with a total residual percent of 7.3 %. This is an indication of successful functionalization of the tubes.
Figure 3.4. Thermogravimetric analysis curves of (a) pristine SWCNT, and (b) diazonium functionalized SWCNT, showing the percent weight loss as a function of temperature and mass change per °C temperature (dMass (mg)/dTemperature (°C)).
The surface morphologies of the diazonium-functionalized CNTs examined by TEM and SEM are shown in Figure 3.5. The SEM image showed large amounts of CNTs entangled together in a “spaghetti” form and the TEM shows the cylindrical structures of the nanotubes. Both images demonstrated retention of structural integrity of the CNTs. The relative abundance of oxygen (O) and carbon (C) atoms on the diazonium functionalized CNTs and the pristine CNTs generated by high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) are shown in Figure 3.5 c & d. The images reveal higher amounts of oxygen in the functionalized CNTs than in the pristine CNTs. An estimated weight percent of oxygen in functionalized CNTs was 5% compared to 0.54% in the pristine CNTs. The oxygen content may be attributed to the carboxyphenyl moieties grafted onto the sidewalls of the SWCNTs, and suggests successful functionalization.
Figure 3.5 (a) SEM image of diazonium functionalized CNTs shown the surface morphology, (b) TEM image revealing the tubular structure of the functionalized CNTs, (c1 & c2) HAADF STEM image showing the relative abundance of carbon atoms and oxygen atoms, respectively, in the diazonium functionalized CNTs, and (d1 & d2) HAADF STEM image showing the relative abundance of carbon atoms and oxygen atoms, respectively, in the pristine CNTs.
3.4.2. Identification of optimal pH conditions for CRP biosensors

To maximize the performance of the developed biosensor, the sensor’s response was examined under various buffer pH conditions. Each of the buffers used was 10 mM with total ionic strength maintained at 150 mM, adjusted with addition of sodium chloride. The high ionic strength was very important as the goal was to develop a sensor that could be used for analysis of biological samples and that might avoid the negative impact of high salt concentration on the performance of many such sensors. The pH values of the buffers tested ranged from 3.9 – 9, these were chosen based on the isoelectric point (pI) of CRP, which has been variously reported to be between 5 and 6, and as high as 7.4\textsuperscript{30,31}. Concentrations of CRP from \(8.33 \times 10^{-14}\) to \(8.33 \times 10^{-9}\) were examined using stepwise addition. Table 3.1 shows the results of the sensor’s response (\(\Delta E\) (mV)) to the various CRP concentrations studied. Highest sensitivities were obtained with a buffer pH < 5; above pH 5, no measurable signals were obtained. This increase in sensitivity at low pH was attributed to the rate of charge formation and accumulation as CRP bound to the antibody on the biosensor electrode, since the protein (CRP) is charged at pH below its pI. Lack of signal at pH above 5 is an indication of no charge formation at pH values proximate to the pI of CRP. However, an equilibrium signal at a single concentration for the reaction could not be obtained easily at pH \(\leq 4.5\), especially at the higher CRP concentration range; pH 4.75 was, therefore, chosen for further studies. At pH \(\geq 9\), equilibrium signal could not be obtained for the buffer system; therefore, no further experiments were run in buffers with pH above 8.5. This could be as result of unfavorable condition for anti-CRP antibody at such basic medium.
Table 3.1 Change of potential as a function of concentration of CRP in buffers of various pH values.

<table>
<thead>
<tr>
<th>[CRP (M)]</th>
<th>pH 3.9</th>
<th>pH 4.5</th>
<th>pH 4.75</th>
<th>pH 5.0</th>
<th>pH 5.4</th>
<th>pH 6.4</th>
<th>pH 7.07</th>
<th>pH 8.56</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.33 x 10^{-14}</td>
<td>-1.1</td>
<td>-0.5</td>
<td>-0.4</td>
<td>-0.3</td>
<td>0.2</td>
<td>-0.2</td>
<td>-0.1</td>
<td>0</td>
</tr>
<tr>
<td>4.17 x 10^{-13}</td>
<td>-2.2</td>
<td>-1.3</td>
<td>-0.9</td>
<td>-0.4</td>
<td>0.4</td>
<td>-0.3</td>
<td>-0.1</td>
<td>0</td>
</tr>
<tr>
<td>8.33 x 10^{-13}</td>
<td>-3.1</td>
<td>-2</td>
<td>-1.2</td>
<td>-0.7</td>
<td>0.7</td>
<td>-0.5</td>
<td>-0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>4.17 x 10^{-12}</td>
<td>-3.5</td>
<td>-3</td>
<td>-1.4</td>
<td>-0.9</td>
<td>0.8</td>
<td>-0.6</td>
<td>-0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>8.33 x 10^{-12}</td>
<td>-4.1</td>
<td>-4</td>
<td>-2.4</td>
<td>-1.3</td>
<td>0.9</td>
<td>-0.8</td>
<td>-0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>4.17 x 10^{-11}</td>
<td>-6</td>
<td>-6.5</td>
<td>-5.2</td>
<td>-1.7</td>
<td>0.8</td>
<td>-0.9</td>
<td>-0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>8.33 x 10^{-11}</td>
<td>-8.6</td>
<td>-10.1</td>
<td>-8</td>
<td>-2.3</td>
<td>0.7</td>
<td>-1.1</td>
<td>-0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>4.17 x 10^{-10}</td>
<td>-16.8</td>
<td>-18.4</td>
<td>-12.6</td>
<td>-4.5</td>
<td>-0.5</td>
<td>-1.3</td>
<td>-0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>8.33 x 10^{-10}</td>
<td>-24.8</td>
<td>-26.3</td>
<td>-16.7</td>
<td>-7.8</td>
<td>-1.7</td>
<td>-1.5</td>
<td>-0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>4.17 x 10^{-9}</td>
<td>-36.8</td>
<td>-42.6</td>
<td>-26.1</td>
<td>-15.4</td>
<td>-6</td>
<td>-1.8</td>
<td>-0.3</td>
<td>1</td>
</tr>
<tr>
<td>8.33 x 10^{-9}</td>
<td>-47.6</td>
<td>-57.6</td>
<td>-35.9</td>
<td>-26.5</td>
<td>-9.7</td>
<td>-2.1</td>
<td>-0.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

3.4.3. Performance of the biosensor based on sensitivity and detection limits

The performance of the biosensors was characterized for CRP concentrations from $6.25 \times 10^{-12} - 8.33 \times 10^{-9}$ M in a 10 mM buffer of pH 4.75 and ionic strength of 150 mM; appropriate for physiological ionic strength. Figure 3.6a shows the typical time response obtained for the biosensors developed. The total analysis time for the CRP detection as a function of how long it takes the signal to reach equilibrium (with a change less than 0.1
mV per minute) after addition of thrombin to the electrochemical cell was shorter (ca 3.5 min) lower concentration (< 500 pM) than for higher concentration (ca 8 min). However, immediate and substantial changes were observed at the point of addition of CRP to the electrochemical cell. Three antibody sensors were independently evaluated for performance in this study, each tested three times for a total of nine analyses. After use, the antibody sensors were regenerated by soaking the electrode tip in a 10 mM phosphate buffered saline solution for 30 min prior to rinsing thoroughly with Milli-Q water.

As shown in the calibration curve (Figure 3.6b), two segments of different slopes were obtained with the useful linear interval from $5 \times 10^{-11}$ M to $4.17 \times 10^{-9}$ M showing an average sensitivity, in terms of slope, of $-14.65 \pm 0.83$ mV per log concentration of CRP with regression coefficient $\geq 0.99$. Using the classical potentiometric sensor criteria of the intersection of the two straight lines, a calculated limit of detection of $4.96 \times 10^{-11}$ M was obtained. According to the American Heart Association and the United States Centers for Disease Control, CRP concentrations below 1.0 mg/L (equivalent to $8.33 \times 10^{-9}$ M) represent a low risk, $1.0 - 3.0$ mg/L (equivalent to $8.33 \times 10^{-9} - 2.5 \times 10^{-8}$ M) is an intermediate risk, and $3.0$ mg/L – $10$ mg/L (equivalent to $2.5 \times 10^{-8} - 8 \times 10^{-8}$ M), is a high risk$^{24, 25}$. Therefore, the ability to detect CRP at a low concentration is crucial to early detection. The very low limit of detection obtained in this study will enable the application of the sensor for biological samples after dilutions of up to 150:1.
Figure 3.6 Calibration curve for CRP detection in the concentration range of $6.25 \times 10^{-12} - 8.33 \times 10^9$ M. (a) Time response showing change in potential as a function of analyte’s concentration. (b) Calibration curve obtained from three different sensors with the error bars representing the standard deviation of the potentiometric measurements.
3.4.4. Repeatability and reproducibility

The repeatability of the antibody sensor was evaluated based on the slopes of the calibration curves obtained for three independent experiment performed with a single sensor in the useful linear range studied, over a period of 12 days, by regenerating the sensor electrode surface as described above. The results showed a relative standard deviation of 4.6% for the slopes. For a single concentration of $6.25 \times 10^{-10}$, the sensor gave a relative standard deviation of 5.3% for five different measurements, confirming the good repeatability of the biosensor electrode. To examine the consistency of the performance of different sensors fabricated with the same technique, reproducibility test was performed. Three independent antibody sensors were fabricated and each evaluated using three regeneration cycles over the CRP concentrations range studied. The overall relative standard deviation obtained from the slopes of the non-regenerated (first time use) and the regenerated sensors over the useful linear range of the calibration curves was 5.6%, an indication of good reproducibility of the sensor fabrication technique.

3.4.5. Selectivity

Selectivity is a crucial parameter for the assessment of the working performance of a biosensor and indicates the specificity of a biosensor for detection of the target analyte. Thrombin, a multifunctional serine protease and human serum albumin (HSA), the most abundant protein in human blood plasma with concentration up to 4% w/v, were used evaluate the possibility of the CRP to respond to other proteins. Two types of experiment
were performed for this purpose. First, the CRP biosensor was used separately for detection of each of these proteins, and second, the sensor was used for detention of CRP in the presence of other proteins. For the first experimental set up, the sensor’s response to HSA was examined in the concentration range from $1 \times 10^{-10} – 5 \times 10^{-6}$ while for thrombin, concentrations from $1 \times 10^{-10} – 1.5 \times 10^{-6}$ were used. In the second set up, the response of the sensor to CRP in the presence of $2.5 \times 10^{-6}$ M HSA was evaluated over the CRP concentration range studied.

As shown in Figure 3.7a, the sensor displayed a high specificity for binding of the target analyte (CRP) compared with the other proteins that only show slightly significant signal at very high concentrations, and may be as a result of nonspecific interactions of the non-target proteins with the sensor electrode. As shown in the figure, thrombin did not show any significant response at concentrations less than $2.5 \times 10^{-7}$ M, despite that fact that it will be highly charged in at the buffer pH used. For HSA, no significant change in signal was observed below $7.5 \times 10^{-7}$ M. The results of possible negative effects of sample matrix as demonstrated with the detection of CRP in the presence of HSA (Figure 3.7b) showed a reduction in the sensor sensitivity (slope) by 13% with increase in the limit of detection to $6.75 \times 10^{-11}$ M. This may not be unconnected to reduction in the rate of diffusion of CRP to the sensor electrode surface as a result of the presence of HSA. It was observed during the experiment that the presence of HSA in the buffer solution significantly increased the time needed to obtain equilibrium signal form the buffer solution. However, because of the very low detection limit achieved in this study, a dilution up to 1: 150 will decrease the
effect of nonspecific interaction of other proteins with the sensor and enhance its specific detection ability.

Figure 3.7 CRP sensor response to (a) non-specific protein targets (thrombin and HSA) compared with the specific binding to CRP. (b) specific binding of CRP in the presence of $2.5 \times 10^{-6}$ M HSA compared with the sensor response to CRP in buffer only.
3.4.6. Control experiments

Experimental controls were set up to ratify that the signals obtained with the sensors were due to binding of CRP to the immobilized antibody on the CNT-electrode surface. A Glassy carbon electrode with CNTs deposited on its surface was used as the indicator electrode to detect CRP in the concentration range studied (6.25 x 10^{-12} – 8.33 x 10^{-9} M). This was used to determine the possibility of using CNT alone for detection of CRP at such low concentrations. Additionally, a sensor was fabricated following all the steps described previously but with the deposition of CNTs and diazonium treatment omitted. Since the immobilization of the antibody require the presence of carboxyl group on the CNTs for covalent attachment, this set up was used to determine the effect of non-covalent attachment or adsorption of antibody to the electrode surface.

As shown in Figure 3.8, the presence of CNTs alone on the GCE surface generated significant signal at CRP concentrations above 2 x 10^{-10} M and gave a slope of -4.93 mV per log concentration of CRP in the linear section but with a lower regression coefficient. However, the same trend of response was observed when this electrode was used with thrombin hence the signal was attributed to the non-specific binding or adsorption of highly charged protein onto the surface of carbon nanotubes. The other control sensor behave in a similar trend with fairly increased responses to CRP at concentrations above 2.0 X 10^{-11} M and with sensitivities of -4.04 mV per log of CRP concentration. This is attributed to the presence of low amount of antibody adsorbed non-covalently to the surface or covalently linked to the surface through few reactive species, especially carboxylated groups, present
on the electrode surface\textsuperscript{32,33}. These results indicated that the CRP antibody sensors displayed much higher specific interaction ability to the CRP molecules than the non-functionalized sensors and also shows that diazonium treatment of CNTs on the electrodes surfaces enhances the attachment of antibody to the sensor electrodes surface.

Figure 3.8 Comparison of CRP sensor’s responses with those of the control sensors.
3.4.7. Assessment of the new technique of antibody sensor fabrication with the commonly used whole antibody functionalized sensors

In order to ascertain the efficiency of the sensors prepared with this new immobilization technique in comparison with existing techniques, another sensor was prepared by immobilizing CRP whole antibody to the CNT sidewall using the amide linkage. Briefly, CNTs on glassy carbon electrode were functionalized with diazonium salt solution as described earlier. The carboxylic acid of the 4-carboxybenzene moieties on the nanotube sidewalls were activated by placing electrodes in an aqueous solution of 200 mM N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and 50 mM sulfo-N-Hydroxysulfosuccinimide (sulfo-NHS), prepared with activation buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid sodium salt (MES), 0.5 M NaCl, pH adjusted to 5.76 with HCl) at room temperature for 30 min. Immediately afterwards, electrodes were briefly dunked in the activation buffer and placed in a solution 150 µg/mL of whole antibody for CRP, prepared in 10 mM phosphate buffered saline (PBS) of pH 7.33 (containing 140 mM NaCl and 5 mM KCl) for 6 hr at room temperature. Upon completion, the electrode was washed sequentially with PBS and Milli-Q water before drying under a gentle stream of N₂. The sensor was stored in a fridge at 4 °C when not in use.

The performance of the resulting biosensor was evaluated under the same condition and CRP concentration range used for the newly developed biosensor. As shown in Figure 3.9, the response and sensitivity of whole antibody sensor is similar to that of reduced F(ab)2 sensors. However, a significant drop in the sensitivity (slope) was obtained. This show a
percent drop of over 30% in slope and the detection limit increased from less than 50 pico molar to greater 65 pico molar concentration of CRP. This shows the high efficiency of the newly developed sensor. It is not clear why the whole antibody sensor gave lesser sensitivity but may be as a result of uncontrolled orientation of the antibody on the nanotubes sidewalls. Studies have shown that random-coupling of antibodies lowers their antigen binding capacities by 2–3-fold compared to the capacities of well-oriented antibodies\textsuperscript{5,7,8}.

Figure 3.9 Comparison of the performance of whole antibody and reduced F(\(ab\)2) CRP sensors under the same experimental conditions.
3.5. Conclusions

A new technique for effective immobilization of antibody on CNT in a controlled orientation for construction of highly sensitive potentiometric biosensor was reported here. This biosensor was used for detection of C-reactive proteins in a physiological relevant high ionic strength buffer, and at clinically relevant low concentrations of the protein. This biosensor demonstrated high sensitivity and selectivity for CRP in the concentration range of $6.25 \times 10^{-12} - 8.33 \times 10^{-9}$ M with limits of detection < 50 pM. We also demonstrated the dependence of the sensitivity of potentiometric biosensors on the pH of the electrolyte buffer. The detection mechanism is similar to the one proposed for the aptamer biosensor in the previous chapter and is based on the formation of an electrical double layer capacitor as a result of accumulation of positively charged CRP molecules as they interact with the antibodies on the sensor to yield corresponding accumulation of negative charge on the sensor electrode surface. The rate of positive charge formation is a function of the buffer electrolyte pH relative to the pI of the analyte protein. The higher the concentration of positively charged CRP captured, the more negative the electrode side of the interface becomes, supplying the other side of the capacitor. Hence the decrease in potential as the concentrations of positively thrombin is increased.
3.6. References


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(30) Min-Ho Lee; Dong-Ho Lee; Suk-Won Jung; Kuk-Nyung LeeYoung Soo Park; Woo-Kyeong Seong. Measurements of serum C-reactive protein levels in patients with gastric cancer and quantification using silicon nanowire arrays. Nanomedicine: Nanotechnology, Biology and Medicine 2010, 6(1), 78–83


CHAPTER 4

PREPARATION OF WATER SOLUBLE CARBON NANOTUBES AND ASSESSMENT OF THEIR BIOLOGICAL ACTIVITY IN EMBRYONIC ZEBRAFISH

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4.1. Abstract

Carbon nanotubes (CNTs) are currently one of the most important classes of nanomaterials with unique properties sparking off numerous applications in many fields, including electronics, material science and medicine. However, applications of CNTs in medicine and other biological fields are hampered by their insolubility in aqueous media and concerns regarding toxicity. In this study, seven types of CNTs, including two single-walled, one double-walled, and four multi-walled, were evaluated for possible toxicological effects. Soluble CNTs were prepared by treatment with a mixture of acids (D$_2$SO$_4$ and DNO$_3$), washed with Milli-Q water and oven dried. Transmission electron microscopy, thermal gravimetric analysis, and other techniques were used to characterize the prepared CNTs. CNT toxicity was assessed using the embryonic zebrafish. Results showed that none of the CNTs studied caused significant adverse developmental effects. These results support the potential safe use of CNTs as components of indwelling medical devices and drug delivery tools.
4.2. Introduction

Since discovered by Iijima in 1991\textsuperscript{1}, carbon nanotubes (CNTs) have emerged as one of the most important nanomaterials catching the attention of both industries and researchers in different areas of nanotechnology. The peculiar physical, mechanical and electronic properties of CNTs such as high electrical conductivity, high tensile strength, nanosize diameters and large aspect ratios\textsuperscript{2,3} make it the wonder material of the 21st century\textsuperscript{4-6} and shows great promise for various areas of applications such as molecular electronic devices\textsuperscript{7}, molecular reinforcements in composites\textsuperscript{8}, biomedical engineering\textsuperscript{9,10}, tissue engineering\textsuperscript{11}, drug delivery\textsuperscript{12}, nanoprobe and biosensor technology\textsuperscript{13}.

Applications of carbon-based nanotechnology in biomedical research are being keenly explored by many researchers\textsuperscript{5,11,14-24}. In spite of the versatility of carbon nanotubes, their insolubility in process-friendly solvents which poses difficulty to their processing and manipulation in a facile manner and fears about any potential toxicity has severely hindered their widespread use in the areas of nanomedicine and nanobiotechnology\textsuperscript{25}. The recent developments in chemical modification and functionalization of CNTs have greatly improved the stability, solubility and dispersion of CNTs in water, subsequently opening the path for their handling and processing in physiological environments\textsuperscript{5}.

Toxicity of CNTs is a principal concern, with different groups pointing to their resemblance to asbestos fibers as a major issue\textsuperscript{26}. CNT toxicity in various studies has been credited to various factors like size, concentration, duration of exposure, method of exposure, and even the material used to solubilize/disperse the nanotubes\textsuperscript{4}. Most areas of
CNT toxicity are inconsistent and many studies suggest that elements of CNT toxicity are unsubstantiated\(^4\). The discrepancies appear to arise chiefly due to variances in experimental protocol especially the ability to obtain stable aqueous soluble CNTs as well as removing possible contaminants including metal catalyst residues that are associated with the synthesis of CNTs\(^{27}\).

The ability to solubilize and separate discrete CNTs from their tight bundles would not only help in their use, but would also help in their purification allowing their integration in more nanobiotechnology applications. In this study we have successfully prepared water soluble CNT of different types and evaluated their toxicity using the zebrafish model. To efficiently investigate the interactions between nanomaterials and the biological system, a sensitive, in vivo, rapid throughput model must be used. Cell-culture assays are rapid, high throughput and cost efficient, however they lack the complexity of a whole biological animal. In vivo models possess this complexity, but are traditionally low throughput and cost- and labor-intensive. However, zebrafish are the ideal model that possesses the convenience of cell culture, while combining the power of an in vivo system. It is also a well established in vivo toxicological model\(^{28-31}\). Zebrafish share a high degree of homology to the human genome and many cellular and anatomical similarities to vertebrates. A single female can lay several hundred embryos every 3-5 days that are small, develop externally, and are optically clear. An embryo’s organs develop within 5 days post fertilization and are genetically tractable. The zebrafish requires a significantly less amount of material to assess nanoparticle toxicity at multiple concentrations with replicates.
compared to other in vivo models, such as mice. By using the embryonic zebrafish model, we have developed a rapid process to investigate how nanomaterials induce biological responses\textsuperscript{32-37}. By using this model, our data will position us to understand what physicochemical properties of carbon nanotubes drive the differential biological responses and observe whether these water soluble CNTs are developmentally toxic.

4.3. Experimental

4.3.1. Materials and reagents

Deuterated sulfuric acid D\textsubscript{2}SO\textsubscript{4}: with a concentration of 96-98 wt. % in D\textsubscript{2}O, isotopic purity of 99.5 atom % D, deuterated nitric acid (DNO\textsubscript{3}: with a concentration of 65 wt. % in D\textsubscript{2}O and isotopic purity of 99 atom % D) were purchased from Sigma-Aldrich and were used as received. The nanotubes used, single walled carbon nanotubes (SWCNTs), double walled carbon nanotubes (DWCNTs) and multi-walled carbon nanotubes (MWCNTs) were produced by chemical vapour deposition method (CVD) and were obtained from CheapTubes (112 Mercury Drive Brattleboro, VT 05301 USA) and NanoLab, Inc. (179 Bear Hill Road, Waltham, MA 02451, USA).
4.3.2. Preparation procedure for soluble carbon nanotubes

Pristine carbon nanotubes (CNTs) up to 5 mg/mL concentration were dispersed by ultrasonication (100 W, 42 KHz, Branson 3510 ultrsonication bath, maximum power) in a mixture of D$_2$SO$_4$ and DNO$_3$ (3:1 v/v)$^{38}$ for 4 hours at temperature from ambient to 50°C to obtain a well homogenised colloidal solution (Warning: the acids are highly corrosive and should be handled with care under a chemical hood). The CNTs suspensions thus prepared were thoroughly washed (8 times) with Milli-Q water (18 MΩ) and separated by centrifugation (Clay Adams compact II centrifuge, 3200 rpm) to obtain a pH > 4.5. The CNTs were then filtered, washed again and dried in a vacuum oven. Stable solutions of CNTs were prepared in Milli-Q water by brief sonication for about 90 seconds.

4.3.3. Characterization

Scanning electron microscopy (FEI Quanta 3D Dual Beam SEM) and transmission electron microscopy (Philips CM12 TEM) were used to assess the surface morphology of the nanotubes. The samples were dispersed with methanol and then dropcast on pre-cleaned silicon wafer and spun coated with a thin layer of gold. The electron accelerating voltage and magnification for SEM were 15 KV and 200,000, respectively, while the accelerating voltage and magnification for TEM were 120 KV and 200,000, respectively. Thermal gravimetric analysis (Shimadzu TGA-50 thermogravimetric analyzer) was done to study the mass loss behavior of the CNTs. This was done under flowing Argon gas (20 ml/min) at a heating rate of 5°C/min from ambient temperature to 1000°C. To identify the
attachment of the functional groups on the surface of the nanotubes, fourier transform infrared spectroscopy (FTIR, Nicolet 510P FT-IR spectrophotometer) was used. The FTIR spectra were recorded using pellets of CNTs and KBr made by pressing the powder mixture into pellet. While the surface elemental composition and assessment of the chemical environment of the detected elements were analysed with X-ray photoelectron spectroscopy (XPS) with methanol dispersed sample dropcast on pre-cleaned silicon wafer until the surface is well covered. The CNT stability in Milli-Q water and embryo medium was studied by measureing zeta potential using ZetaPALS ζ-potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY). All samples contained 1 mM KCl.

4.3.4. Toxicity testing

The carbon nanotubes solutions (at a concentration of 100 µg/mL) were vortexed briefly prior to making a 50 µg/mL working solution using embryo medium (EM). Five-fold serial dilutions were prepared using a Caliper liquid handler. Adult Tropical 5D zebrafish were housed and reared at Oregon State University Sinnhuber Aquatic Research Laboratory. Embryos were collected and staged from group-spawned zebrafish39. To increase bioavailability, the embryonic chorion was removed at four hours post fertilization (hpf) as described by Truong et al, 201133. Embryos were rested for 30 minutes prior to initiating CNT exposure. Dechorionated embryos were transferred into individual wells of a 96-well plate with 100 µl of prepared CNT solution. Exposure plates were sealed and wrapped with aluminum foil to prevent evaporation and minimize light exposure. Embryos were exposed
to five concentrations of CNT solutions and a negative control (n=16, two replicates) with the highest concentration at 50 µg/mL down to 0.08 µg/mL. The static CNT exposure continued until 120 hpf. At 120 hpf, each embryo was euthanized with MS 222 and assessed for mortality and morphological malformations according to previously published protocol.

4.4. Results and Discussions

4.4.1. Characterization

Seven types of CNTs (Table 4.1), including two single walled (SWCNT) with different lengths, one double walled (DWCNT), and four multi-walled (MWCNT) with different lengths and diameters, were evaluated. After treatment with a mixture of deuterated sulfuric and nitric acids (D₂SO₄ and DNO₃), thoroughly washed with water and oven dried, the CNTs exhibited solubility and were stable in water at concentrations up to 100 µg/mL. As shown in Table 4.1, all the CNTs studied were negatively charged and stable in Milli-Q water with zeta potential ranging from −46.07 mV to −61.55 mV and showed similar stability in embryo medium with zeta potential ranging from −33.77 mV to −39.61 mV.
Table 4.1. Description of the nanotubes and their zeta potential in Milli-Q water and zebrafish embryo medium.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Type</th>
<th>Outer Diameter (nm)</th>
<th>Length (µm)</th>
<th>Zeta Potential (mV) in Milli-Q water</th>
<th>Zeta Potential (mV) in Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SWCNT</td>
<td>1-2</td>
<td>1-5</td>
<td>−46.45 ± 2.52</td>
<td>−39.61 ± 0.38</td>
</tr>
<tr>
<td>2</td>
<td>SWCNT</td>
<td>1-2</td>
<td>5-30</td>
<td>−53.34 ± 1.60</td>
<td>−37.30 ± 0.44</td>
</tr>
<tr>
<td>3</td>
<td>DWCNT</td>
<td>4±1</td>
<td>1-5</td>
<td>−49.81 ± 1.97</td>
<td>−37.44 ± 1.67</td>
</tr>
<tr>
<td>4</td>
<td>MWCNT</td>
<td>15±5</td>
<td>1-5</td>
<td>−46.07 ± 1.37</td>
<td>−33.77 ± 1.07</td>
</tr>
<tr>
<td>5</td>
<td>MWCNT</td>
<td>15±5</td>
<td>5-20</td>
<td>−59.33 ± 2.82</td>
<td>−37.05 ± 0.71</td>
</tr>
<tr>
<td>6</td>
<td>MWCNT</td>
<td>30±15</td>
<td>1-5</td>
<td>−47.43 ± 1.65</td>
<td>−35.11 ± 1.30</td>
</tr>
<tr>
<td>7</td>
<td>MWCNT</td>
<td>30±15</td>
<td>5-20</td>
<td>−61.55 ± 4.38</td>
<td>−37.76 ± 1.60</td>
</tr>
</tbody>
</table>

The hydrophobicity of unmodified CNT leads to suspension instabilities that result in settling within minutes or an hour depending on the type of modification or surfactant used.

Figure 4.1a showed a well homogenised colloidal solution of the CNT prepared using the described method after 6 months of storage under ambient conditions, with no settling. This ensures that the CNT solutions are stable in homogenous dispersion form throughout the duration of the toxicity testing and that nanotubes aggregation is minimal. The SEM and TEM images obtained of the prepared CNTs (Fig 1b & 1c- for SWCNT, other results are not shown here) demonstrated retention of structural integrity of the CNTs and free of amorphous carbon impurity. This was most likely due to the washing and drying process used to make the samples, rendering them virtually free of contaminants.
Results of TGA show an average maximum oxidation temperature of 610 ± 11 °C and residual percent of 8.2 ± 3.9 % for all the nanotubes studied. Figure 4.2 showed a maximum oxidation temperature at 615°C for pristine SWCNT with a residual percent of 10.5 % and a percent weight loss of 3 % between ambient temperature and 106°C which was attributed to vaporization of water molecules. A maximum oxidation temperature at 600°C with a residual percent of 5.7 % was observed for the deuterated acids treated SWCNT. Weight loss of 6.5 % in the temperature range less than 112°C was attributed to vaporization of water molecules while 22 % weight loss at temperature between 145°C to 350°C with mid point at 257°C is attributed to pyrolysis of the carboxylated carbon residue.
resulted from carboxyl (COO\(^-\)) groups grafted on the nanotubes sidewalls through covalent bonds\(^{41,42}\). This agrees well with the X-ray photoelectron spectroscopy (XPS) results about the proportion of the oxygenated component of the prepared CNTs and also agreed with the possible functional groups grafted on the CNTs as revealed by the FT-IR results.
Figure 4.2. Thermogravimetric analysis curves of pristine and treated SWCNT (after washing and drying) showing (a) percent weight loss as a function of temperature and (b), mass change per °C temperature (dMass (mg)/dT Temperature (°C)).
As shown in Figure 4.3, the percent elemental composition for both pristine and treated SWCNT showed pristine SWCNTs contain 96.3% carbon and 3.7% Oxygen atom (attributed to –OH group of water content), while acid treated SWCNTs contains about 78% carbon and 21% oxygen attributed to carboxylic group and a small amount of water and 1% sulfur. The deconvoluted C 1s XPS spectra revealed a high degree of carboxylated carbons in the acid treated CNTs with none in pristine CNTs. These compositions may be the explanation for the high solubility achieved with the treated SWCNTs. The results show no significant change in chemistry between samples, only a difference in concentrations of sulfur and oxygen and agree with FT-IR results, which revealed the functional groups introduced onto the nanotubes following the treatment.
Figure 4.3. X-ray photoelectron spectroscopy (XPS) data demonstrates the elemental composition of SWCNTs before and after the acid treatment, indicating that only minor changes (attributable to surface fictionalization with carboxylic groups) arise following treatment.

All the nanotubes studied showed similar IR characteristic. Representative FT-IR spectra of pristine and functionalized CNTs are shown in Figure 4.4. Infrared spectroscopy measures the quantity of radiation absorbed versus its frequency. When CNTs are subjected to an infrared radiation, the difference of charged state between carbon atoms induces the formation of an electric dipole; the appearance of these dipoles generates signals that are...
detected\textsuperscript{43}. IR absorption spectrum of D-acid treated CNT display –OH stretching vibrations band at 3460 cm\textsuperscript{-1}, a characteristic of -OH group attributed to carboxylic group and trace amounts of water. The bands at 1727 cm\textsuperscript{-1} and 1641 cm\textsuperscript{-1} were attributed to the presence of carboxylic C=O and C-O stretch respectively. The observed IR absorption peaks from the acid treated CNTs indicate the introduction of carboxyl groups due to surface oxidation\textsuperscript{44}. Most of these absorption bands were not observed in the untreated sample safe for the –OH stretching vibrations band displayed at 3328 cm\textsuperscript{-1}. However, the intensity of the peak is much lower than the one observed in the acid treated sample spectrum; therefore we can infer that it is due to the presence of trace amounts of water in the sample. This agrees with the results obtained from the TGA and XPS analysis.
Figure 4.4. FTIR spectra of untreated and acids treated CNTs (after washing and drying)

4.4.2. Toxicity

To assess the bioactivity of the seven different types of CNTs (two single-walled, one double-walled, and four multi-walled), the embryonic zebrafish model was used. Embryos are exposed to 5 concentrations (0.08, 0.16, 0.4, 2, 10, and 50 µg/mL) of the CNTs from 6 to 120 hpf to assess for developmental toxicity. At 24 hpf, embryos are evaluated for mortality and developmental progression, since at this life stage, not all morphological structures are present. By 120 hpf, 18 endpoints are assessed (17 morphological and 1 behavioral) to determine if static exposure to CNTs throughout development was adversely affecting the development of the embryos. As Figure 4.5 illustrates, regardless of the length
(1-5 or 5-30 µm) of the SWCNTs, there was no significant toxicity observed. When 15±5 nm MWCNT were modified to have an increased length from 1-5 µm to 5-20 µm, no adverse response was induced, but when the length was maintained at 1-5 µm and the diameter increased from 15±5 nm to 30±15 nm, a mild increase in toxicity was observed. MWCNT with a length of 5-20 µm induced less toxicity at a diameter of 30±15 nm than those with 15±5 nm. Regardless of the length, thicker MWCNTs were more toxic. Yamashita\(^45\) observed that long (5 – 10 µm) and thick MWCNTs caused DNA damage and severe inflammatory effects in the lung of mice, but not the short and thin ones\(^45\). These researchers also examined that the long and thick MWCNTS induced the strongest DNA damage while similar SWCNTs caused little effects. The lack of effect from SWCNTs is consistent with what was observed in this study. A study by Fenoglio et al\(^46\) found the opposite trend, where thin MWCNTs was more toxic compared to the thicker ones in both their in vitro (cytotoxicity) and in vivo (LDH activity and total proteins) assays\(^46\). Wang et al\(^47\) reported that a higher toxicity towards alveolar macrophages for short (1-5 µm) CNTs with 40-100 nm diameters than those with 10 – 20 nm\(^47\). The difference in the role of diameter in toxicity may be due to a number of reasons such as metallic content, presence of surface functionalities or defects. Another potential reason for the different conclusions may be a consequence of the diameter of the CNTs affecting the curvature and modifying the interactions (to cells or proteins)\(^48\). Or the thicker MWCNTs exhibit a larger surface area which is exposed and allows for more interactions\(^46,49\). Collectively, our results and these three studies suggest that the nanotube diameter plays a role in the toxicological assessments of CNTs.
Figure 4.5. Mortality and adverse effects induced by seven different types of water soluble CNTs. Dechorionated embryos were exposed to the CNTs from 6-120 hpf and 4 endpoints were evaluated at 24 hpf, and 18 at 120 hpf. Endpoints evaluated are defined as follows: MO24 = mortality observed at 24 hpf; DP24 = developmental progression at 24 hpf; SM24 = spontaneous movement at 24 hpf; NC24 = notochord malformation at 24 hpf. Endpoints evaluated at 120 hpf were: MORT = cumulative mortality; YSE = yolk sac edema; AXIS = axis defects; EYE = eye defects; SNOU = snout defect; JAW = jaw defect, OTIC = otic (ear) defect; PE = pericardial edema; BRAI = brain defect; SOMI = somite defect; PFIN and CFIN = pectoral and cadual fin defect; PIG = pigmentation abnormalities; CIRC = circulation defects; TRUN = trunk defect; SWIM = swim bladder abnormalities; NC = notochord defect at 120 hpf and TR = touch response abnormality.
Of the three types of CNTs tested, the DWCNTs induced mortality at a dose dependent manner, and caused snout malformations. The observation that DWCNTs induces snout malformations and mortality is consistent with a study using nasal cells. DWCNTs caused cytotoxicity to nasal cells at concentrations of 0.5 to 50 µg/mL and at 25 µg/mL; there was an increase of reactive oxygen species\textsuperscript{50}. Not only are the DWCNTs cytotoxic to nasal cells, they are capable of activating Nlrp3 inflammasome and causing inflammation similar to that caused by asbestos\textsuperscript{51}. With the first target for nanoparticles being the nasal cavity, these results are concerning and demonstrate the health hazards DWCNTs is capable of.

A sample blank was assessed to see if the methodology used to create water soluble CNTs had inherent toxicity. Although a low level of mortality (<20%) was observed after exposure to the sample blank, the low percentage of incidence is considered background in the zebrafish developmental toxicity assay. The lack of adverse biological response in the sample blanks demonstrates the methodology use was not toxic. It should be noted that we could not use pristine CNTs in the toxicity test because they form aggregation rapidly because we will not be differential if the lack of a biological response is due to bioavailability or the CNTs themselves. The acid washes caused the surface modification to the CNTs, resulting in them being water soluble and dispersed. Other studies have observed that dispersed CNTs were more toxic than non-dispersed\textsuperscript{52}. As these results demonstrated, the preparation method did not cause any toxicity and in general, these seven different types of water soluble CNTs were not toxic, but the diameter of CNTs is a parameter that may influence toxicity.
4.5. Conclusions

Various factors can be responsible for numerous results published in the literature on CNT toxicity and a number of the observations may not be a direct effect of CNTs. Toxicity studies have suggested that, besides the nano-dimensions of CNTs, many other factors may play roles in their toxicity. Raw nanotubes usually contain significant impurities, such as metal catalysts, which have been shown to contribute to increased toxicity through induction of oxidative stress. Purification procedures, such as strong acid treatment is expected to eliminate the impurities thereby making the CNTs less toxic. Aggregation is another possible factor that can cause false signal when doing toxicity studies of CNT as the functionalization of CNT helps to disperse and water solubilize the tubes and appears to reduce their toxicity. It is therefore important to remove any potential contributor(s) to toxicity effects in order to effectively understand the direct effects due to CNT. In this study we have prepared and characterized water soluble CNTs of different types, the solutions obtained were stable for our observation period of 6 months without any settling which means no aggregation. This has enabled us to effectively study the toxicity effects of the prepared CNTs using the zebrafish model. The results obtained showed that the CNTs studied caused no significant adverse effects on development. This bodes well for the application of CNTs as components of indwelling medical devices such as tissue growth scaffolds, monitoring devices, and drug delivery tools.
4.6. References


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CHAPTER 5

5.1 Summary and conclusions

Nanobiotechnology is revolutionizing the field of biosensors. The unique properties of carbon nanotubes have inspired their application to various biomedical sensing targets, which has contributed to making CNTs one of the most studied materials of the last two decades. In spite of the great potential of CNTs in biomedical and biotechnology applications, their manipulation is hampered by several problems. First, CNTs form bundles composed of hundreds of single nanotubes entwined together leading to formation of large aggregates, thus making it difficult to effectively apply them as originally envisioned: as single nanotube transducers. Second, pristine CNTs lack solubility in process-friendly solvents, which poses difficulties in preparing and manipulating them when building sensors. This has imposed great limitations on the practical use of CNTs. Third, regardless of the production method, metal and carbonaceous impurities remain in the sample and affect CNT properties, behaviors and interactions with biomolecules. Finally, fears of potential CNTs toxicity have severely hindered their widespread use in the areas of nanomedicine and nanobiotechnology. Despite the enormous efforts that researchers have put into solving these problems to improve CNT biocompatibility and allow for their application in biotechnology, many questions remain unanswered. Inspired by some of these challenges, this study was designed to answer some of those unresolved questions, thereby contributing to the CNT knowledge base.
First, we have successfully demonstrated the fabrication and application of a label free solid-state potentiometric biosensor for detection of proteins under physiologically relevant ionic strength conditions. The high salt concentration in the electrolyte ensured compatibility with the usual high ionic strength of any unprocessed biological sample, making the sensor relevant for the analysis of real biological samples. To ensure the electrical properties of the CNTs were preserved and that the desired distribution of reactive species (necessary for attachment of molecular probes on the sidewalls of the CNTs) was achieved, we employed diazonium functionalization of the CNTs. The prepared biosensors exhibited acceptable reusability and reproducibility, and were capable of detecting protein across a physiologically relevant range of concentrations. For our model protein, thrombin, a high degree of linearity was obtained for concentrations ranging from $1 \times 10^{-10}$ M to $1.8 \times 10^{-7}$ M for both the aptamer-modified and the antibody-modified biosensors with a limit of detection in the pico molar range.

Also, a new technique for effective immobilization of antibody probes on CNTs in a controlled orientation was reported. Here, we exploited the sulfhydryl group of the intrinsic disulfide groups in the hinge region of the antibody, common to all antibodies, as a point of attachment to the functionalized CNT using 3-(2-pyridyldithio)propionyl hydrazide (PDPH) as a crosslinker. As the number of disulfide bonds is quite small, a self-assembled monolayer of antibodies on CNTs was envisaged. This approach was intended to ensure the correct orientation of the antibodies and to preserve their activity. To demonstrate this principle, C-reactive protein was used as the model target using anti-C-reactive protein
antibody as biorecognition molecule. This biosensor demonstrated high sensitivity over physiologically relevant concentrations of CRP with limits of detection in the picomolar range. The successful application of the developed biosensors with both aptamer and antibody as bioreceptors, under the same condition of high ionic strength is an indication that the mechanism of detection in this study was a unique one and is independent of the nature of the bioreceptors. We also demonstrated in these studies the dependence of the sensitivity of potentiometric biosensors on the pH of the electrolyte buffer and proposed a novel mechanism for protein detection by the biosensor. Our success in these studies may extend the range of possible applications of potentiometric biosensors for detection of disease biomarkers in real unprocessed biological samples.

Lastly, we successfully prepared highly hydrophilic carbon nanotubes, with good stable suspension in physiological medium using deuterated acids, and evaluated their possible toxicological effects. The prepared functionalized nanotubes were well characterized to evaluate their physical morphology and elemental composition. The results showed that none of the CNTs studied caused significant adverse developmental effects. This is an indication that some of the previously reported toxicity effects of CNTs may be due to the other factors. For example, aggregation is a possible factor that can cause false signal when doing toxicity studies of CNT as the functionalization of CNT helps to disperse and water solubilize the tubes and appears to reduce their toxicity. It is therefore important to remove any potential contributor(s) to toxicity effects in other to effectively understand the direct effects due to CNT. Impurities associated with CNT production could be another possible
factor. The results in this study support the potential safe use of CNTs as components of indwelling medical devices such as tissue growth scaffolds, monitoring devices, and drug delivery agents.

5.2 Future work

These studies have given us the platform to demonstrate the ability of CNT based potentiometric biosensors to selectively detect disease biomarker proteins at physiologically relevant concentrations and high ionic strength with little or no influence of the sample matrix. Detection of some disease biomarkers such as cancer cell at its earliest stages will by no doubt increase the survival rate of patients and help in determining if revolutionary drugs are effective. As part of the future work, applying this principle for development of biosensors for detection of cancer cell biomarkers will be one of the primary focuses. Next to that is the development of the sensors for environmental monitoring of organic pollutants.

Although the presented potentiometric sensors are among the most affordable in terms of instrumental approach, the challenge remains when dealing with their use for point of care personal devices which requires large scale production. The cheaper way of mass production of such sensors is to use CNT screen print electrodes, which are commercially available, as a replacement for glassy carbon electrode. This will greatly enhance the portability of the devices and bring the cost of production down tremendously. The alternative is to develop paper based CNT sensor electrode which can be used as disposable electrodes.
CHAPTER 6

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