Applications of Biosensor-based Analysis: A Review and The Synthesis of Biphenyl Modified Deoxyguanine as a New Class of Aptasensors

By
Olasunkanmi Olaoye

A Research Paper
Presented to
The University of Guelph

In partial fulfilment of requirements
For the degree of
Master of Science
In
Chemistry

Guelph, Ontario. Canada.

© Olasunkanmi O. Olaoye, August 2015
ABSTRACT

Applications of Biosensor-based Analysis: A Review and The Synthesis of Biphenyl Modified Deoxyguanine as a New Class of Aptasensors

Olasunkanmi O. Olaoye
University of Guelph, 2015
Advisor: Richard A. Manderville

The broad concept of biosensors is very simple, yet their design and application today employs a great deal of sophistication. They basically have a biological recognition component within them and are responsible for the sensing, detection, or/and quantification of target samples. Their biological recognition component are mainly enzymes, nucleic acids, cells, or some naturally occurring biological product. The design of biosensors has also incorporated artificial biological recognition components e.g. aptamers, modified molecules, and polymers. Their mode of detection may be for single molecular targets or a diverse range of molecular target groups, a concept which has seen extensive applications in life sciences. Their applications also extend to medicine and pharmaceuticals, food processing and safety, and environmental screening.

Aptasensors are a rapidly growing aspect of biosensors, and their applications is almost as wide as the entire biosensor scope itself. The understanding that nucleic acids can assume stable secondary structures, and that they can easily be synthesized and functionalized to afford structures beyond natural evolution, has opened the door for aptamers as a biological recognition agent in several biosensing applications. Aptasensors as a class of biosensors use aptamers which are highly selective recognition nucleic acid agents. Aptasensors can be incorporated on very sophisticated designs, such as in micro and nano scale platforms, which allows for their integration in
miniaturized devices. Their detection is also very rapid, specific and sensitive, and can offer reduced costs, high throughput, and a minimized material consumption.

Aptasensors has also rapidly evolved, and is now widely used in environmental monitoring, pharmaceuticals, medicine, agriculture, and food safety.

This research paper reviews the extensive field of biosensor designs, and their applications in several life disciplines. It also reviews the emerging areas of aptasensor development, as having massive potential in becoming the future of biosensor applications. Finally, the design of a new, fluorescent aptasensor detection based on modified nucleic acid aptamers is presented, and the optical properties of this analogue is discussed with regard to its potential as a recognition and detection component in an aptasensor design.
ACKNOWLEDGEMENTS

I say a very big thank you to Dr. Richard Manderville who has been my advisor during the period of this project. First, I saw you as a tutor, then as a boss, and afterwards as a great guide. But in the end, I came to understand that you are an exceptional gentleman. I am very grateful for giving me the chance to work in your laboratory, encouraging me in my very difficult moments, and challenging me to push for greater things. You are an inspiration, and I will always be grateful for the chance to work with you. Thank you Richard.

My thanks also goes to Dr. Wojciech Gabreylski, for accepting to be on my advisory committee without hesitation. I enjoy the moments we chatted, and how you encourage me with your professional advice. I also thank Dr. Mario Monteiro, for accepting to chair my seminar and final project presentations. You were also very kind to me during my search for a research supervisor and I am very grateful. I want to say thank you to Dr. Marcel Schlaf, who mentored me during my early days at the University of Guelph. You allowed me in your group, a period which formed my first learning experience, and you also took it upon yourself to ensure I get funded for my research project. You were awesome Marcel, thank you so much. I want to also thank Dr. Paul Rowntree, and the Chemistry Department for supporting and funding my research project. I say thank you to Karen Ferraro, who helped to tie up my loose ends, and had me for some amazing chess games.

I cannot but appreciate the intellectual effect the Manderville group members had on me and on my research. I came in with no understanding of what I had to do, and you showed me the way. You answered my questions, gave me advice, and even offered your time and expertise to help me get going. I have learnt a lot from you guys, and I say a very big thank you.
My deep-felt appreciation goes to the team I met at Catalyst Centre, University of Guelph. Erin, Melissa, David, Steve, Gregor, Jaleh and Andrea. Sharing few hours each day with you had a lot of positive effect in my life. You took me in as a friend and shared your wonderful moments with me. Thank you so much. I want to especially thank Erin for your love and support. You took me a step further, and regarded me as part of your family. Your kids, Cam and Will, I think are the coolest boys in the world. Your husband, parents and siblings, you were all very wonderful to me. A lot would not have happened without you. Thank you so much.

I am also grateful to my family, my parents and siblings back in Nigeria. You stood by me all the way, especially at my very darkest hours. You encouraged and spurred me, and I am forever grateful. To my big family at Guelph SDA church, I also say thank you. My friends, Tolulope and Dr. Dele, thank you for the amazing dinners and long night chats.

Finally, I say thank you to God for being the holder and sustainer of my life. Nothing I am, or will ever be, if not for You. Thank you to everyone, mentioned or not, who had even the briefest stint with me during my time in the University of Guelph. I will miss you all.
TABLE OF CONTENT

Chapter One: Introduction to Biosensors .................................................... 1

1.1. Sensory Agents .................................................................................. 3
    1.1.1. Biologically Derived Sensory Agents ........................................... 3
    1.1.2. Synthetic Analogues ................................................................. 6

1.2. Transducers ....................................................................................... 9
    1.2.1. Optical Transducers ................................................................. 9
    1.2.2. Electrochemical Measurements ................................................ 13

1.3. Biosensor Configurations ................................................................. 16
    1.3.1. Performance Evaluation of Biosensors ....................................... 20
    1.3.2. In vivo Biosensors ..................................................................... 21

1.4. Introduction to Aptasensors .............................................................. 22

Chapter Two: Biosensors for Medical Applications ................................. 28

2.1. Transduction Methods Employed in Medical Biosensors ..................... 28
    2.1.1. Electrochemical Biosensors ....................................................... 28
        2.1.1.1. Label-Based Electrochemical Biosensors ............................. 30
            2.1.1.1a Enzyme Labeled Electrochemical Biosensors .................. 31
            2.1.1.1b DNA Labeled Electrochemical Biosensors ..................... 31
            2.1.1.1c Nanomaterial Labeled Electrochemical Biosensors ......... 31
        2.1.2. Piezoelectric Biosensors for Medical Applications .................. 34
            2.1.2.1. Piezoelectric Immunosensors ......................................... 34
            2.1.2.2. Piezoelectric Nucleic Acid Biosensors ............................ 35
            2.1.2.3. Piezoelectric Aptamer Biosensors .................................. 38
        2.1.3. Nanomaterial-Based Biosensors for Medical Applications ...... 39
        2.1.4. Enzyme-Based Biosensors in Medical Applications ............... 43

2.2. Use and Applications of Medical Biosensors ...................................... 46
    2.2.1. Detection and Characterization of DNA and RNA .................... 46
        2.2.1.1. Optical-Based DNA Biosensors ......................................... 47
        2.2.1.2. Electrochemical-Based DNA Biosensors ........................... 49
        2.2.1.3. Other Transduction Approaches to Medical Biosensors ...... 54
    2.2.2. Disease Biomarker Detection .................................................. 54

2.3. Biosensors for Cancer Detection: Affibodies as Alternatives .............. 59

2.4. Drug Discovery and Testing .............................................................. 62
    2.4.1. Electrochemical Biosensors for Drug Discovery ....................... 63
    2.4.2. Optical and Piezoelectric Biosensors for Drug Discovery .......... 63

2.5. Non-Invasive Measurements ............................................................ 66
    2.5.1. Non-Invasive Biosensors Using Saliva as Analyte ..................... 66
    2.5.2. Non-Invasive Biosensors Using Sweat as Analyte ..................... 68
    2.5.3. Wearable Biosensors ............................................................... 68
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.4. Clinical Applications of Non-Invasive Measurements</td>
<td>70</td>
</tr>
<tr>
<td>Chapter Three: Biosensors in Food Safety and Environmental Health</td>
<td>82</td>
</tr>
<tr>
<td>3.1. Biosensors in Food Safety</td>
<td>82</td>
</tr>
<tr>
<td>3.1.1. DNA Biosensors in Food Safety</td>
<td>84</td>
</tr>
<tr>
<td>3.1.1.1. Genosensors for GMO Detection</td>
<td>84</td>
</tr>
<tr>
<td>3.1.1.1. Genosensors for Food Pathogens</td>
<td>89</td>
</tr>
<tr>
<td>3.1.2. Other Biosensor Approaches to Food Pathogen Detection</td>
<td>93</td>
</tr>
<tr>
<td>3.1.3. Biosensors for Detecting Pesticides in Foods</td>
<td>94</td>
</tr>
<tr>
<td>3.1.4. Biosensors for Detection of Natural Toxin</td>
<td>98</td>
</tr>
<tr>
<td>3.2. Biosensors in Environmental Health</td>
<td>104</td>
</tr>
<tr>
<td>3.2.1. Biosensors in Soil and Water Applications</td>
<td>105</td>
</tr>
<tr>
<td>3.2.1.1. Biosensors for Detecting Soil Contaminants</td>
<td>105</td>
</tr>
<tr>
<td>3.2.2. Biosensors for Detecting Endocrine Disruptors and Other</td>
<td>111</td>
</tr>
<tr>
<td>Environmental Pollutants</td>
<td></td>
</tr>
<tr>
<td>3.2.2.1. Biosensors for Endocrine Disruptor Chemicals (EDCs)</td>
<td>112</td>
</tr>
<tr>
<td>3.2.2.2. Biosensors for Other Environmental Pollutants</td>
<td>115</td>
</tr>
<tr>
<td>Chapter Four: Aptasensors as Bioanalytical Tools</td>
<td>132</td>
</tr>
<tr>
<td>4.1 SELEX and its Recent Optimizations</td>
<td>133</td>
</tr>
<tr>
<td>4.1.1. Chemistry of SELEX</td>
<td>133</td>
</tr>
<tr>
<td>4.1.2. Modifications of SELEX</td>
<td>138</td>
</tr>
<tr>
<td>4.1.3. Advantages and Limitations of SELEX</td>
<td>140</td>
</tr>
<tr>
<td>4.1.4. Applications of Commercial Aptamers</td>
<td>143</td>
</tr>
<tr>
<td>4.2. Aptamer-Based Biosensors</td>
<td>145</td>
</tr>
<tr>
<td>4.2.1. Electrochemical Aptasensors</td>
<td>146</td>
</tr>
<tr>
<td>4.2.1.1. Labeled Approach to Electrochemical Aptasensors</td>
<td>147</td>
</tr>
<tr>
<td>4.2.1.2. Label Free Electrochemical Aptasensors</td>
<td>150</td>
</tr>
<tr>
<td>4.2.1.3. Applications of Electrochemical Aptasensors</td>
<td>153</td>
</tr>
<tr>
<td>4.2.2. Optical-Based Aptasensors</td>
<td>158</td>
</tr>
<tr>
<td>4.2.2.1. Fluorescence-Based Aptasensors</td>
<td>160</td>
</tr>
<tr>
<td>4.2.2.2. Colorimetric Aptasensors</td>
<td>167</td>
</tr>
<tr>
<td>4.2.2.3. Chemilluminescence Approaches to Aptasensor Designs</td>
<td>168</td>
</tr>
<tr>
<td>4.2.3. Other Transduction Methods in Aptasensor Design</td>
<td>169</td>
</tr>
<tr>
<td>4.3 Future Perspectives</td>
<td>170</td>
</tr>
<tr>
<td>Chapter Five: Synthesis and Optical Properties of Fluorescent Aptamer</td>
<td>181</td>
</tr>
<tr>
<td>5.1. Fluorescent Aptamers</td>
<td>182</td>
</tr>
<tr>
<td>5.1.1. Principles of Fluorescence</td>
<td>182</td>
</tr>
<tr>
<td>5.1.2. Fluorescent Oligonucleotides</td>
<td>183</td>
</tr>
<tr>
<td>5.1.3. Research Background</td>
<td>184</td>
</tr>
<tr>
<td>5.1.4. Research Objective</td>
<td>187</td>
</tr>
<tr>
<td>5.2. Experimental Procedures</td>
<td>188</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Table 2.1</th>
<th>Piezoelectric sensors used in real sample analysis</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.2</td>
<td>Different biosensor designs for drug detection</td>
<td>66</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>PNA probe sequence and target regions</td>
<td>88</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Electrochemical genosensors for screening and detection of specific GMOs in food</td>
<td>89</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Enzyme based biosensors using nanomaterials for pesticide detection</td>
<td>96</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>List of important mycotoxins and phycotoxins</td>
<td>100</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Some environmentally relevant EDCs</td>
<td>113</td>
</tr>
<tr>
<td>Table 3.6</td>
<td>Important PAHs to environmental health</td>
<td>117</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Modifications of SELEX procedures for generating aptamers</td>
<td>140</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Examples of Commercial Clinical Aptamers</td>
<td>145</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Labels employed in aptasensor designs</td>
<td>150</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Optical aptasensors for detection of small biomolecules</td>
<td>160</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Characteristic absorption and emission wavelengths of common fluorophores</td>
<td>162</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Absorption and emission maximums of the $^{\text{FBPV}}dG$ in various solvents</td>
<td>197</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Absorption and emission maximums of the $^{\text{CF3PV}}dG$ in various solvents.</td>
<td>200</td>
</tr>
<tr>
<td>Table 5.3</td>
<td>Absorption and emission maximums of the $^{\text{CF3PV}}dG$ in various solvents.</td>
<td>201</td>
</tr>
</tbody>
</table>
List of Figures

| Fig 1.1 | Forms of sensors and transducers currently employed in biosensor designs | 3 |
| Fig 1.2 | Simplified overview of MIP preparation process | 7 |
| Fig 1.3 | Block diagram showing basic component of a simple spectrofluorimeter | 10 |
| Fig 1.4 | Block diagram showing basic component of a simple UV-Vis spectrophotometer | 11 |
| Fig 1.5 | Principle for a simple surface plasmon resonance measurement | 13 |
| Fig 1.6 | Design of gold CD electrochemical transducer based biosensor for Interleukin-6 | 16 |
| Fig 1.7 | A BOD biosensor design and use in a free-flow wastewater detection | 18 |
| Fig 1.8 | Set-up and typical result of a DNA microarray biosensor | 19 |
| Fig 2.1 | Classic scheme for DNA detection using electroactive labels or redox enzymes | 30 |
| Fig 2.2 | Schematic representation of amplified detection of DNA | 37 |
| Fig 2.3 | MWCNTs non-covalently attached to electrode surfaces used in electrochemical biosensor design and applications | 41 |
| Fig 2.4 | Some electroactive labels used in electrochemical-based DNA biosensors | 51 |
| Fig 2.5 | A combimatric electrosense reader | 53 |
| Fig 2.6 | 2-D and 3-D immunoassay showing the advantage of simplicity, multiplicity, and reduced reagent and sample quantity in 3-D particle-based assay | 57 |
| Fig 2.7 | Organosilica particles and flow cytometry as particle-based detection of biomarkers | 59 |
| Fig 2.8 | Refined solution NMR of Z-domain in protein A | 62 |
| Fig 2.9 | Wireless configuration of a body sensor network | 69 |
| Fig 3.1 | Construction and detection levels of GMOs | 85 |
| Fig 3.2 | Deoxyribozyme biosensor for GMO detection | 86 |
| Fig 3.3 | Principle of G-Quadruplex DNAzyme sensor for detection of GMO | 87 |
| Fig 3.4 | Aptsensor for multiplex detection of E. coli and S. enteriditis | 92 |
| Fig 3.5 | Scheme for AChE/Au/NP/cr-GS biosensor for paraoxon detection | 96 |
| Fig 3.6 | SWV detection of MC-LR using an aptamer functionalized GSPE | 102 |
| Fig 3.7 | Design of colorimetric aptasensor for OTA detection | 103 |
| Fig 3.8 | Concept of GOx and urease biosensor for glucose and heavy metal detection | 108 |
| Fig 3.9 | 8-17 DNAzyme and constructs for FRET studies on heavy metal detection based on cation induced folding | 110 |
| Fig 3.10 | Schematic representation of a tyrosinase-based biosensor for detection of bisphenol A in a polluted aqueous solution | 114 |
| Fig 3.11 | Chemical structure of environmentally relevant PAHs | 116 |
| Fig 3.12 | Schematic for the generation of antibody specific to PAHs using benzo[α]pyrene as an example | 118 |
| Fig 4.1 | Scheme for the in vitro target-specific aptamer selection using SELEX | 134 |
| Fig 4.2 | Modified nucleoside and nucleotides | 136 |
| Fig 4.3 | Increase in aptamer based biosensor publications in the last 8 years. Distribution by country | 146 |
| Fig 4.4 | Electrochemical aptasensor for thrombin based on voltammetric turn-on and quenching | 147 |
| Fig 4.5 | Platinum nanoparticles as signal amplifiers in electrochemical aptasensors | 147 |
| Fig 4.6 | Schematic of FIS aptasensor for the detection of lysosome | 151 |
| Fig 4.7 | A reusable graphene-based electrochemical aptasensor for detecting cancer cells | 152 |
| Fig 4.8 | Graphene based electrochemical aptasensor for the detection of S. aureus | 154 |
| Fig 4.9 | Detection of OTA using PEG on a boron-doped diamond microcell | 157 |
| Fig 4.10 | Dyes and fluorophores used for fluorescent labelling of aptasensors | 164 |
| Fig 4.11 | Schematic of the FRET-based kanamycin aptasensor | 165 |
| Fig 4.12 | Amplified fluorescence polymerization aptasensor based on CSDA for detection of proteins | 166 |
| Fig 4.13 | Exo III-assisted signal amplification recycling in a label-free colorimetric aptasensor detection of PDGF | 170 |
| Fig 5.1 | Jablonski diagram of the energy levels of a fluorescent molecule | 182 |
| Fig 5.2 | Modified nucleobase analogues and their base pairing | 184 |
| Fig 5.3 | Modified furan and cyanophenyl dG nucleosides showing syn- and anti-conformations. | 186 |
| Fig 5.4 | Bezo[b]thiophene and an indole modified deoxyguanosine nucleobase | 186 |
| Fig 5.5 | Biphenyl modified dG with a vinyl linker | 187 |
| Fig 5.6 | Emission Spectra of FBPVdG in solvents of different polarities | 196 |
| Fig 5.7 | Excitation and emission spectra of FBPVdG in 100% MOPS pH 7 with slit width set to emm: 5.0, exc: 5.0 | 198 |
| Fig 5.8 | Emission spectra of FBPVdG in different viscous environment (MOPS pH 7 buffer + different concentrations of glycerol) | 199 |
| Fig 5.9 | Emission spectra of FBPVdG in 80% glycerol + 20% MOPS pH 7 buffer at different temperatures | 199 |
| Fig 5.10 | Structure of CF3PVdG as synthesized by Xing Li at the Manderville Laboratory | 200 |
| Fig 5.11 | Emission spectra of CF3PVdG in solvents of different polarities | 200 |
## List of Schemes

<table>
<thead>
<tr>
<th>Scheme 5-1</th>
<th>Synthesis pathway for 1''',1'''-Biphenyl, 4'''-fluoro, 4'''-vinyl-dG</th>
<th>194</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 5-2</td>
<td>Proposed synthesis of FBPV\textsuperscript{dG} DNA phosphoramidite monomer</td>
<td>203</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
<td></td>
</tr>
<tr>
<td>AchE</td>
<td>Acetylcholine Esterase</td>
<td></td>
</tr>
<tr>
<td>AFB1</td>
<td>Aflatoxin B1</td>
<td></td>
</tr>
<tr>
<td>BAW</td>
<td>Bulk Acoustic Wave</td>
<td></td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
<td></td>
</tr>
<tr>
<td>BSN</td>
<td>Body Sensor Network</td>
<td></td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
<td></td>
</tr>
<tr>
<td>CCD</td>
<td>Charged Couple Device</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Compact Disc</td>
<td></td>
</tr>
<tr>
<td>ChO</td>
<td>Choline Oxidase</td>
<td></td>
</tr>
<tr>
<td>CNF</td>
<td>Carbon Nanofibers</td>
<td></td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon Nanotubes</td>
<td></td>
</tr>
<tr>
<td>CSDA</td>
<td>Continuous Strand-Displacement Amplification</td>
<td></td>
</tr>
<tr>
<td>CVD</td>
<td>Chemical Vapour Deposition</td>
<td></td>
</tr>
<tr>
<td>(\text{Ac}^p\text{dG})</td>
<td>4''-Acetylphenyl-Deoxyguanosine</td>
<td></td>
</tr>
<tr>
<td>(\text{CF}_3\text{PV}\text{dG})</td>
<td>4''-Trifluorophenyl, 1''-Vinyl-Deoxyguanosine</td>
<td></td>
</tr>
<tr>
<td>(\text{FBPV}\text{dG})</td>
<td>1''-Biphenyl, 4'''-Fluoro, 4''-Vinyl-Deoxyguanosine</td>
<td></td>
</tr>
<tr>
<td>dG</td>
<td>Deoxyguanosine</td>
<td></td>
</tr>
<tr>
<td>DME</td>
<td>Dropping Mercury Electrode</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
<td></td>
</tr>
<tr>
<td>DWCNT</td>
<td>Double-Walled Carbon Nanotubes</td>
<td></td>
</tr>
<tr>
<td>EDC</td>
<td>Endocrine Disruptor Chemicals</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunoassay</td>
<td></td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
<td></td>
</tr>
<tr>
<td>FET</td>
<td>Field Electric Transistors</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>FIS</td>
<td>Faradaic Impedance Spectroscopy</td>
<td></td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>Fluorescence Polymerization</td>
<td></td>
</tr>
<tr>
<td>FRET</td>
<td>Forster Resonance Energy Transfer</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
<td></td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically Modified Organisms</td>
<td></td>
</tr>
<tr>
<td>GNP</td>
<td>Gold Nanoparticles</td>
<td></td>
</tr>
<tr>
<td>GNS</td>
<td>Graphene Oxide Nanosheet</td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>Graphene Oxide</td>
<td></td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose Oxidase</td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
<td></td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
<td></td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
<td></td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency on Research on Cancer</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>LNA</td>
<td>Locked Nucleic Acids</td>
<td></td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidase</td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>Microcystins</td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>Molecular Imprinted Polymers</td>
<td></td>
</tr>
<tr>
<td>MORFS</td>
<td>Phosphoramidite Morpholino Oligomers</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>Micro Ribonucleic Acid</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
<td></td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-Walled Carbon Nanotubes</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)</td>
<td></td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectrometry</td>
<td></td>
</tr>
</tbody>
</table>
NOS  Nopaline Synthase
NP   Nanoparticles
OTA  Ochratoxin A
PAH  Poly Aromatic Hydrocarbons
PCR  Polymerase Chain Reaction
PDGF Platelet-Derived Growth Factor
PEG  Polyethylene Glycol
pH   Potential of Hydrogen
PNA  Peptide Nucleic Acids
PQS  Piezoelectric Quartz Crystal
PVA  Poly Vinyl Alcohol
QCM  Quartz Crystal Microbalance
RGO  Reduced Graphene Oxide
RIA  Radio-immunoassay
RNA  Ribonucleic Acid
ssDNA Single Strand Deoxyribonucleic Acid
ssRNA Single Strand Ribonucleic Acid
SAM  Self-Assembly Monomers
SAW  Surface Acoustic Wave
SELEX Systemic Evolution of Ligands by Exponential Enrichment
SERS Surface Enhanced Rahman Spectroscopy
SiNW Silicon Nanowires
SiRNA Small Interfering Ribonucleic Acids
SNP  Single Nucleotide Polymorphism
SPR  Surface Plasmon Resonance
SPCE Screen Print Carbon Electrode
SPE  Screen Print Electrode
SWCNT Single-Walled Carbon Nanotubes
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEA</td>
<td>Tetra Ethyl Amine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetra Hydro Furan</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-Layer Chromatography</td>
</tr>
<tr>
<td>TBA</td>
<td>Thrombin Binding Aptamer</td>
</tr>
<tr>
<td>TPPTTS</td>
<td>Triphenylphosphnine 3,3’,3’’ Trisulfonyl Acid, Trisodium Salt</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>USATSDR</td>
<td>United States Agency for Toxic Substances and Disease Registry</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
</tbody>
</table>
Chapter One: Introduction to Biosensors

Biosensors are usually small, portable designs which can selectively quantitate a specific or a broad range of biochemical/chemical analyte of interest. Biosensors are made up of two parts: the recognition agent otherwise known as the bioreceptor or sensory agent, and the transducer. The sensor is the sensitive biologically derived material or component such as cell receptors, antibodies, enzymes, and nucleic acids that binds and selectively recognizes an analyte of interest, thus sensing a physical variable change in the analyte. The transducer on the other hand is the detector, and it transforms the signal generated when the sensory agent interacts with the analyte into another signal (i.e. transduction) that is easily measured and quantified. The signal generated by the transducer is usually in either electrochemical, optical, piezoelectric, or physicochemical forms.

The earliest biosensor known was developed in 1962 by Clark Jr and Lyons.\(^1\) They used this biosensor to measure blood oxygen content during cardiovascular surgery, using an electrochemical cell system. Since then, there has been huge strides in the field of biosensors, and modern, mobile devices now characterize this field. The value of commercial biosensors has grown significantly from US$5bn in 2004 to over US$13bn in 2012, exceeding projected forecasts.\(^2,3\) Point-of-care biosensor applications account for almost half of the biosensor market, and over 32% of the commercial biosensor industry is based on home care, chip-based, glucose biosensors.

The massive growth in biosensors has attracted the interest of world’s major R&D and diagnostics companies.\(^2\) A peculiar feature of biosensors is that they can allow analyte measurements without prior purification such as from complex matrixes of milk, blood, food and environmental samples, and fermentation broths. Some biosensors are designed for a specific
analyte and as such are highly selective; while some are used for broad-based detections, such as cell-based biosensors used in general toxin testing. There should be a great deal of compatibility between the sensory agent and the transducer, such that the signal generated by the sensory agent can be accurately and completely interpreted by the transducer.

The design of modern biosensors demands that they are small-sized, relatively cheap, energy-saving, and can offer improved detection for the intended target. These features are required for biosensor design for use in point-of-care and personal home-use tests. Their response time should also be very fast preferentially in the order of seconds, and their detection limits should be very low. These features have made biosensors useful in food and agriculture, environmental testing, health and medicine. The disposable biosensor strips used for glucose testing, which as discussed above is the most successful biosensor known to date, has found widespread use in home care monitoring of diabetic patients, and this biosensor accounts for more than 85% of the available commercial biosensor market.

Several improvement in the glucose biosensor technology have been developed, and there is a positive outlook and a strong future for these biosensors, and even for those outside the field of clinical diagnostics. Some improvements in biosensor designs has led to DNA sensor arrays used in genetic testing, biosensors used in multiple detection of drugs, and lactic acid biosensors used in detecting substance misuse in sports and athletics. Areas for improvement still lies in the accuracy and reproducibility of results, in sensitivity and stability. The several sensory agent and transduction means used in biosensor designs are classified in Fig. 1.1.
1.1. Sensory Agents

1.1.1. Biologically-Derived Sensory Agents: These are naturally occurring sensors obtained from living systems. They may be in isolated or complex forms. Enzymes are a widely used natural sensor because they offer very good detection limits and sensitivity. Enzymes are also suitable for
a wide variety of substrates, and can be easily incorporated with several transduction methods. In the design of an enzyme-based biosensor, the choice of a transduction method largely depends on the physical property of the analyte.\textsuperscript{4} Usually, enzymes are immobilized to the transducer surface and the immobilization method depends on enzyme binding and kinetic parameters. Immobilization methods include physical or chemical approach. Chemical immobilization methods employ formation of covalent bonds between the enzyme functional groups and the support material, and it is achieved by crosslinking and non-polymerizing methods. Physical immobilization, on the other hand, does not involve covalent bonds thus retaining most of the chemical properties of the enzyme. Physical immobilization is achieved by adsorption, entrapment, and microencapsulation.\textsuperscript{5} Enzyme-based biosensors sometimes involve more than one substrate, and in this case one of the substrate is tethered to the transducer surface, such that the property of a second, freely flowing substrate is not considered. For the disposable home glucose biosensors discussed above, a freely flowing dehydrated cofactor substrate is used such that it dissolves completely when it comes in contact with the blood sample, migrating to the glucose oxidase sensor (GOx) which is immobilized to a transducer, for detection.\textsuperscript{6}

Antibodies are another class of biologically derived sensory agent, and they provide recognition through their specific binding to the analyte of interest. Unlike enzyme sensors, they do not catalyze reactions. Antibodies provide the selective binding properties of immunosensors, and they are large glycoproteins produced in living animals from an immune response. Antibodies are often referred to as bivalent, as they have two similar binding sites per molecule. The selective binding features of an antibody towards an immunogen (substance that generates the immune response) also allows for its binding with non-immunogen species that have structural similarities.
Antibody-based biosensors exploit the selective binding and recognition of antibodies for the detection and quantitation of haptens and antigens (species similar to or also known as immunogens). Immunosensors is the term often used for antibody-based biosensors. The class of antibodies that are biologically generated is known as polyclonal antibodies, and they are directly extracted from the serum. Several transduction methods can also be used for an antibody-based biosensor and for some designs, a labeling strategy e.g. fluorophore and enzymes, are employed for signal generation. When non-label designs are required, mechanical transduction means such as piezoelectric and surface wave acoustic transducers are preferred as they can directly generate their signal based on surface changes when the antibody binds the analyte.

Single stranded DNA (ssDNA) are the main natural nucleic acids used as recognition agents in biosensor designs, and their recognition is based on specific binding to a complementary sequence. This approach is useful in the detection of genetic sequences, single base mismatch, and DNA damage. Advancements in ssDNA sensors has also led to a DNA array technology for simultaneous multiple detection of analytes. Fluorophores or redox sensitive labels are required when optical or electrochemical transducers are used. However, label free ssDNA sensors have also been achieved using several transduction means. 

Other natural sensors used in biosensor design include tissue-based and cell-based biosensors. Tissue-based sensors act similarly to enzyme-based sensors, as tissues with a high concentration of a specific enzyme are usually employed such as the mushroom tissue which has a large abundance of the enzyme tyrosinase. The specificity of tissue sensors may be lower, but they offer the advantage of a cheaper source and require no enzyme purification step as the tissue contains mainly the enzyme of interest. Cell-based biosensors are based on microorganisms, mainly yeast and bacteria. Cells can be physically immobilized by entrapment or adsorption, or in
some cases by size-selective dialysis membranes. Transducers transmit cellular responses from changes in cellular metabolism, a feature usually exploited in cell-based biosensor designs for the environmental screening of pollutants. Commercially available cell-based biosensor used for environmental screening are available, and are applied in the measurement of parameters such as biochemical oxygen demand (BOD), acute toxicity assessment in water bodies and endocrine disruptors.\textsuperscript{8,9}

1.1.2. Synthetic Analogues: Artificial sensors that mimic the properties of natural biosensors have also been studied and applied. These include aptamers, nucleic acid analogues, MIPs, monoclonal antibodies, and artificial ion channels. These biomimetic sensors can offer improved properties such as better stabilities at temperatures and pH values where natural biosensors may become denatured or inactive, and the flexibility of making several variants that cannot exist naturally.

Aptamers are single strand RNA (ssRNA), ssDNA and oligopeptides that demonstrate a high binding selectivity and affinity for their target. They have been extensively used to detect a broad range of targets in applications such as in the determination of DNA sequences, peptides and proteins, heavy metals, small organics and even whole organisms. They are usually engineered by a process called the Systemic Evolution of Ligand by Exponential Enrichment (SELEX), which is discussed in subsequent chapters. After the aptamer sequence (sometimes up to 80 bases long) is identified, the vital recognition sequence is cropped to improve functionality and facilitate further synthesis.\textsuperscript{10}

Nucleic acid analogues are polymeric materials that structurally mimic natural nucleic acids as they can recognize complementary strands and also store biological information. They are synthesized from modified nucleobases, and are very similar in terms of structure and function to aptamer based sensors. They may also show improved stabilities, binding, and functional
characteristics. Examples include locked nucleic acids (LNA), peptide nucleic acids (PNA), phosphoramidite morpholino oligomers (MORFs) and hexitol nucleic acids.\textsuperscript{11}

While polyclonal antibodies are directly extracted from the serum, monoclonal antibodies are biochemically synthesized, a feat first accomplished in a Nobel Prize effort by Milstein and Köhler.\textsuperscript{12} Monoclonal antibodies offer several advantages over polyclonal antibodies e.g. longer activities eliminates the need for continuous animal sacrifice, identical affinity sites allows specific binding to a single antigen site, and no interference or cross-reactivities from similar binding antibodies which is experienced in polyclonal antibodies.\textsuperscript{13}

Molecular Imprinted Polymers (MIPs) are synthetic cross-linked polymeric materials, with a three-dimensional structure having synthetically fabricated sensory sites. A template mimicking the required target, is used to induce this sensory site. A control called Non-Imprinted Polymer (NIP) is used to confirm the activity of MIPs, as NIPs are synthesized under similar conditions, but with the exemption of the template. A descriptive process for the synthesis of MIPs is shown in Fig. 1.2.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{mip_process.png}
\caption{Simplified overview of MIP preparation process. Hayden \textit{et al}, 2006}
\end{figure}

MIPs have been shown to be an attractive option to antibody-based biosensors. The synthesis of MIPs takes place in less polar aprotic organic solvents such as chloroform, to favour electrostatic interactions and hydrogen bonding between the monomers and templates. A modifier
such as acetonitrile is used to minimize template solubility issues. Templates are bound to the monomers covalently or non-covalently. Non-covalent binding is however more frequently employed, as removal of covalently bound templates, a process called washing, may involve additional chemical reactions. Also, the washing process in covalently bound templates sometimes removes only a fraction of the template depending on its size, and at these times, the MIP produced usually has low activity. Alternative methods to washing for covalently bound templates include electro-polarization or UV-assisted polymerization, and this produces the MIP directly to the surface of the transducer. These approaches have been used in the determination of target agents such as paracetamol, caffeine, and melamine. MIP-based biosensor have also been designed with protein templates (such as haemoglobin, bovine serum albumin (BSA), and lysosome) with virus templates, and various templates used for food and environmental-screening biosensors.

Artificial ion membranes are used in biosensor designs for measuring responses to the discovery of naturally existing ion channels in cell membranes, in chemically altered channels, and in reconstructed channels and membranes. Ion channels show selective ion permeability, and will preferentially allow only ions with a specific mass or charge to pass. Ion channels have a biological gated passage, and electric or chemical stimuli control the opening and closing of this gate. Analytical applications that uses chemically modified ion channels have been extensively reviewed. A chiral L-histidine biosensor using a polyethylene terephthalate nanochannel has been developed. The chiral selectivity was imposed by modification with a chiral cyclic oligosaccharide, β-cyclodextrin. Ion channel based biosensors have also been reviewed for their applications in drug screening, modern medical care, food safety, environmental monitoring, and biowarfare control.
1.2. Transducers

Transduction is the process where energy is converted from one form to another. In analytical chemistry however, transduction means the conversion of a detectable change, such as mass and concentration, into a suitable, recordable electronic signal usually in voltage. The recorded electronic signal is directly related to the change in the analyte species measured. There are cases when the species measured is not the analyte itself, but whose measurement is directly proportional to the analyte of interest, such as when detecting the activity of enzymes by the formation of products, or by the disappearance of the reactant.

Transduction can be achieved in several ways, and the choice of method largely depends on the peculiarity in the measurable properties of the analyte matrix. Biosensors have thus employed several transduction means. Optical transducers convert light to current, and then to voltage; electrochemical transducers convert chemical energy into current and then into voltage, or sometimes directly into voltage; while thermal transducers convert heat into current, and then into voltage.

1.2.1. Optical Transducers: Optical measurement makes use of light that is either absorbed or emitted in the UV and visible region of wavelengths between 10 nm to 780 nm. The visible and near UV regions are the useful areas, while the far UV is not generally analytically useful. The mostly used optical transduction methods in bioanalysis and biosensor designs include fluorescence, UV-Visible absorbance, turbidimetry, chemiluminescence and bioluminescence.

Fluorescence involves the excitation of electrons in a molecule, and the detection of the corresponding emission i.e. the loss of the imparted excitation energy by the release of a photon. Fluorescence spectroscopy uses a light source with very high intensities such as a xenon arc lamp.
There is an incident monochromator for the excitation of the analyte, and an emission monochromator for detection of emitted light. During excitation, the electrons in the molecule absorbs energy at their characteristic absorption wavelength, and moves from their ground energy levels to an excited state. However, by a series of radiationless deactivation, the excited electrons loses a photon and returns back to its ground state. The difference between the emission and excitation wavelengths is called the Stokes shift. A block analogy of a spectrofluorimeter design is shown in Fig. 1.3. The arrangement shows that the absorption and emission monochromator can be placed at right angles, and only the emission from the analyte of interest at its characteristic wavelength passes through. This provides for improved precision and interference elimination.

Quantum yield is a parameter used to measure the fluorescence of a species and it is the ratio of the number of molecules that emits a photon to the number of molecules of the same species that are in the excited state. The quantum yields value ranges between zero and one; and a value of one means all molecules in the excited state emit a photon, which is the highest order of fluorescence. Factors such as temperature, pH, dissolved oxygen content, and solvents can affect
fluorescence, and it has been shown that structurally rigid functional groups and aromatic molecules with extensively conjugated π-electron systems have high orders of fluorescence.\textsuperscript{21} Self-absorption is a fluorescence phenomenon which occurs when the relative concentration of fluorescent molecules is high, and as such, emitted photons are absorbed by nearby molecules who are still in the ground state. This leads to decreasing intensities of fluorescence as concentration increases. Another phenomenon related to fluorescence is called quenching, and this occurs when there is a nearby molecule whose absorbance wavelength corresponds to the emission wavelength of the analyte. Quenching is a fluorescence principle that has been applied to bioanalysis and extensively in the field of biosensors.

UV-Vis absorbance is closely related to fluorescence. While fluorescence measures photon emission, UV-Vis absorbance measures how much light is absorbed by a compound. A simple UV-Vis spectrophotometer, unlike the spectrofluorimeter, has only one monochromator which is used for scanning light to the appropriate wavelength required for the analyte of interest. The light source employed here is usually a tungsten filament lamp. The sample is placed in a quartz, polystyrene or glass cuvette, a material that is transparent to the wavelength of interest. The intensity of the incident light beam given by the symbol $P_0$ is first determined using a blank.

![Fig. 1.4: Block diagram showing basic component of a simple UV-Vis spectrophotometer](image)

In blank solutions, the total amount of light absorbed at a corresponding wavelength is assumed the initial value, as a blank contains all other components of the sample except the analyte. When the analyte is introduced, the absorbance intensity, $P$ is then detected. The transmittance, $T$, is given as the ratio $P/P_0$, and may vary between 0 and 1 when expressed as a simple ratio, or from
0 to 100 when expressed as a percentage. Like fluorescence, an absorbance of 1 is maximum, showing that the total amount of light passing through the analyte is absorbed. Absorbance varies linearly with the concentration of the analyte and it is represented by the Beer-Lambert equation

\[ A = \varepsilon bc \]

where \( A \) is the absorbance, \( b \) is distance in cm travelled by the incident light beam through the analyte, \( c \) is the concentration in M of the analyte, and \( \varepsilon \) is the molar absorptivity in M\(^{-1}\)cm\(^{-1}\). Absorbance thus has no unit. The wavelength of choice for analyte concentration can be determined from an absorbance spectra which is the plot of absorbance vs wavelength. The peak intensity in an absorbance spectra corresponds to the wavelength where the molar absorptivity of the analyte is at a maximum, and this property, plus the likelihood of interfering species, is used to determine the wavelength of choice for absorbance studies. Because analyte quantitation is usually done in aqueous solutions, and because the protonation state of molecules affects their energy levels, molar absorptivity is usually dependent on pH of the sample solution. It is also dependent on ionic strength of the solution, nature of the solvent, and temperature.

Surface plasmon resonance (SPR), is an optical detection tool that has been widely incorporated into the field of biosensors, and has recently found several uses such as in clinical studies for the detection of single nucleotide polymorphisms (SNPs).\(^{22,23}\) The SPR approach as relies on the impact of a bound sample material to a metal layer, mostly gold, as this binding changes the refractive index which in turn is expected to change the angle of reflection of an incoming polarized monochromatic light. As incident light falls on one side of the glass prism, it is refracted inwardly and reflected by the metal gold film, before travelling out through the other side of the glass prism where its intensity can be monitored with a charge-coupled device (CCD).
When the reflected light falls on the gold surface, it has the ability of exciting the electrons of the bound material, and continuous variation of the incident light angle reaches a critical angle point where a surface plasmon resonance is created. At this point, the intensity of reflected light is at a minimum. The angle where this occurs depends on the refractive index change imposed by the bound layer. Thus, association and dissociation studies are done using SPR, where one of the binding component is immobilized on the gold surface, and changes in plasmon resonance on association or dissociation of a second binding partner can be monitored. The detection limit of this change is in the order of microdegrees.

Due to constraints imposed by temperature and flow rates, another method employed for these studies uses a second chip with a complete flow cell to provide for reference, and difference in measurements between these reference and the sample is detected. The set-up for this approach is shown in Fig. 5. Current limitation of this approach include the nonspecific binding of species in solution as they tend to stick to the chip surface and not interact with the already surface-bound partner. Another limitation is that these chips are expensive. Future advances in the manufacturing
process is expected to reduce costs, and thus improve the practicability of this transduction method for widespread use in biosensors.26

1.2.2. Electrochemical Measurements: Many biological processes involve the transfer of electrons in which a species is oxidized and the other is reduced. These kind of reactions is the principle that electrochemical measurements are based on. Electrochemical means of transduction is in fact widespread in the field of biosensors. Biological redox reactions are part of natural aerobic processes, respiration in living organisms, which are mainly enzyme catalyzed and thus can be detected with electrochemical biosensors. In electrochemical cells however, electron transfer is made to occur with two electrodes, where oxidation and reduction occurs. Each electrode is placed in a different solution, where one of the solution is the analyze of interest, and each electrode is connected by a copper wire. Electrons flow from the reduced solution to the oxidized one, and a connecting salt bridge moves ions the other way round to compensate for the loss in electrons. The transduction process is the conversion of ionic current into an electronic current, and this occurs on the surface of the electrodes. The design of electrochemical cells have evolved, and very advanced methods have been developed allowing for the design of miniaturized electrochemical biosensors. An example is the disposable substrate biosensors, where the electrodes are screen printed unto a flat plastic, and used for quantifying blood glucose and lactate levels.27

Amperometry measurements involves applying a potential $E$, to control the redox reaction ratio occurring at the indicator electrode where the reaction of interest is occurring. When the redox ratio differs from that existing in solution, there is a current flow, and this current can be measured. Stirred or flowing solutions are generally employed to enhance mass transport, as reactions takes place only on electrode surfaces, so there is a need for analyze transport to the
surface of the indicator electrode. When the potential $E$ applied is sufficient enough, all analyte transported to the indicator electrode reacts, and under this condition, current generated and measured is directly proportional to the concentration of the analyte. The Clark oxygen electrode is an example of a widely-used amperometry-based analysis. The Clark electrode converts oxygen to hydrogen peroxide, and has been used to detect and quantify enzymes such as oxidases that consume molecular oxygen.$^{28}$

Potentiometric transducers measures the value of cell potential $E$, using a high-impedance voltmeter, while ensuring that the overall cell reaction do not occur to a significant extent. The combination pH electrode$^{29}$ is an example of a potentiometric measurement which uses two reference half-cells immersed by the means of a concentric tube into a single probe. A glass membrane separates one of the half-cells from the external solution and allows for a selective proton transfer scheme. There is a glass membrane which traps a weakly buffered solution that allows only dissolved gas from the analyte sample to pass through. This device has allowed for $(\text{CO}_2/\text{HCO}_3^-)$ and NH$_3$/NH$_4^+$ detection and quantification.

Impedimetry measurements is used to monitor the conductivity or ionic strength of a solution from a biochemical reaction, and it is used for weakly buffered solutions where ions are consumed or produced. A small sinusoidal voltage is applied to both electrodes in solution and prevents redox reactions from happening at the electrode surfaces. The migration of ions can hence be safely monitored allowing for a reliable measurement and quantification of the magnitude of ion current generated. Examples of impedimetry measurements involve the detection of human serum albumin (HSA) to determine liver function, and of foodborne pathogens.$^{30,31}$
1.3. Biosensor Configurations:

There is a vast array of biosensor configuration that has found commercial application. It is almost impossible in this short report to provide an extensive review of the several biosensor configuration types in use today. However some interesting reviews\textsuperscript{32-34} are available for further reading. There is advancing technology in the discovery of new sensory agents and transducer methods which has continued to spur the growth of biosensor analysis.

Electrochemical cells are extensively studied as transduction means in biosensor configuration and development. Electrodes used are relatively inert, such as carbon, platinum, and gold. Platinum and gold are useful in that they allow the spontaneous accumulation of self-assembled monolayers (SAM). SAM provide a simple and mild means for biomolecule immobilization during biosensor configuration. An example is in the use of a recordable compact disc (CD), because it contains a gold layer, to detect interleukin-6 (IL-6), through a gold-array biosensor configuration.\textsuperscript{35}

(a) Design of gold-array from commercial CDs

(b) Signal amplification strategy in a commercial gold CD electrode antibody-based biosensor

Fig. 1.6: Design of gold CD electrochemical transducer biosensor for Interleukin-6. Tang et al, 2012
The CD was first immersed in a solution of concentrated nitric acid to remove the outer layers and expose the gold surface. Layers of hot toners was then transferred to the gold surface, first to protect the gold, and later to define wells and insulate conducting tracks.\textsuperscript{36} This IL-6 biosensor employs a sandwich-type antibody recognition sensor, using covalently immobilized mercaptopropionic acid SAM and anti-human IL-6. A horseradish peroxidase (HRP) is used for signal amplification, and sample addition allows the detection reaction to occur. This approach is shown in Fig. 1.6.

Cellular sensors as discussed above are commonly applied in biosensor development for environmental studies and measuring conditions of cell viability. This is usually accomplished by exposing the living cells to the analyte, and changes in the cellular function and endpoints such as metabolism, reproduction, and mortality are observed. An example of this is a biochemical oxygen demand (BOD) sensor that uses a heterotrophic microbial species, which has been described for use in monitoring wastewater in a flow system.\textsuperscript{37}

BOD and toxicity biosensors, which both can employ a cellular sensory agent, are related in behaviour and design. BOD sensors uses immobilized microorganisms in a carbon-free ambience to minimize any background metabolic activity, so that any observed increase in metabolic activity is adjudged to be from the analyte. The reverse is the case for toxicity biosensors, as they allow a maximum in background metabolic activity, and the presence of a toxin in the analyte would reduce or quench the metabolism process. The BOD sensor reported by Liu \textit{et al}\textsuperscript{38} uses a Clark-type oxygen electrode, shows great reproducibility and stability, has a low BOD detection limit of 0.5 mg/L, and has the advantage of been able to discriminate between different mixtures and organic compounds. Oxygen-rich sources shows high signals due to basal microbial metabolism,
and lower signals with carbon-rich sources due to microbial oxygen consumption. The design of this biosensor and its use in a free-flow wastewater detection is shown in Fig. 1.7.

![BOD sensor diagram](image)

**Fig. 1.7: A BOD biosensor design and use in a free-flow wastewater detection. Lui et al, 2011**

A mechanical system of transduction called microcantilevers has been applied as biosensor for virus detection. Microcantilevers are mass sensitive transducers that can be cheaply produced using integrated circuits and electronics based on modified semiconductor device production. The bending amplitude and vibrational frequency responds to surface mass, and can also respond to changes in density, viscosity, and flow rate. The most common recognition or sensor used with microcantilevers involve optical methods. Microcantilever biosensors can be designed using a magnetic vibration system which can detect changes in vibration frequency through an optical means. The use of optical sensors helps to eliminate electrical interference that is associated with vibrational frequency. This design of microcantilever-based biosensor was employed by Timurdogan et al\textsuperscript{38} for the detection of hepatitis A and C virus in serum, as the hepatitis antibody was immobilized to the surface of the micrcantilever using a SAM based succinimidyl propionate
for covalent attachment. The biosensor proved to be very selective, and showed low detection limits of 0.1 ng/mL.

A DNA microarray biosensor has been applied in the analysis of genetic expressions in a species population, by evaluating the relative expression of different mRNA sequences. These DNA-array biosensors allowed for simultaneous analysis of several thousand genes. A fluorescent dye was used as label, and this is employed to detect the hybridization of an immobilized probe with its specific target sequence. For most DNA microarray based biosensor, there is a need for sample pretreatment, and very active gene samples will give bright fluorescent spots. Dimmer fluorescent spots occurs when there are few mRNA producing genes, and no fluorescence shows

(a) DNA microarray biosensor; green spot shows gene expressed in normal cells, red spot shows gene is expressed in cancer cells

(b) A 4,000 probe spotted DNA array enlarged inset shows spot details

Fig 1.8. Set up and typical result of a DNA microarray biosensor. Hu et al, 2014

a completely inactive gene, as no hybridization has taken place. In an application of this biosensor, two fluorescent dyes are used, each with a different color for a different gene, and this was used
to differentiate between tumor and normal cells. A schematic of this type of biosensor design is shown in Fig. 1.8 (a).

DNA microarray biosensors are widely used in cancer diagnosis, mostly employing a complex gene expression pattern for recognition and differentiation, as this gives a more standardized cancer diagnosis platform. DNA microarray biosensors have also been commercialized as DNA chips, having large detection capabilities with some having up to 4,000 or more spots that can use different DNA probes. The use of high resolution detection devices and the small spot size allows for correct spot identification. An example of result from a DNA chip biosensor is shown in Fig 1.8 (b).

Enzymes, antibodies, and nucleic acids are presently used in the design of multiplex-screening sensors, and their design employs mostly optical and electrochemical transduction means.

1.3.1. **Performance Evaluation of Biosensors**: There are some parameters that are used in evaluating biosensor performance depending on their intended use. Detection limits, sensitivities, recovery time, response time, relative stabilities, and elimination of interferences, are some of the usual parameters measured. The detection limit and sensitivity of a biosensor has been discussed earlier. The recovery of a biosensor is defined as the time taken for a biosensor to return to 95% of its baseline signal after analyte removal, while the response time of a biosensor is defined as the time taken for the biosensor to achieve 95% final detection value on the addition of an analyte. Biosensors with very low recovery and response times, especially those in the order of seconds have found commercial applications, and use in *in vivo* devices. The stability of a biosensor is
evaluated in two aspects: operational and storage stability. The operational stability is the time it takes to reach 50% signal reproducibility of a biosensor under conditions of repeated usage, while the storage stability involves the same evaluation but related to storage times, and both parameters are evaluated under various conditions of temperature, pH, and humidity. A broader discussion on evaluating the performance of biosensors is available for further reading.  

**1.3.2. In vivo Biosensors:** There are increasing studies on the use of biosensors for *in vivo* applications. Most of these studies are largely related to glucose monitoring, an example being the artificial pancreas. The artificial pancreas allows for effective glucose monitoring and regulation *in vivo* by employing a glucose biosensor attached to a switching mechanism, which allows automatic insulin delivery when low glucose levels are detected. This artificial pancreas has been experimentally tested in chimpanzees, dogs, and rats with good results. However, for realization in human applications and satisfactory clinical studies, the artificial pancreas must show exceptional stability and reproducibility in the human internal environment which can be antagonistic. It must also offer patient comfort, painless treatment options, and provide continuous analyte information. Two properties of *in vivo* biosensors necessary to achieve these conditions are: biocompatibility and inertness. A biocompatible material is defined as a “non-viable material used in a medical device, intended to interact with biological systems.” Biocompatibility is usually the ability of a material to be identified as an endogenous substance by the host body, and not generate any inflammatory response, or an immune rejection. On the other hand, inertness is the biosensors ability to not react with biological fluids or materials present in the host environment.

A significant medical challenge with *in vivo* biosensors and implantable devices is biofilm formation, a process which leads to inflammation or the signaling of an immune response. The
longest duration achieved in avoiding inflammatory responses for *in vivo* devices used a poly vinyl alcohol (PVA) hydrogel to create a long term anti-inflammatory drug release. This reduced any inflammatory effect for a period of one month. Another challenge with *in vivo* devices is in calibration, as sensitivities decrease over time, meaning that calibration curve slopes, and generated signals will also decrease.\(^\text{45}\) Despite these challenges, several implantable biosensors are been designed and applied for use such as in detecting *L*-glutamate, *D*-serine, pyruvate, lactate, choline, and acetylcholine.\(^\text{50-52}\)

### 1.4. Introduction to Aptasensors:

The use of aptamers in bioanalysis has greatly evolved, and they are now commonly used as recognition agents for many several bioanalysis. Aptamers have a molecular recognition scaffold which allows them to bind specifically to their desired targets (proteins, nucleic acids, and biomolecules) with excellent affinity and specificity. Aptamers are synthetic oligonucleotides sequences, and they are isolated from a library of about \(10^{15}\) different sequences in a process called SELEX. The SELEX process and an extensive discussion of the application of aptasensors in bioanalysis is covered in chapter 4. Just like other sensory agents, the specific binding of aptamers depends on their 3-dimensional folding and conformational plasticity when in contact with a binding partner. Aptamer sequences are usually between 40-100 nucleotides long, and thus can act as either receptors for small molecules, or ligands for macromolecules. The ability to modulate aptamer sequences makes them easily combined with other functional nucleic acids, signaling agents, and a variety of carriers. They can also be preferentially designed for enhanced specificity and selectivity to an application type. Because aptamers are highly sensitive, they can respond to small changes in their microenvironment, and these changes can be made to generate a signal when
a transducer is present. These properties have made aptasensors versatile recognition agents in a wide array of biosensor designs.

Aptasensors have also been used in a multi-array biosensor application, and they represent an intense field of scientific research. The vast majority of aptasensors rely on fluorescent labels for optical transduction because they employ very fast, simple, and sensitive designs which can easily be miniaturized. However, there is increasing efforts in aptasensor design with other signalling means such as electrochemical and physical transduction.\textsuperscript{53-55} Aptasensors have been reported for use in a multiplex biosensor analysis for the detection of different protein analytes.\textsuperscript{56,57} Yan \textit{et al}\textsuperscript{58} combined tiles of self-assembled DNA arrays hybridized with aptasensors. These aptasensors was labelled with a blue fluorescent dye which on hybridization with the DNA tile formed a broad array of colours, and enabled the DNA to be characterized.

Aptasensors, though largely regarded as superior recognition agents to other sensors, still has its own setbacks. One drawback of aptasensors is that many aptasensor-target pairs have not been extensively studied. The most studied aptamer is the anti-thrombin aptamer, HD1 also called TBA.\textsuperscript{59} The amount of studies done on this aptamer accounts for over 80\% of all aptasensor studies done so far.\textsuperscript{59-61} Therefore, there is need for more studies on other aptamer-target binding pairs. Despite this drawback, there is still a large body of evidence that predicts the future of aptasensors as indispensable in bioanalysis, research, and diagnostics.\textsuperscript{62,63} Tissue and cell based targets for aptamer binding is also expected to be explored significantly. The expected research challenges will therefore be in identifying and studying more selective aptamers-target bindings for applications in medicine, food safety, and environmental screening.
References


42. Ligler FS. *Analytical and bioanalytical chemistry.* 2003; 377 (3): 469.


Chapter Two: Biosensors for Medical Application

2.1. Transduction Methods Employed in Medical Biosensors

This section introduces the various transduction methods that has seen application in medical biosensor design. The several compatible signal receptor and transducer designs will also be discussed. Further sections will discuss the extensive applications of these biosensor design as it relates to medical analysis.

2.1.1. Electrochemical Biosensors: Since the advent of glucose biosensors, electrochemical biosensors has become very significant in the advancement of point-of-care medical diagnostic systems as they can afford very sensitive, easy to operate, real-time bioanalytical devices. There has been further improvement in the design of electrochemical biosensors, such as in biomolecule immobilization, nanomaterials innovation for enhanced signal transduction, and hybrid materials.\textsuperscript{1,2} Electrochemical biosensors in point-of-care medical devices offer significant design advantages such as low manufacture costs, simple designs, minimal power requirements, ease of miniaturization and a facile user interfaces.\textsuperscript{3} These features made electrochemical biosensors the first commercialized biosensor (the first commercial glucose biosensor used a Clark & Lyons-type oxygen electrode), and even modern biosensors employ the underlying design concepts of this first commercial biosensor. Modern electrochemical biosensors designs incorporate several sensory agents, and are extensively applied in food analysis, environmental monitoring, bio-warfare, health monitoring, and quality control.\textsuperscript{3-7} Electrochemical transduction is based on either voltammetry, impedimetry, potentiometry, amperometry or conductometry. Further discussion on these transduction techniques are provided in these texts.\textsuperscript{8,9} Electrochemical biosensors can be classified as label-based and label-free.
2.1.1.1. **Label-Based Electrochemical Biosensors:** Three types of biological labels can be used in electrochemical biosensor designs. These include oligonucleotides, enzymes, and nanomaterial sensors.

2.1.1.1a. **Enzyme-Labeled Electrochemical Biosensors:** Enzyme-based electrochemical biosensors are used extensively in health analysis, environmental detection, food safety and quality assurance.\(^{10-12}\) This kind of biosensor however suffer from slow rates of electron transfer, which applies to most electrochemical biosensors that incorporate biomolecules.\(^{13}\) One way this challenge has been attenuated is by using conductive wires or electroactive mediators to shuttle electrons between the electrode surface and the enzymes redox sites.\(^{14}\)

Redox enzymes are also used extensively for electrochemical signal amplification, and they are useful in detecting biomolecule binding interactions such as in DNA and antibodies. A secondary enzymatic redox reaction usually generates the signal in an electrochemical sensor for DNA detection. One way of achieving this involves the hybridization of the target DNA to one end of immobilized probe, while a second labeled probe is hybridized to the other end, and a signal is recorded when the second labelled probe binds the DNA target. Campbell et al\(^ {15}\) applied this approach for the determination of hydrogen peroxidase, using horseradish peroxidase (HRP).

![Fig. 2.1: Classic scheme for DNA detection using electroactive labels or redox enzymes](image)

*Campbell et al, 2002*
The use of labels mostly require that they are stable over a wide pH range and have easy facile surface modification which can be achieved by covalent immobilization of their functional residues.\textsuperscript{16}

\textbf{2.1.1.1b. DNA-Labeled Electrochemical Biosensors:} These have been applied to study DNA interactions as alternatives to conventional approaches such as Raman spectroscopy, x-ray crystallography, or even fluorescence studies. The challenge with these methods is the requirement of large samples and their low sensitivities, to which electrochemical biosensors provides significant advantage. Though direct detection of the redox activity of guanine and adenine is possible by electrochemical means,\textsuperscript{17} most common electrochemical biosensors used for DNA detection use labels, the simplest being electroactive intercalators.\textsuperscript{16} These intercalators are mostly planar aromatic structures, and interact with double strand and single strand DNA differently such that there is a detectable change in its redox properties on hybridization. Examples of DNA intercalators that have been used include cobalt bipyridine, anthraquinone derivatives, and methylene blue.\textsuperscript{18,19}

\textbf{2.1.1.1c. Nanomaterial-Labeled Electrochemical Biosensors:} Nanomaterials such as metal nanoparticles, silica nanoparticles, and semiconductor nanoparticles are used as labels in electrochemical biosensors as they can provide comparable signal amplifications to enzymes, metal ion, and redox probe labels. Metal nanoparticles is well studied being the easiest to prepare, and it also has beneficial chemical properties. Gold nanoparticles (GNP) is the most used metal nanoparticles because it has a high surface energy, large surface area, and can adsorb biomolecules strongly. Dequaire \textit{et al}\textsuperscript{20} reported that GNP electrochemical biosensor have a very high sensitivity for goat IgG. GNP was also shown to be able to detect DNA hybridization events.\textsuperscript{21} Quantum dots
which are semiconductor nanoparticles are also used in biosensors and has been shown by Kerman et al.\textsuperscript{22} to correctly identify bases involved in single nucleotide polymorphisms (SNPs).

2.1.1.2. **Label-Free Electrochemical Biosensors:** Although most analytical techniques involve various labeling forms such like enzymes, fluorescent or radioactive labels, label-free approaches are also widely used as they offer the advantage of a fast analysis time, simple analyte preparation, and broader range of targets.

2.1.1.2a. **Label-Free Enzyme Biosensors:** Label-free electrochemical biosensors based on enzymatic detection usually involve active protein binding to the electrode surface, for which several electrochemical transduction methods can be applied. In these cases, either the substrate or the product formed is electroactive, and their redox process at the electrode surface generates a current signal which is detected and is proportional to the initial concentration of analyte. Ignatov et al.\textsuperscript{23} used this technique for detecting and quantifying antioxidant activity using a cytochrome C modified gold electrode. More recently, Duonin et al.\textsuperscript{24} showed that a solution based enzyme assay, rather than immobilization to electrode surfaces, is possible, and they used this scheme to quantify acetylcholinesterase (AChE) from the oxidation reaction of the product, thiocholine, using gold electrode. Other approaches to a label-free enzyme based electrochemical biosensor have also been achieved.\textsuperscript{25}

2.1.1.2a. **Label-Free DNA Biosensors:** Guanine (G) and adenine (A) are the most electroactive DNA bases, and they have oxidation signals of 1.0V and 1.3V respectively as measured on carbon electrodes. These signals are vital in hybridization studies where intensities decrease on annealing to complimentary bases.\textsuperscript{26} Label free DNA biosensors based on electrochemical transduction allow mass production of miniaturized devices.
Palaček et al.,\textsuperscript{27} using a dropping mercury electrode (DME), was the first to discover that DNA bases can be electroactive at electrode surfaces, making them suitable for label free electrochemical biosensors. These biosensors are also useful for detecting DNA damage and mutation studies. Ravera \textit{et al.}\textsuperscript{28} has reported the detection of drug-DNA interactions through electrochemical means by monitoring redox activities of G and A on carbon electrodes. Wang \textit{et al.}\textsuperscript{29} used peptide nucleic acid (PNA) due to its strong affinity for oligonucleotides to detect DNA point mutations using an electrochemical based biosensor. Kara \textit{et al.}\textsuperscript{29} studied induced DNA damage with electrochemical detection by indirect and direct irradiation with iodine-31 and technetium-99m. Roy \textit{et al.}\textsuperscript{30} detected DNA interactions using a label-free DNA sensor based on a nanogap. The design employs a DNA and its probe immobilized on opposite sides of the nanogap, and their hybridization creates a bridge across the gap. This bridging gave rise to conductance changes in the nanomaterial allowing a signal to be generated.

\textbf{2.1.1.2a. Label-Free Nanomaterial Electrochemical Biosensors:} Since their discovery by Iijima in 1991, carbon nanotubes (CNT) are of increasing interests due to their excellent electronic, optical, chemical, and mechanical characteristics.\textsuperscript{31} CNT can promote the electrochemical reactivity and electron transfer redox reactions of proteins and biomolecules. CNT modified surfaces also accumulate nucleic acids without any surface fouling effect. Electroactive amino acids such as tyrosine, cysteine, and tryptophan have been detected by Tang \textit{et al.}\textsuperscript{32} using CNT fibers on carbon electrode surface. Zhang \textit{et al.}\textsuperscript{33} however used a layer-by-layer nanomaterials made up of CNT and GNPs as DNA sensors using a thiolated DNA probe. Hung \textit{et al.}\textsuperscript{34} achieved improved sensitivity of Zhang’s biosensor, achieving over 1000-fold enhanced sensitivity due to additional GNPs electrodeposited on the CNT.
2.1.2. **Piezoelectric Biosensors for Medical Applications:** Piezoelectric transduction is based on changes in resonance frequency and molecular mass when analytes are adsorbed or desorbed from a piezoelectric crystal surface. These changes are brought about by the crystal vibration which is induced by an electric field. Several similar transduction techniques exist, but the general term used is piezoelectric transduction. Some of these similar techniques include SAW/BAW – surface/bulk acoustic wave, QCM – quartz crystal microbalance, PQC – piezoelectric quartz crystal, and TSM – thickness shear mode. Though there are variations between these transduction means, they all exploit the same piezoelectric properties of crystals, and will be termed generally as piezoelectric biosensors with specificity provided when necessary. The first study to detect the piezoelectric properties of crystal was done in 1880 by Jacques and Pierre Curie who discovered the generation of an electric potential across crystals when subjected to mechanical stress, and observed that the potential generated was proportional to the magnitude of stress applied. The term piezoelectric was coined from a Greek word, *piezen*, which means to press, or cause a stress. Piezoelectric biosensors have the advantage of providing label-free, real-time transduction, and useful for detecting a wide scope of biomolecular interactions.

2.1.2.1. **Piezoelectric Immunosensors:** Aside from piezoelectric transducers advantage of offering label-free biosensors design, another well studied feature in the development of piezoelectric biosensors is in their antibody immobilization protocols, and this knowledge has been transferred to other systems of biosensor development. Caneva et al.\(^3\) designed a piezoelectric immunosensor for the detection of metalloproteinase-1 (MMP-1) matrixes by immobilizing MMP-1 specific monoclonal antibody to a quartz crystal surface. Their approach used a layer by layer self-assembly technique, and the surface roughness before and after antibody deposition was determined by atomic microscopy, which was used to characterize the MMP-1 target. Several
Table 2.1: Piezoelectric sensors used in real sample analysis

2.1.2.2. Piezoelectric Nucleic Acid Biosensors: Nucleic acid biosensors, also called genosensors, refers to analytical devices that incorporates either an oligonucleotide with a known base sequence, or a more complex nucleic acid structure, incorporated with an appropriate signal transducer. These kind of biosensors are now useful in medicine for determining genetic features of patients such as genotypes and SNPs. Nucleic acid-based biosensors used in medical field employs several
transducer methods including label-free piezoelectric transducer. Some reviews are available on design and important aspects of this transduction step.\textsuperscript{47}

Although several means are available for clinical genotype and SNP tests such as fluorescence in situ hybridization (FISH), real-time polymerase chain reaction (PCR), chromosome analysis, and flow cytometry, they all have some limitations such as high expense and poor precision. Piezoelectric genosensors is a viable alternative as it offers real-time, label-free, low costs devices. The first piezoelectric nucleic acid biosensor for DNA was reported in 1988,\textsuperscript{48} and since then several other types have been designed for use in clinical testing of different targets such as Staphylococcus epidermidis, Escherichia coli, hepatitis B virus, human cytomegalovirus, and human papilloma virus (HPV).\textsuperscript{49-52}

The amplification step required for DNA analysis is usually done by PCR, but this presents some drawbacks in clinical analysis such as longer waiting times for results and high costs of analysis. Though studies are few in this area, some works have however been reported to analyze genomic DNA directly using piezoelectric genosensors. A piezoelectric genosensor for detecting Mycobacterium tuberculosis (MTB) in genomic DNA has been reported,\textsuperscript{53} and this was applied in the clinical analysis of over 200 sputum samples.

For clinical genomic DNA detection, two issues are very important to the design of an applicable biosensor – sensitivity and selectivity. Several signal amplification protocol has been applied to piezoelectric genosensors for enhanced selectivities such as the use of enzymes, metal nanoparticles and semiconductor nanoparticle\textsuperscript{54,55} The use of nanoparticles involves sandwich-type assays where a signaling probe bearing the amplification molecule and another immobilized probe are hybridized to the target. An example of this sandwich biosensor was used by Ye \textit{et al}\textsuperscript{55} to detect single point mutations. The disadvantage of this sandwich-type piezoelectric genosensor
is that each DNA target must bind to two probes, one immobilized, and the other a signaling probe, and this limits the flexibility in genosensor design, which also increases analysis costs.

Fei et al\textsuperscript{56} used hairpin probes to afford a high selectivity and sensitivity in the detection of DNA target hybridization using a piezoelectric genosensor, and they used GNPs for signal amplification. Hairpin DNA probes combines a single stranded loop which has the recognition sequence, and a double stranded stem, and their functionality largely depends on the length of the stem, and the amplitude of the loop. Thus when using hairpin DNA probes, their design will be crucial to their functionality, sensitivity and selectivity. The recognition probe as used by Fei and colleagues was immobilized to a QCM, and would dissociate in the absence of the DNA target when exposed to endonuclease. This dissociation would not allow for any signal detection.

However, hybridization of the probe in the presence of the DNA target opens the hairpin and dissociates the cleavable double strand region. Treatment with endonuclease in this case does
not affect signal detection, and continual introduction of GNPs with the immobilized hairpin probe results in many-folds signal increase. This approach was used in the detection of DNA base mismatches.

2.1.2.3. Piezoelectric Aptamer Biosensors: Clinical based diagnosis relies more on antibody-based biosensors whose production may be difficult and expensive, and may also involve the use of animals. Attention is rapidly shifting towards affinity molecules generated by evolutionary molecular biology methods, of which the major category applied to clinical biosensors are nucleic acid aptamers. Aptamers are ssDNA or ssRNA oligonucleotides, which have high binding and specificity for molecular targets and may range from small organic molecules to macromolecules such as proteins. Nucleic acid aptamers have been explored for therapeutic effects, such as the Eyetech/Pfizers Mucagen\textsuperscript{TM}, approved by the FDA in 2004 for the treatment of diabetic macula oedema and other age related macular degeneration.\textsuperscript{57} Other therapeutic applications of aptamers include cell targeting such as in flow cytometry, cancer cell detection, biomarker discoveries, and the identification of molecular variations among patients.\textsuperscript{58-60}

Aptamers are also been explored for use in medical biosensors, and piezoelectric aptamer biosensors for medical applications has been developed. Most of these piezoelectric biosensors are based on the detection of immunoglobulin E (IgE) target.\textsuperscript{61,62} This is because aptamers for IgE and their interactions are well studied and understood, as well as the high molecular weight of IgE makes it suitable for the detection of changes in mass which is useful in a piezoelectric transducer. Recent piezoelectric aptamer biosensors with transducer means such as TSM and QCM. have afforded very low detection limits, good correlation and recovery in various serum samples.\textsuperscript{61} Piezoelectric aptamer biosensors for thrombin detection is also well studied, and several studies have been reported with similar results to the IgE biosensor.\textsuperscript{63,64}
2.1.3. Nano-biosensors for Medical Application: The performance of biosensors has greatly been improved by the electrochemical, electrical and chemical properties of nanomaterials. The main nanomaterials that have been used are metal nanoparticles, graphene, carbon nanotubes, and GNPs. Electrochemical biosensors is the main application of nano-biosensors, and this designing has broadened the sensing abilities, selectivities and detection limits of electrochemical biosensors. Nano-biosensors can detect and interact with minute compounds and biomolecules. These advantages have made nanomaterials useful in biosensor design for disease diagnosing and physiological target recognition. The two main transduction means involved in nanomaterial-based biosensors involve optical and electrochemical measurements.

Optical nano-biosensors usually have their optical fibers coated with specific fluorescent dyes, and these dyes will fluoresce with intensities proportional to analyte concentration. Two methods have been achieved for this fluorescence signaling: fluorescence intensity sensing and fluorescent lifetime sensing. Fluorescent intensity sensing relates analyte concentration to intensity of fluorescence, while in fluorescent lifetime sensing, analyte concentration corresponds to the phase of fluoresced light. Nanomaterials have been shown to enhance the recognition and transduction potential of optical biosensors. For example, a titanium dioxide nanoparticles was used by Chatni et al.\textsuperscript{65} to optimize signal output in optical dyes which will improve detection ranges and intensities when applied to biosensors, while GNPs fluorophore has also been shown to enhance the output fluorescent signals of biosensors.\textsuperscript{66,67}

Surface Enhanced Raman spectroscopy (SERS) is another method incorporated in nano-biosensors to enhance optics-based sensing. SERS is a surface sensitive technique based on the frequency changes of excited and scattered light as influenced by the structure and composition of a molecule when attached to a metal surface or nanomaterial. Metallic nanoparticles thus enhance
Raman signals by particle surface plasmon excitation, which is useful in the detection of biomolecules. Applications of this SERS protocol includes the detection of glucose concentrations and various virus identities using a silver nanoparticle.\textsuperscript{68,69}

Electrochemical measurements in nano-biosensors use voltammetry or amperometry based detections. Voltammetry biosensors that are nano-inspired have been extensively studied for use in clinical diagnostics.\textsuperscript{70} Most amperometry nano-biosensor use an enzyme as recognition agent to convert the analyte e.g. glucose or ethanol (which are mostly electrochemically inert) into an electroactive molecules e.g. H$_2$O$_2$ or NADH, whose formation can be detected and is proportional to the concentration of analyte converted.\textsuperscript{71} Aside from glucose and ethanol, amperometric nano-biosensors have also been used to characterize other relevant clinical target substrate such as glutamate, lactate, dopamine, and ATP.\textsuperscript{72-74}

The nanomaterial used for most biosensor design is usually from carbon e.g. carbon nanotubes, noble metals, or a combination of both. CNTs are classified based on the number of present cylindrical carbon rings (or walls) which are usually separated at a distance of 0.34 nm. CNTs are broadly categorized as single walled carbon nanotubes (SWCNT), double walled carbon nanotubes (DWCNT) or multi walled carbon nanotubes (MWCNT). SWCNT have diameters less than 3 nm, and provide an electrical interface suited for biosensor designs. Non-covalent attachment of nanoparticles to electrode surfaces uses SWCNT suspension solutions for the preparation of the electrode surfaces prior to deposition. To create this SWCNT solution suspension, functionalization of the SWCNT with hydrophilic functional groups, chemical modifiers and functionalized polymers is necessary to enhance solubility.\textsuperscript{75} Non-covalent attachments of SWCNT to electrode surfaces has also been achieved by spinning or casting the SWCNT solutions unto the surface of the biosensor. These method proves very useful for detecting
and quantifying H₂O₂, NADH and dopamine. Other approaches that have been used to achieve SWCNT incorporation into biosensors involve direct covalent attachment, electrodeposition, and chemical vapor deposition (CVD) growth of the nanomaterial, and each method has been applied to biosensor designs for health and clinical diagnosis.

MWCNTs attachment to electrode surfaces, like SWCNTs, involves similar attachment protocol involving covalent and non-covalent attachments. Non-covalent attachments include methods such as paste-electrode techniques, polymer entrapment, abrasive immobilization, and chemical vapor deposition. The paste-electrode approach is usually achieved by suspending MWCNTs in binders such as Teflon, epoxy, or in direct packing methods. Abrasive immobilization is done by rubbing the carbon electrode with the MWCNT on a filter paper. This method has shown high sensitivity for some biomolecule target such as glucose, dopamine, serotonin, and H₂O₂. Polymer encapsulation approach affords the encapsulation of the MWCNT on the electrode surface using materials like silicate sol-gels, chitosan, and dimethylformamide. The advantage of this method sometimes involve the repelling of electrochemical interferents such as uric or ascorbic acid which are normally found in bodily fluids.

For covalent means of attachment, cross-linking agents such as MPS (3-mercaptopropyl trimethoxysilane) can be used for the immobilization of MWCNT to electrode surfaces. This forms a S-Au bond with gold electrodes, and shows good reproducibility due to the firm anchorage of the nanotube to the electrode. Similar to SWCNT, MWCNT can also be covalently immobilized by CVD growth on the electrode surface. This can offer an advantage of adequate control of carbon nanotube placements, which might be important in in situ biosensor designs. Advantages of the
different incorporation techniques used for CNTs in medical biosensor design can be found in the following reviews.⁷²,⁸³

Metal nanoparticles (MNPs) are frequently used in biosensor designs as they have great catalytic and conducting properties. MNPs can assist in electroactive species catalysis, biomolecule labeling, biomolecule adsorption, electron transfer, and can also show enhanced SPR suited for SERS-based optical biosensors as discussed earlier.⁸⁴,⁸⁵ GNPs are the mostly used MNP for medical biosensor design as they afford excellent biocompatibility and catalytic characteristics.⁸⁶

Methods of preparing MNPs involve liquid phase dispersion, and electrodeposition. Electroactive metals like silver, palladium, and platinum have also been used as MNP in medical biosensor designs. These MNPs have been applied clinically for the detection and determination of choline, acetylcholine, hydrogen evolution reactions, and amino acids.⁸⁶

Other nanomaterial types that have been employed in medical biosensor design include graphene, carbon-metal hybrids, and graphene-metal hybrids. Hybrids nanomaterials show improved properties especially when incorporated in electrochemical nano-biosensors. They offer
lower detection limits, higher sensitivities, shorter response times, and the combined ability of both nanomaterials to catalyze redox reactions of electroactive intermediates such as NADH and H$_{2}$O$_{2}$. They are prepared in a similar processes as described for SWCNT, MWCNT and GNPs.

2.1.4. Enzyme-Based Biosensors in Medical Applications: Enzyme-based medical biosensors offer advantages such as an unsurpassed selectivity, rapid turnover rates, and very low detection limits. The most successful medical biosensor used today relies on an enzyme, glucose oxidase (GOx) which is a highly selective receptor for only glucose, and it is employed in home care biosensor devices used in monitoring blood glucose levels in diabetic patients. The use of enzymes in biosensors however has its own drawbacks such as the unsatisfactory enzyme purities obtained due to isolation costs or difficulty, activity loss from enzyme immobilization, enzyme fouling from chemical analyte species or substrates, and interferences from biological samples (e.g. saliva, blood, and electrochemically active solutes) present in biological matrices.

Enzyme based biosensors employ mostly electrochemical transducer means. Several amperometric and potentiometric transducers have thus been applied in enzyme-based medical biosensor designs. Amperometric biosensors monitors the generation or consumption of an electroactive specie when the enzyme and analyte interacts, e.g. the monitoring of the depletion/consumption of O$_{2}$ or H$_{2}$O$_{2}$ in a GOx-based glucose biosensor as shown by the equation below.

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{Glucuronolactone} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2e^- + 650 \text{ mV vs Ag/AgCl}
\]

Some initial challenges with the glucose biosensor design was the interference from electroactive species whose oxidization potential was around +650 mV, and may sometimes lead
to errors in results. This interference was prevented by applying a permeable selective coating to
the sensor, as this minimizes concentration of interfering species at the electrodes. Some protective
coating materials employed included cellulose acetate, Nafion, and diaminobenzene, and these
offered biocompatibility and the selective exclusion of interfering species.\textsuperscript{88,89} These designs were
regarded as first generation sensors and were also used for multi-analysis, but could not be
integrated into home use biosensors.

Second generation glucose biosensors used a mediator which acts an electron transfer agent
between the enzyme and the electrode. The electron mediators should be stable and readily interact
with enzymes in both its oxidized and reduced forms. It should also eliminate ambient oxygen
competition, be non-toxic to internal surfaces, and have as low oxidation potential as possible.
Lower oxidation potentials offer an advantage of further interference elimination from
electroactive species with higher oxidation potentials. The first home glucose sensor kit,
Exatech\textregistered, is based on this chemistry.\textsuperscript{90} Higher sensitivities and lower interference has been
obtained with newer devices such as Pelikan\textregistered, which require only \( \mu \text{L} \) of blood samples.\textsuperscript{91}

Third generation glucose biosensors have also been developed. Their designs eliminated
the need for a mediator, and direct enzyme immobilization to the electrode surface is achieved
using a modified polymer. Polyvinyl pyridine/imidazole modified with osmium electron transfer
relays has been used to immobilize GOx to electrode surfaces. Other chemical and physical
processes have also been used for enzyme immobilization and these techniques have been
reviewed by Davis and Higson.\textsuperscript{92}

Another alternate approach to enzyme biosensor designs are the enzyme inhibition
biosensors which has been useful for the environmental determination of pesticides such as DDT
and organophosphates. These pesticides at low concentrations can inhibit enzyme activity e.g.
acetylcholinesterase (AChE). The lowered enzyme activity reduces the redox reactions at the electrodes and drops the signal recorded. The decrease in signal is proportional to the presence and concentration of the target pesticide.

There are several challenges associated with enzyme-based biosensors. The most notable of these challenges is enzyme stability. GOx is the most stable enzyme used in medical biosensors and its activity can last for several months under proper storage conditions. Many other enzymes have lower stabilities, some less than a day, and this leads to test-to-test variations, especially in point of care and personal home diagnostics. This challenge is often attenuated by standardization on a regular basis with a known standard. As discussed earlier, interference from electroactive species can also affect the sensitivity of enzyme based biosensors. Interference can be minimized by the use of permeable selective membranes, mediators, pre-measuring the interference signal to determine its contribution, or spiking the solution with known amounts of the suspected interferent to determine if it changes result outcomes.

Enzyme leaching represents another challenge, and this leads to sensitivity decreases when the same electrode is repeatedly used for a number of tests. A disposable biosensors does not have this limitation, but for continuous use enzyme biosensors, a strong immobilization protocol such as cross-linking may be employed. Polymers such as polyaniline are often used for enzyme immobilization and may lead to wrong polymer deposition. This results in an irreversible electrode insulation leading to lower recordable signals and may be avoided by polymer deposition at low pH values after consideration of the effect of this on enzyme denaturing. Enzyme denaturing by some analytes such as toxic heavy metals may lead to loss of enzyme activity. For enzyme inhibition based biosensor for pesticide detection as described above, this becomes very troublesome, such as when the total sample toxicity is to be determined. A useful way of
eliminating this challenge is by purification of the sample and removing known enzyme pollutants, such as in the solid phase extraction for heavy metals.

A similar challenge is the biofouling of enzymes from biological samples such as blood, leading to a slow downward slide in response signal. Sometimes, this downward creep continues till it reaches a plateau, where signal may now stabilize. At this time, the electrode may be appropriately preconditioned such that the biosensor can be exposed to the sample for a defined period where the plateau region is expected to be reached, before sample measurements are recorded. A second way to avoid biofouling is to use electrodes once, and take measurements as quickly as possible. A third option is to improve surface biocompatibility by sensor modification with appropriate polymeric materials e.g. Nafion, and polydiaminobenzene. The broad pH ranges in biological samples limits enzyme biosensors for medical use, as they can be easily denatured by changes in pH. pH variations can be especially troublesome when potentiometric electrodes are employed. Though solution buffering may be employed, buffer ions tend to swamp electrode surfaces thus giving rise to unwanted interference. A better option is to use two electrodes, and one will be a pH electrode, such that the two readings can be recorded and subtracted to cancel out pH fluctuations.

2.2. Use and Applications of Medical Biosensors

2.2.1. Detection and Characterization of DNA and RNA: Biosensors for DNA and RNA determination is primarily based on nucleic acids sequence specific hybridization as a recognition course. Usually, the recognition probe, which is a complimentary nucleic acid sequence to that of the DNA or RNA of interest, is immobilized to a solid surface where hybridization occurs when
the sample matrix containing the analyte is introduced. Signal transduction is achieved by either optical, electrochemical, or mass-sensitive approaches. Biosensors for DNA is more largely explored, as DNA is more stable than RNA. Both biosensor system still suffer from lack of adequate simple portable device design that can incorporate the sensing protocol. Fluorescent labels are usually used, and early studies employed enzymes or radioisotopic labels. Immobilization protocols of DNA to various surfaces have been explored. The strong interaction of gold-thiol has been exploited to attach modified strands of nucleic acids, but the challenge encountered is that the DNA tends to be immobilized lying flatly thus inhibiting sequence availability for hybridization. Alkane thiol dilution was introduced to solve this limitation, as only the thiolated DNA ends attach to the gold surface, which allowed for better accessibility and hybridization. Cationic surfaces such as polyethylenimine solution pretreated glass slides and aminosilyl modified glass surfaces have also been explored for DNA immobilization through electrostatic interaction.

2.2.1.1. Optical-Based DNA Biosensors: This employs optical signal transduction in the form of photons i.e. light energy, and can offer the advantage of reducing interference from electroactive species as observed in electrochemical biosensors. The use of fiber optic cable or glass chips have helped miniaturize the design of optical DNA biosensors. DNA microarray is an example of an optical DNA biosensor. The design of a DNA microarray biosensor involves the immobilization of several different oligonucleotide spots, and the addition of labeled complimentary oligonucleotides results in hybridization that can be visualized by fluorescence microscopy. This allows for a rapid, multiple sequence detection of DNA. Some of the medical applications of a DNA microarray biosensor is described. These applications involve medical comparisons such as genotype of different or related species, pathogenic and non-pathogenic cells, healthy cells and
cancer cells. These biosensors offers the promise of human genome re-sequencing at very affordable cost and a high throughput rate. SPR biosensors usually combines electrochemical and optical signal transduction approach to afford real time, label free detection of DNA hybridization. The principles of SPR has been introduced in chapter one, and a detailed reviewed has also been done by Frances Ligler.

An improvement in SPR biosensors was achieved by Scarano et al. by depositing different arrays of sensing species to the SPR chip, and obtained a surface imaging of the SPR chip rather than obtaining just a simple SPR curve. This introduces versatility as various oligonucleotide sequence binding can be spotted and separately visualized in a single procedure. A microarray SPR chip has also been achieved by Manera et al. using avidin-biotin attachment of oligonucleotides, and this array has been used to detect ssDNA of mycotoxin producing fungi that are present and capable of proliferating in cereals. The SPR imaging system was shown to be a good choice for real-time, multi-array determination of DNA hybridization because of the high characterization sensitivities afforded by the ultra-thin metal films which were adsorbed to a gold surface. An SPR imaging biosensor that attaches L-DNA to SPR chips was used by Hayashi et al. for distinguishing three genotypes of the MDR1 gene which differs only by a single nucleotide. Their technology involved the fusion of two PCR techniques, one allele-specific, and the other L-DNA tagged. The PCR products was thus labelled with L-DNA and hybridized with a complementary sequence L-DNA immobilized to SPR gold surfaces. The hybridization was hence detected using SPR imaging. Other studies have used various optical PCR approaches to detect point mutations, and single nucleotide polymorphisms (SNP). A major disadvantage of SPR biosensing techniques is its relative insensitivities especially for the detection of small moieties like RNA (18-24 bases long).
Methods have been developed to enhance selectivity depending on the substrate of interest, such as the attachment of a polymer chain to the RNA following hybridization, where the polymer chain is capable of binding to GNPs.\textsuperscript{105,106} The evolution of fiber optics has developed optical sensing techniques for remote sensing. The integration of fiber optics into oligonucleotide array biosensors has been explored, and allowed for monitoring individual location transmission on sensor chips, and for the determination of a large array of individual analyte from a complex biological matrix.

2.2.1.2. \textbf{Electrochemical-Based DNA Biosensors}: The advantages of an electrochemical biosensor has been described and this includes low costs and simplicity, use of inexpensive print-screen electrodes means disposable devices eliminating challenges of sensor cleaning, contamination, and regeneration, and easy miniaturization. Electrochemical DNA biosensors require the immobilization of a ssDNA (recognition DNA) that will hybridize at the immobilized electrode surface producing a detectable change. Electrochemical DNA biosensors usually involve a label, or label-free sensing protocol.

In a label-based biosensor, a substrate such as an enzyme that can generate an electroactive specie such as $\text{H}_2\text{O}_2$ is labeled to the probe DNA. A simple labeling approach involves labels that will either bind to ssDNA or dsDNA, e.g. $\text{Co(phen)}_3^{3+}$ binds strongly to dsDNA and not to ssDNA, which makes it able to detect hybridization at the electrode surface. This label, along with ferrocene based polythiophene, has been used in this simple labeling protocol for the detection of DNA related to cystic fibrosis, and \textit{Mycobacterium tuberculosis}.\textsuperscript{107,108} Other labels that have been used in this approach include methylene blue and Hoechst which were able to detect single base mismatches.\textsuperscript{109} Another approach that has been used is attaching the label to the sample DNA. In this case however, the sample modification may lead to contamination or sample damage. Another
approach that improves sensitivity and does not modify sample uses an immobilized DNA probe with partial complimentary sequence to the DNA sample. On sample introduction, there is partial hybridization at the electrode surface, and a second labeled DNA with complimentary sequence to the sample DNA is introduced giving rise to a second hybridization. Common labels used are enzymes such as glucose oxidase, and horseradish peroxidase.\textsuperscript{110}

Nanomaterials based labels have also been proposed for DNA detection and they can enhance the generated electrochemical signal. In this approach the probe DNA is immobilized to a magnetic bead coated with streptavidin, the sample DNA was then introduced and this hybridized to the probe DNA, the beads were removed and GNPs was attached to the hybridized sample DNA using biotin-avidin chemistry.\textsuperscript{111} The labeled DNA was separated and detected by the gold in an electrochemical striping detection approach, and this enhanced the selectivity and sensitivity of the biosensor. Another method is the use of magnetic nanoparticles substrate for the probe DNA to bind PCR products. The capture and separation of the product, and then modification with an enzyme affords electrochemical detection at very low levels. This approach was used by Lermo \textit{et al}\textsuperscript{11} to detect single base mismatches and Salmonella pathogens.
Label-free approaches have the advantage of simplicity in design, and are more amenable to application in real-time modes. The first electrochemical DNA biosensor using label-free sensor would be Palaceks’ dropping mercury electrode used for oligonucleotide detection based on the oxidation of guanine. Subsequently, adsorptive transfer stripping was used to detect DNA, and this could differentiate between ssDNA and dsDNA and also afforded lower detection limits. The limitation of this approach is the broad adsorptive spectrum which was mitigated by allowing the hybridization process occur on a magnetic bead, separation and DNA removal allows the monitoring of guanine oxidation on mercury.

Conductive polymers are also used in label-free electrochemical biosensors due to their electronic conductance ability between electrode surfaces and biological moieties such as nucleic acids, antibodies and enzymes. AC impedance on composite films of ssDNA can also be used to detect hybridization. This allows system frequency scanning to obtain analysis of its most complex electrical behavior. A polymethylthiophene matrix was used to immobilize DNA probes,
and could selectively detect its complimentary DNA sequence. Impedance spectra changes was directly related to conformational changes during hybridization. These systems achieved impedance changes for specific complimentary ssDNA without any labeling techniques employed.\textsuperscript{114}

The detection of genomic DNA however, is not as straightforward as that of short ssDNA, as the annealing of genomic DNA leads to imprecise nucleotide base pairing on recombination. A method for label-free electrochemical genomic DNA detection was achieved by Robert \textit{et al}\textsuperscript{115}, by adsorbing spots of genomic DNA to carbon substrates and then scanning image by electrochemical microscopy which can capture images of electrochemical activity at microscopic levels. The increase in current at the adsorbed spots shows that hybridization had occurred.\textsuperscript{115} Nanomaterials are also proposed as options to improve the sensitivity and selectivity of label-free detection. An array of silicon nanowire was functionalized with peptide oligonucleotides by Cai \textit{et al},\textsuperscript{116} and the resistance of the nanowire array was shown to change based on the presence and concentration of the target DNA, offering low detection limits for DNA characterization. This approach requires no label, offers real-time data, and differentiates adequately mismatched DNA target. CNTs are also of increasing interest due to the possibility of making the electrical conductors and their size similarity with many biomolecules. Hence CNTs can act in direct contact between biomolecules and electrode surfaces.\textsuperscript{18} Similar carbon nanomaterials such as carbon nanofibers (CNF), have also been used.

Wang \textit{et al}\textsuperscript{117} used CNF arrays on glassy carbon to immobilize oligonucleotide and determine DNA hybridization. Field electric transistors (FETs) are also of interest in label-free electrochemical DNA biosensor design. FETs are made of p-type silicon containing two n-type regions, with a source and drain implant in the p-type silicon. When FET is in contact with a
sample solution, various charged species or ions can alter the source-drain current, and this scheme has exploited oligonucleotide polyanionic nature for DNA characterization and determination.\textsuperscript{118} CNTs have been incorporated with FETs for the detection of several biological interactions. An example is hybridization studies of DNA immobilized on SWCNT incorporated FET, and the presence of metal salts was shown to enhance sensitivity.

This approach has led to miniaturized electrode design with dimension size less than 100 µm, and has been fabricated for label free capacitance-based DNA hybridization detection. An example of this is the commercial array, Combimatrix, which has 12 544 electrodes/cm, with each electrode sized 44 µm, and surrounded by a counter electrode made of platinum grid. This chip apparatus (Fig. 2.5), and is suitable for electrochemical analysis or fluorescence imaging. The use of a PC software can simultaneously allow for multi-analysis of the microelectrodes, and readings of entire chip can take as little as 15 seconds. Using this approach, nucleic acids determination have thus been achieved and developed for genotyping of various health related pathogens such as Eschericia coli, Bacillus anthracis, Bacillus subtilis, and Yersinia pestis.\textsuperscript{119}
2.2.1.3. Other Approached Used in Medical Biosensors: Other approaches to the design of biosensor for medical applications have used piezoelectric means such as QCM or SAW sensors, or a microcantilever DNA sensor. Several biosensor systems have immobilized oligonucleotides unto QCM surfaces. Though these systems are commercially available, they do not offer the sensitivities of electrochemical and optical biosensors for DNA detection, and are therefore not widely used. Cantilever-based sensors are also an attractive transducer for DNA detection, and an array of nanocantilevers with diameter range of 100 nm – 1 µm have been employed to immobilize and detect biological molecules such as oligonucleotides and antibodies. Cantilevers are attractive for use in biosensors because they can be selectively functionalized on a particular side. Gold binding is mostly employed, and gold-thiol chemistry can afford the attachment of several biomolecules. Binding of these biomolecules leads to an internal system stress and subsequent cantilever bending. Detection is thus achieved using an optical laser. This approach has been employed for multi-RNA detection, differential gene expression, and cancer biomarker sensor.

The sensitivity of these sensor types is very high, and prospects in nanotechnology engineering offers even further improvements. These systems can detect single molecules in simultaneous multi-analyte capacity, and can detect complementary DNA sequence even in the presence of more than a 100-fold non-complementary DNA sequence. Research has intensified in this biosensor field, as commercial microcantilever biosensors (Cantisens®) have been developed.

2.2.2. Disease Biomarker Detection: Disease biomarker based clinical diagnosis has become very important to improving patient health and reducing healthcare costs. The goal is to enable physicians use disease biomarkers to detect, personalize, and monitor diseases at the very onset when it can be easily managed, and treatment outcomes can be more favourable. Biomarkers are
biological features which can be evaluated and measured as indicators for normal biological processes, or as a response to a stimulated intervention. Biomarkers in humans include metabolites, cell-free DNA and RNA, circulating tumor cells, proteins and peptides present in biological fluids like urine, blood, and cerebrospinal fluids. Very small changes in biomarker composition and/or concentration indicates the pathological or the physiological state of the patient.

Proteomic biomarkers are the most extensively studied, and this has advanced disease diagnosis and monitoring. Proteomic biomarker assays usually rely on antigen-antibody interaction studies, and protein concentrations in cells, blood and tissues represent largely the screening basis for most diseases like cancer and infectious epidemics. In the last decade, human genome sequencing has played a key role in protein sequence discovery relevant for use as disease biomarkers. Validation of the role of these protein collections in the occurrence of these diseases are crucial, and a validation process involves understanding if the protein is sensitive and specific, if changes can be detected easily, and adjudging its relevance to disease progression and treatment. This is where the need for efficient detection technologies comes to play, and detection kits must be able to detect very minute changes in these biomarkers.

The current available technology for disease biomarker detection are immunoassays, microarrays, PCR-based technologies, chromatography, mass spectrometry and gel electrophoresis, and immunohistochemistry.

Immunoassays involves the specific binding of antibodies to antigens, and provides quantitative analysis for the presence and levels of biomarkers in biological samples. Antigens invoke the immune response that leads to antibodies production, and antibodies are proteins with a specific binding for its antigen. Genetic engineering advancements have allowed the selective control and modification of protein expressions, and antibody engineering has allowed the
production of only the Fab functional antibody region. The use of antibodies as diagnostic tests for analyte quantification in biological samples principally rely on two methods which are radioimmunoassays (RIA) and enzyme-linked immunosorbent assay (ELISA). While RIA involves radio labelling the antibody for signal generation, ELISA methods offers safer and more sensitive methods as it uses an enzyme label. There are still some limitations though such as the occasional shielding of antibody binding region following immobilization, antibody denaturing from the electrostatic or hydrophobic interactions, fouling from non-specific interactions with non-target proteins also present in sample matrix, and the cross-reactivity of capture antibodies resulting in decreased specificity. Proteomic microarrays however offer promise of multiplex protein assays, and can be used for protein interactions with several other biomolecules such as DNA, oligonucleotides, and peptides. Genome wide protein analysis has been achieved with this method for proteins that interact with phospholipids. Due to the availability of public human genome data, sequence characterization has been accomplished for detection of complex disease markers using protocols such as enzymatic PCR technology. Quantitative real time PCR also provides analysis and amplification of a desired genomic fragment with no need for sample manipulation.

The need for newer technologies for disease marker detection has necessitated increased emphasis and focus on biosensors. An emerging area is in particle-based biosensors which is considered to be a workable alternative to the presently employed molecular diagnostics.\textsuperscript{122} Particle based biosensor employ similar chemistry and sample preparation as in microarray technology, but offers the advantages of a 3D- assay such as in a simple device setup and design, reduced reagent and sample quantity required, and applicable to multiple analysis studies. Particles are specifically labelled using encoding technology for the multiple target mixture in solution
which can all be detected simultaneously. The 3-D nature of the solution suspended particles also minimizes steric constraints that is usually found in 2-D assay platforms, and this allows higher throughput analysis, efficient assay kinetics and reduced incubation times.\textsuperscript{123}

(a) Single-plex 2-D assay

(b) Multiplex 3-D assay

![Image of 2-D and 3-D immunoassay showing the advantage of simplicity, multiplicity and reduced reagent and sample quantity in 3-D particle-based assay. Gines G. 2014]

The first design of particle based biosensors was in 1981 using latex particles fluorescent isothiocyanate (FITC) labelled latex particles, and was used to determine phagocytosis in macrophages and neutrophils.\textsuperscript{124} There was rapid interest immediately, and in the same year, Steinkamp \textit{et al.}\textsuperscript{125} used fluorescent particles for measuring blood cell count, and Lisi \textit{et al.}\textsuperscript{124} used a particle assay for quantifying human immunoglobulin (IgG). Now, particle-based assays are commercially available which has been used for detection of various targets including autoimmune cells and cytokines. Luminex Corp. USA is probably the market leader in this technology, and their product xMap uses a polystyrene microsphere particle with two incorporated fluorescent dyes of different ratios, and the resulting 100 particle system has distinct fluorescent signatures which allows for the multi-detection of various targets.\textsuperscript{126}

These particle based designs however have limitations which are usually related to chemical stability of the particles as they are mostly carboxyl group functionalized polystyrene.
Another limitation is their optical instability in solution, as fluorescent dye molecules physically incorporated into these particles are released uncontrollably in solution which eventually eliminates the unique particles optical codes. The polystyrene-functional group used have compatibility issues with most reagents used for protein purification, hence this also limits their versatility.

Two techniques has been introduced as improvements to particle based assays namely organosilica particles and flow cytometry. Organosilica particles are monodispersed with an adjustable diameter from 40 nm – 10 µm. They also have large surface areas which allows inflow of fluorescent dyes when they are covalently bounded to thiolated groups allowing the varied dye ratios to produce unique optical signatures. Organosilica particles are also stable, useful in solvent-based protocols, and can overcome the challenges in proteomic assays such as low sound to noise ratios and biofouling.\textsuperscript{127}

Flow cytometry has also been used in particle-based assays to enable particle analysis across several fluorescent protocols and allowing the analysis of thousands of replicate particles. This provides better data quality and higher assay replication than in traditional assays. With the use of fluorescent bead standards, it is possible to calibrate flow cytometry for adsorbate quantification, and this allows the comparison of the fluorescence from the fluorophore labelled particle to emission from unidentified fluorescent materials that are also bound to the surface.\textsuperscript{128} The principle here is based on the theory that fluorescent intensity of the particles must be same as that bound to cell surface. Kozak \textit{et al}\textsuperscript{129} used this theory to quantitatively determine protein adsorbed on to particles.
Other forms of biosensor design that has found application as use in detection of disease biomarker include semiconducting silicon nanowires (SiNWs). SiNWs are used in nano-biosensor for the detection of biomarkers of prostrate and breast cancer in whole blood, in impedimetric biosensors for the detection of stroke and cardiac biomarkers, in SAW biosensors for bacteria spore detection such as in E.coli and Bacillus thuringiensis, in magnetic sensors for detection of biomarkers of ovarian cancer, amongst other biosensor applications. There is increasing emphasis on the use of biosensors for disease marker detection. Since disease markers are mainly proteomic biomarkers, and the ability of biomarkers, depending on design approach, to detect low level proteins is very appealing and would potentially revolutionize the field of disease diagnostics.

2.3. Biosensors for Cancer Detection: Affibodies as Alternatives

The discovery and detection of biomarkers for cancer has been a major research area. The intensive research and discovery of new biomarkers implicated in cancer formation has led to more
assays for their detection. Currently, immunosensors are the major focus of research for the
detection of cancer biomarkers. This is because antibodies are highly specific and sensitive to the
target bio-analyte. Commercial immunosensors are also available for detection of several cancer
biomarkers such as Calbiotech PSA ELISA kit, CA15-3, and the ADVIA Centaur assay. These
tests offer some advantages over standard detection practices such as their non-invasive and easy
procedures, and they can be applied to throughput assays for multiple tests. The presence of a
biomarker does not necessarily indicate the presence of cancer or any biological outcome, but their
levels are more indicative and can be used as disease diagnostics. Hence, immunosensors are more
employed for cancer prognosis, by monitoring biomarker levels in comparison to a set of
biomarker basepoint. This is a more effective way of disease prognosis, as levels can indicate if a
treatment plan is effective or not. This approach is applied to the determination of human HER2
serum levels, as an alternative to other protocols like fluorescence in situ hybridization (FISH)
and immunohistochemistry (IHC). This is especially useful in HER2 monitoring after tumor
removal to detect disease relapse, in which case the detection material for FISH and IHC is no
longer available.

The biggest drawback in use of antibodies have been discussed and this also applies to
immunosensors for biomarker detection. This include costs, stability, the need for animals, and
unexpected interference. Heterophilic anti-animal antibodies (HAIA), shown to be present in
healthy serums by up to 40%, can give rise to crosslinks between capture and detection antigens
when used in sandwich assays, which can lead to false-positives. These limitations have
necessitated the search for a cheap, sensitive alternatives to antibodies for use in cancer biomarker
assays. Alternatives that have been used include antibody fragments, non-antigen protein
scaffolds, and aptamers. There is extensive research to evaluate the suitability of these alternatives,
not only in addressing the limitations of antibodies, but to assess if they can match the sensitivity and specificity of antibodies in cancer biomarker assays. Another alternative that has been proposed are affibodies.

Affibodies are antibody mimetics, which are small engineered proteins that can bind target proteins or peptides in a manner similar to monoclonal antibodies. Genetic engineering of proteins binding domain can afford a Z-domain (an IgG binding domain), and this has allowed for the engineering of several high-affinity antibody alternatives. Affibodies are very robust three-helical peptides, containing only 58 amino acids and no disulfide group functionalization, hence are produced in simpler organisms and require no animal systems for their production as is the case with antibodies. The small structures of affibodies allows for their production using solid-phase peptide synthesis (SPPS), and as such animals are not required for its production. The Z domain of affibodies is mutation tolerable, allowing synthetic variations for a large library of targets. Chemical synthesis has allowed the engineering of high affinity, specific affibodies which are useful for the characterization of various biomolecules such as Taq, RSV, IgA and HER2. Affibodies can also be linked to each other to create what is known as multi-affibodies. Their modification usually exploit their non-cysteine composition by genetically altering the amino acid groups far away from their binding sites to allow for a specific fluorophore or enzyme label binding.

The synthesis of affibodies can also incorporate fluorescent or electroactive labels such as biotin, and enzymes. HER2 affibodies have been used in imaging techniques for the direct detection of tumors expressing HER2. Radiolabels are employed in this case, and aside from the cost issues, this also poses significant health risks. HER2 affibodies using radioactive labels however provide very good results. The use of affibodies have also been explored in
microarrays,\textsuperscript{137,139} but really there is not much use of affibodies for biosensor design found in literature.

Affibodies can be incorporated to use several transduction means such as enzyme labelled electrochemical biosensors, optical biosensors using fluorescent molecules, SPR approaches, and also in piezoelectric biosensors.\textsuperscript{137,140} Based on the potential advantages in engineering design and specificity that affibodies have over antibodies, it is safe to expect that more research will be directed into this area. This assumption is further based on their ability to be incorporated into several existing biosensor design and their compatibility with various transduction means. Thus, a possible emergence of affibodies-based biosensors, as an alternative to immunosensors, for cancer biomarker detection and in other clinical analysis may not be too far away.\textsuperscript{137}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig28.png}
\caption{Refined solution NMR of Z-domain in staphylococcal protein A: rainbow spectra gradient used to color protein chains from N-terminal to C-terminal. Justino C.L., 2015.}
\end{figure}

\section*{2.4. Drug Discovery and Testing:}

The interaction of pharmaceutical agents with biomolecules is central to drug design and discovery. To understand these interactions, standard instrumental analytical chemistry tools are
employed such as liquid chromatography, mass spectrometry, and capillary electrophoresis. Biosensors have become another focus during drug research and discovery, and several biosensors have been used to analyze the effect of prospective therapeutic agents on prospective biological targets such as DNA, oligonucleotides, enzymes, antibodies, and various cell populations.\textsuperscript{141}

\subsection*{2.4.1. Electrochemical Biosensors for Drug Discovery:} Electrochemical biosensors remain the most studied and employed biosensors for medical applications. Enzyme-based biosensors using voltammetry or amperometry transduction is widely used in drug research and discovery. Electroactive enzymes commonly employed are the CYP450 enzyme superfamily, as they are redox active enzymes. Their electron transfer to the electrodes in biosensor designs use proteins like cytochrome P450 reductase (CPR) or flavodoxin, unlike in biological systems where NADPH serves as the electron transfer agent. This approach was used by Mie \textit{et al.}\textsuperscript{142} in the electrochemical assay of testosterone and ketoconazole metabolism using CYP3A4. Most electrochemical biosensor systems that uses an electroactive enzyme for signal amplification monitors the production or consumption of H$_2$O$_2$. The enzyme monoamine oxidase (MAO) has been used in many electrochemical assay with this approach. MOA acts on amine groups in drugs and produces H$_2$O$_2$, and this reaction was exploited by Volotskaya \textit{et al.}\textsuperscript{143} in an amperometric biosensor for detecting nitrofuran and anti-depressant drugs such as pyrazidol and desipramine.

\[
\begin{align*}
R-C-NH_3 + O_2 + H_2O & \rightarrow R-CHO + H_2O_2 + NH_3 \\
\end{align*}
\]

Acetylcholinesterase (AChE) is another enzyme used in electrochemical biosensors, and it is responsible for the degradation of the neurotransmitter, acetylcholine. AChE was co-immobilized with another enzyme, choline oxidase (ChOx), which oxidizes the choline produced
by AChE to generate H₂O₂, and the generated H₂O₂ is subsequently oxidized at the electrode. Deng et al.¹⁴⁴ used this approach for detecting the activity of drugs such as nicotine using ChOx. Other enzymes used for drug testing using electrochemical biosensors include the enzyme laccase used in the assays of adrenaline, morphine and methyldopa;¹⁴⁵-¹⁴⁷ tyrosinase inhibition by the drug pipemidic acid was studied, and horseradish peroxidase (HRP) was used to study several metabolism of drug substrates such as acetaminophen,¹⁴⁸ clozapine, promazine and rifampicin.¹⁴⁹-¹⁵¹

Electrochemical biosensors have also employed the use of DNA to study drug activity. The design and incorporation of DNA into electrochemical biosensors has been discussed earlier. A dsDNA is usually employed to study drug-DNA interactions, and signal generation is by current changes due to the oxidation of guanine when the immobilized dsDNA interacts with the drug sample. Several examples of drugs that have been studied with this protocol include anticancer drugs such as cisplatin,¹⁵² methotrexate¹⁵³ and leuprolide,¹⁵⁴ and the antibiotic tetracycline.¹⁵⁵

2.4.2. Optical and Piezoelectric Biosensor for Drug Discovery: Optical and piezoelectric biosensors are also an attractive tool for understanding drug activity and their impact on biomolecules. Although research focus on biosensor designs for drug discovery relies mostly on electrochemical transduction, other approaches have also been explored such as optical and piezoelectric means. Optical biosensor for drug screening is mainly based on a fluorescent signal generation by SPR, as drug-DNA interaction and immunosensing reactions can be easily controlled at the surface of the sensor. Recognition is achieved either by a direct binding assay where a DNA or antibody is immobilized to the sensor, or by inhibition assays where the presence of the drug decreases the signal generated when the drug interacts with the recognition agent.
Inhibition assays have been applied to the screening of the presence of some veterinary drugs in animal residue samples, and was able to detect the presence of antimicrobial drugs in animal milk, eggs, serum, and kidney. Piezoelectric biosensors are also an attractive alternative in biosensor design for drug studies and detection. The main approach employed is the quartz crystal microbalance (QCM), which affords a label free, viscosity changes detection, and is suitable for incorporation with other techniques.

Examples of different electrochemical, optical and piezoelectric biosensors that have been studied for use in drug detection is summarized in Table 2.2.

<table>
<thead>
<tr>
<th>Biosensor</th>
<th>Drug</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrochemical Voltametric/ Amperometric Biosensor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4/ CPR-microsomes/ Thiol coating/ AuE</td>
<td>Testosterone, Ketoconazole</td>
<td>Mie et al, 2009</td>
</tr>
<tr>
<td>MAO/ SPPtE</td>
<td>Furagin, Furazolidone, Furadonine (Dopamine)</td>
<td>Volotskaya et al, 2011</td>
</tr>
<tr>
<td>ChOx/ DM/ PAAm/ PtE</td>
<td>Choline, Nicotine, L-glutamate</td>
<td>Deng et al, 2013</td>
</tr>
<tr>
<td>Pox/ Cl/ Glyoxal/ CPE</td>
<td>Adrenaline (H2O2)</td>
<td>Ozel et al, 2015</td>
</tr>
<tr>
<td>Lac/ CA/ BMIN (Ti2)/ CPE</td>
<td>Methylodopa</td>
<td>Moccellini et al, 2011</td>
</tr>
<tr>
<td>Lac/ PQQ-GDH/ Clark E</td>
<td>Morphine</td>
<td>Bauer et al, 1999</td>
</tr>
<tr>
<td>Tyr/ APCPG/ AuE</td>
<td>Pipemidic acid (catechol)</td>
<td>Lete et al, 2015</td>
</tr>
<tr>
<td>HRP/ PAA/ GCE</td>
<td>Acetaminophen</td>
<td>González-Sánchez et al, 2011</td>
</tr>
<tr>
<td>HRP/ MMPs/ sCPE</td>
<td>Clozapine (H2O2)</td>
<td>Ben Yoav et al, 2014</td>
</tr>
<tr>
<td>P. alcaligenes/ CM/ Clark E</td>
<td>Caffeine</td>
<td>Babu et al, 2007</td>
</tr>
<tr>
<td><strong>Electrochemical DNA biosensors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA/GCE</td>
<td>Methothrexate</td>
<td>Rafique et al, 2013</td>
</tr>
<tr>
<td>DNA/GCE</td>
<td>Leuprolide</td>
<td>Dogan-Topal et al, 2011</td>
</tr>
<tr>
<td>Biotinylated DNA Aptamer/ Streptavidin/ SPAuE</td>
<td>Tetracycline</td>
<td>Kim et al, 2010</td>
</tr>
</tbody>
</table>
Table 2.2: Different Biosensor Designs for Drug Detection

<table>
<thead>
<tr>
<th>Method</th>
<th>Drug/Clinical Relevance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Biosensors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb/FO</td>
<td>Cocaine</td>
<td>Toppozadat et al., 1997</td>
</tr>
<tr>
<td>Metronidazole/SPR-chip</td>
<td>Nitroimidazole</td>
<td>Thompson et al., 2009</td>
</tr>
<tr>
<td>BSA/SPR-chip</td>
<td>Naproxen, Creatinine, D-glucose, Mannitol, Urea</td>
<td>Kim et al., 2004</td>
</tr>
<tr>
<td>QCM-based biosensors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECs/Gold-QCM</td>
<td>Taxol and Nocodazole</td>
<td>Marx et al., 2007</td>
</tr>
<tr>
<td>HAS/Bilirubin/QCM</td>
<td>Cocaine</td>
<td>Zhang et al., 2008</td>
</tr>
<tr>
<td>BS/ QCM</td>
<td>Trastuzumab</td>
<td>Elmlund et al., 2015</td>
</tr>
</tbody>
</table>

Au=gold electrode, MAO=monoamine oxidase, sPPE=screen-printed platinum electrode, ChOx=choline oxidase, DM=dialysis membrane, PAAm=polyacrylamide microgels, PtE=platinum electrodes, Pox=peroxidase, CPE=carbon paste electrode, lac=lactase, CA=cellulose acetate, BMIN (Tf)2=1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)limide, PQQ=pyrroloquinolinequinone, GDH=glucose dehydrogenase, tyr=tyrosine, APCPG=3-aminopropyl modified controlled poreglass, HRP=horseradish peroxidase, PAA=polyacrylamide microparticles, GCE=glassy carbon electrode, MMP=phenyl methosulfate, sCPE=solid carbon paste electrode, sPAuE=screen-printed gold electrode, PPR=polypyrrole, PVS=polyvinylsulfate, mAb=monoclonal antibodies, FO=fiber optic, ECs=endothelial cells, HAS=human serum albumin, BS=bovine serum

2.5. Non-Invasive Measurements:

Due to the rising increase in health problems and chronic diseases in humans, the need for fast, inexpensive, real-time, and non-invasive diagnostics is rapidly increasing. The need for detection of metabolites and biomarkers other than in blood samples has also been explored, especially for patients with difficulty in collecting blood, and in patients with a need to monitor daily parameters like urea and glycaemia. Sweat, saliva, skin tests are other biological matrixes other than blood used in non-invasive biomarker detection.

2.5.1. Non-Invasive Biosensors Using Saliva as Analyte: The saliva has been a known sample used in drug monitoring, especially in chronic diseases and for drugs with a very narrow therapeutic window. Blood was used commonly as samples, but it has been shown that concentration of most drugs in the body can also be detected from its concentration in the saliva.163
The determination of body alcohol levels is used to estimate intoxication degree, and alcohol intoxication is a leading cause of traffic accidents, injuries and deaths. A widely used protocol for real-time blood alcohol levels detection is by breath analyzers. An alcohol sensor based on an oxygen electrode has also been used in an alcohol biosensor using saliva as test samples.\textsuperscript{164} This approach is based on the enzymatic activity of alcohol oxidase on ethanol, converting it to H\textsubscript{2}O\textsubscript{2}, thus generating a signal at the electrode based either on oxygen consumption or peroxide generation.

\[
\text{ethanol} + \text{O}_2 \xrightarrow{\text{alcohol oxidase}} \text{H}_2\text{O}_2 + \text{acetaldehyde}
\]

Lactate measurements is undertaken in critically ill people, to prevent sudden cardiac arrests; and also in diabetic patients and in sports medicine. An electrochemical probe for lactic acid concentration in human saliva has been developed and it is based on the enzymatic activity of lactate oxidase (LOx).\textsuperscript{165}

\[
\text{lactate} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{lactate oxidase}} \text{pyruvate} + \text{H}_2\text{O}_2
\]

These biosensors are examples of non-invasive electrochemical biosensor which is further discussed \textit{vide supra}. The use of biosensors for non-invasive measurements based on saliva samples can employ different transduction protocols such as the electrochemical means as discussed above. Another means is by optical methods such as in the SPR techniques used for the detection of steroid hormones\textsuperscript{166} and interleukin-8\textsuperscript{167} (a biomarker in cancer prognosis). Other biological health-related substances that has been determined in the saliva by the use of a biosensor include detection of an auto-antibody biomarker for breast-cancer,\textsuperscript{168} glucose,\textsuperscript{169} and \(\alpha\)-amylase.\textsuperscript{170}
2.5.2. Non-Invasive Biosensors Using Sweat as an Analyte: Sweat is also a biological sample useful in the determination of body concentration of several biological substances, as their metabolites are also excreted via sweat. The ‘gold standard’ for the diagnosis of cystic fibrosis is still in the analysis of chloride concentrations in sweat, an approach that has been used for several years. Several metabolites found in sweat are used to characterize determine blood levels of their original compounds, and this is an important aspect in non-invasive clinical analysis.

Lamas et al\textsuperscript{171} developed a disposable lactase biosensor based on amperometric detection, using LOx immobilized on screen-printed carbon electrodes. This biosensor was used to detect lactate in blood and sweat, and the analysis in sweat gave better detection limits. Recent clinical biosensors based on sweat analysis are usually integrated into textiles and woven cotton, such that they are wearable, providing a continuous, real-time assessment of the detected target. This approach has been used to monitor stress activity in sports and everyday lifestyle, and in some health conditions.\textsuperscript{172,173} However most of these biosensor design are used as disposables, as activity usually cannot be regenerated.

2.5.3. Wearable Biosensors: The advancements in computing intelligence, wireless sensor networks and biosensor designs has opened grounds for innovations such as in wearable medical biosensors. Electronic textiles, as mentioned above, and body sensor network (BSN) provide the tools for these wearable biosensors. The design of wearable biosensors must meet the condition of a required functionality, yet must be inconspicuous and easy to use. It must also be inexpensive, wearable without imposing any discomfort, and maintain the required performance level. Several sensor-transduction combinations can been used once they can be miniaturized into cheap, comfortable, and wearable devices.\textsuperscript{174} BSN are multiple sensors that can be worn at different parts of the body, and they wirelessly transmit data to another body device, which is accessible at all
times to the user. Optimization of BSN approaches is expected to lead to improvement in the field of wearable sensors. A multi-tier BSN system architecture is shown in Fig. 2.9.

Textiles are also a growing integration platform for wearable biosensor design due to their non-intrusive nature. An example of this is the smart shirt, as designed by Park and Jayaraman.\textsuperscript{175} Smart shirt is a simple fabric (cotton, polyester, and silk) with interconnected ‘plug and play’ sensors at different locations measuring conditions such as pulse oximetry (SpO\textsubscript{2}), heart rate, ECG, respiration rate, and voice recording. Generated signals are transmitted via a personal status monitor.

Further discussion on the design and applications of these wearable sensors is found in the following review.\textsuperscript{176,177}
2.5.4. Clinical Applications of Non-Invasive Measurements: One of the use of wearable biosensors has been in the monitoring of responses and adverse effects especially in neurodegenerative diseases such as Parkinson’s disease. Parkinson’s disease is characterized by postural balance, tremor, and rigidity, and therapeutic treatments worsen as disease progresses. A common adverse effect of therapeutics in Parkinson’s is known as dyskinesia. Symptom monitoring may become unproductive as drug adverse effect occurs several hours after dosage, hence patient self-reports is relied on. Patient reports are usually flawed by recall bias, or inability to differentiate between normal disease tremor symptoms, or dyskinesia as a result of the drugs adverse effect. This has increased emphasis on wearable biosensor for a more adequate clinical evaluation of Parkinson’s disease symptoms vs its adverse effects to therapeutics. Wearable biosensors for monitoring patients’ symptoms in Parkinson’s was first explored in the early 1990’s, but it was until the 2000’s that an accelerometer based biosensor that can track symptoms severity was designed and reported. However more recently, Son et al have reported a biosensor that can multifunction and measure various responses and symptoms simultaneously.

Another clinical application for wearable biosensors is in the early detection and monitoring of cardiovascular diseases (CVD), a leading cause of death globally. The symptoms of CVD may often be confused with heartburn or indigestion, and in most cases, this improper evaluation proves very costly. The need for a real-time, constant monitoring of the symptoms for CVD has been explored, and wearable biosensors has also been proposed as a possibility. Abnormal heart rhythms, which is termed cardiac arrhythmia, is the most common symptom of sudden cardiac arrests, and it is usually monitored by using an electrocardiogram (ECD). Patient self-tests such as in wearable biosensors is being proposed and explored as a solution. Rai et al recently proposed a wireless nanomaterial-based textile biosensor for monitoring cardiac
arrhythmia as a solution to sudden cardiac attacks and the numerous deaths from this. This wearable sensor gives alerts directly to patient personal care agents or physicians, when symptoms are detected.

Wearable biosensors thus promise a revolutionary and innovative approach to enhancing patient/doctor interaction, especially for complex health diseases where it is impracticable for the doctor to be available at all required times. These approaches will continue to be a research focus, in a bid to improve on its limitations, and make it more useful than currently used technologies will be extensively explored. A recent review on the advancement of wearable biosensors, and their clinical applications is found in this text.\textsuperscript{181}

References


8. Wang J. \textit{Analytical electrochemistry}. NJ: John Wiley & Sons; \textbf{2006}.


53. Domínguez CM. Analytical chemistry. 2015; 87 (3): 1494.


84. Wu DY. Science China Chemistry. 2015; 58 (4): 574.


147. Özel RE. Analytical letters. 2015; 48 (7): 1044.


Chapter Three: Biosensors in Food Safety and Environmental Health

3.1. Biosensors for Food Safety:

Food safety has become an important aspect of food production and processing. Contaminated foods are the most prevalent cause of public health, and their fatality rate can be very high. Contaminants present in food include chemical compounds (toxic metals, pesticides, and veterinary drug residues), microbiological species (viruses, bacteria, and parasites), natural toxins (mycotoxins and seafood toxins), exogenous and packaging materials. Contaminants find their way into food through environmental exposures, while some are just deliberate additives. Several microorganisms can find their way into food during growth, preparation or cultivation, can accumulate during storage, or from the natural constituents of the food. Microbiological species and natural toxins are today the greatest concern in food contaminants. Some bacteria of concern today include *Salmonella* spp, *Escherichia Coli, Clostridium Perfringens, Campylobacter jejuni, Bacillus cereus*, and *Staphylococcus aureus*. These bacteria can multiply rapidly in food, especially warm, protein-rich foods such as milk, eggs, meat, poultry, and fish. even after they have been processed. They can invade cell linings and produce toxins, which leads to illnesses.\(^1\)

Mycotoxins are mainly produced by *Penicillium, Aspergillus* or *Fusarium* species, and the most studied mycotoxins are aflatoxins, ochratoxin A, zearalenone, patulin, and the fumonisins.\(^2\) Some of these mycotoxins are highly harmful even at very low concentrations. Aflatoxins is a widely known human carcinogen, ochratoxin A causes renal pathologies, and zearalenone is estrogenic causing infertility and breeding problems. These toxins are found mostly in cereals, while patulin is found in fruits.
Conventional detection of food contaminants employs culture media which tests for the microorganisms’ ability to multiply under various conditions. In this approach, a single cell is grown into a colony by pre-enrichment and selective enrichment processes, and their presence in the colony is confirmed by biological screening, before a serological test is done. Additional tests may also be done for identification and confirmation. Commercial antibody kits are a cost effective approach in the laboratory culture of cells. These culture assays though are highly sensitive, they are plagued by their assay time, which can take up to 96 h.

Other approaches developed to reduce analytical times included chromatography, bioluminescence, infrared or fluorescence spectroscopy, impedimetry, and flow cytometry. The ideal detection procedure for food pathogens should be simple, cheap, and user-friendly. It should also offer similar sensitivities as is applicable for analyte detection. These requirements has necessitated the need for newer technologies to be explored.

Immunosensors for microbiological and toxin contaminants have been greatly studied, and has been shown to be a specific, sensitive, and highly reproducible approach. Many test kits are available using polyclonal or monoclonal antibodies, and techniques such as ELISA and RIA can detect not just the microorganism, but also the toxins they produce. They however still suffer from their use of animals for antibody generation, or the longer assay period required when monoclonal antibodies are used.

Nucleic acid detection have shown to be more specific and sensitive than immunosensors. The selectivities of DNA-based assays is further enhanced by PCR technology which allows for the amplification of a defined nucleic acid sequence. Hence targets can be amplified for a more sensitive detection. This method usually gives the advantage of rapidity, very low detection limits, higher sensitivity, specificity, and accuracy. New designs of nucleic acid diagnostics in DNA chips
and microfluidic lab-on-chips techniques offer the added advantage of portability and multiple testing. The various applications of these biosensors are described vide infra.

3.1.1. DNA Biosensors in Food Safety: DNA biosensors has been widely applied in the detection of pathogenic microorganisms, toxins, and genetically modified organisms (GMO) present in food. Two aspects of this application, in the detection of food microorganisms and GMO will be discussed in this section. Its use in the detection of food toxins food is covered in section 3.1.4.

3.1.1.1. Genosensors for GMO Detection: GMOs are synthetic organisms with altered genetic information such as natural evolution cannot allow. This genetic modification is achieved by identifying and extracting specific beneficial genes of organisms e.g. bacteria, and inserting them into a plant genome. Herbicide-resistant soybean is a classic example of a very important genetic modified crop, and this provides huge benefits in agriculture and food production. GMOs enhances the nutritional value of food, or generates disease and pest resistant crop strains. Though GMOs improve food yield and quality, their presence in food is still a source of concern, as their complete benefits and effects is not clearly defined. This has made regulation agencies impose proper labeling of GMO foods for consumer awareness. This has also made the fast, real-time analysis of GMOs in food very vital.

GMO inserts are made up of three parts which are the promoter, trait gene, and terminator. A promoter, such as 35S from Cauliflower Mosaic Virus (CaMV), is needed for high gene expression levels in plants; while a terminator, such as the nopaline synthase (NOS), is the gene specific sequence that acts as a stop signal during gene transcription. The promoter and terminator flank the trait gene sequence which is responsible for providing the desirable property in the plant.
Two classic approaches are used to detect GMOs in foods which are the protein based approach for the detection of the new proteins that have been encoded by the GMO, or a DNA based approach to detect the DNA sequence of the modified gene. This target genes are regarded as event specific DNA targets, as they are unique sequences of the new modified DNA. Event-specific target detection offers the highest level of specificity in DNA assays for GMOs, as these are unique to a particular GMO. Other DNA targets can be used as preliminary or screening tests such as the species/taxon, element, and construct specific targets. (Fig. 3.1).

![Diagram](image)

Fig. 3.1: Construction and detection levels of GMOs. Arugula and Simonian, 2014

Although several genosensors have been designed, only those with optical, electrochemical and piezoelectric transduction means have been used for detection of GMOs.5 Most studies done on GMOs are screening studies, usually targeting the 35S; while GMO specific assays use the NOS or PAT gene as targets. Christopoulos and coworkers6 achieved a nanoparticle genosensor
for the visual detection of GMOs. Their design used a dry reagent dipstick configuration for visual detection, which enabled the detection of the 35S promoter sequence in screening studies, or the NOS gene for GMO specific assays. The target sequence after PCR amplification is hybridized with a biotinylated DNA probe on a gold nanosurface, and capture of this hybrid at the test zone by an immobilized streptavidin gives a characteristic red line signal. May et al\(^7\) reported a deoxyribozyme molecular beacon with a fluorophore-quencher system for the FRET determination of GMOs. Detection was achieved by fluorescence, when target hybridization to the loop structure introduces a conformational change that separates the fluorophore and the quencher (Fig. 3.2).

Several other approaches have detected GMOs using a genosensor. Minunni et al\(^8\) used a SPR affinity genosensor for the detection of 35S and NOS gene sequences in GMOs. The sensor used SPR immobilized synthetic oligonucleotides, and a PCR model for amplification of sample sequences. Mutlu et al\(^9\) applied a piezoelectric QCM-based genosensor for screening of GMOs by detection of the 35S promoter gene. This DNA biosensor was used to detect GMOs in tobacco.
plants. Future applications of these biosensors is in mobile, easy to use devices that can be used for field and off-site tests. Such approach has been reported for the visible detection of GMOs in food using a G-quadruplex based biosensor. This was used to detect the 35S promoter sequence. The probes employed were two G-rich sequences that both hybridizes to the DNA target, and fold at their G-rich sequence into a G-quadruplex DNAzyme. This DNAzyme catalyzes the H$_2$O$_2$ mediated oxidation of the label (ABTS$^{2-}$) into its colored radical anion ABTS$^-$. 

![Diagram of G-quadruplex DNAzyme sensor](image)

**Fig 3.3: Principle of G-quadruplex DNAzyme sensor for detection of GMO, Qui et al, 2014**

Another area where continuing work is expected in genosensor designs for GMOs is in the multiplex analysis of samples. Since GMOs have multiple specific events, sensors that can provide real-time multi-analysis is required, and this biosensor type was recently reported by Liao et al.$^{11}$ They achieved the electrochemical detection of GMOs using a 16-sensor gold electrode array chips, with surrounding auxillary and pseudo-reference electrode. PNAs have also been used in a multi-array plaform for GMO detection in food. Germini et al.$^{12}$ used PNA probes deposited on
commercial slides for the detection of real samples such as in Roundup Ready soybean, Bt11, Bt176, Mon810, and GA21 maize. They found out that their most successful probe is a 15-mer PNA sequence (Table 3.1) linked by two 2-(2-aminoethoxy)-ethoxyacetic acids spacers. An extensive review of GMO genosensors design and applications is covered in these texts.\textsuperscript{5,13,14} Table 3.2\textsuperscript{15-22} also gives a list of recent genosensor designs for screening and detection of specific GMOs in food and plants.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>Sequence (H-NH2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>soybean lectin</td>
<td>LL-GAT CAA GTC GTC GCT</td>
</tr>
<tr>
<td>MZ</td>
<td>maize zein</td>
<td>LL-TTT ATA GAT GTA TGC</td>
</tr>
<tr>
<td>MON810</td>
<td>MON 810 maize</td>
<td>LL-CGC TCA CTC CGC CCT</td>
</tr>
<tr>
<td>RR</td>
<td>RR soybean</td>
<td>LL-AAA CCC TTA ATC CCA</td>
</tr>
<tr>
<td>Bt11</td>
<td>Bt11 maize</td>
<td>LL-ATA TCT ACT GAC AAA</td>
</tr>
<tr>
<td>Bt176</td>
<td>Bt176 maize</td>
<td>LL-ACA CCT GTG TGC CGC</td>
</tr>
<tr>
<td>GA21</td>
<td>GA21 maize</td>
<td>LL-CGA ACT TCT TGT TGC</td>
</tr>
</tbody>
</table>

* L is 2-(2-aminoethoxyl)ethoxyacetic acid spacer group

Table 3.1: PNA probe sequence and target regions

<table>
<thead>
<tr>
<th>Target-gene</th>
<th>Electrode Type</th>
<th>Detection Method</th>
<th>Sample Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S</td>
<td>Au microelectrode</td>
<td>EIS (label-free)</td>
<td>Synthetic DNA</td>
<td>Lien et al, 2010</td>
</tr>
<tr>
<td>Bt</td>
<td>Au</td>
<td>DPV with an anthraquinone-based indicator</td>
<td>Synthetic DNA &amp; reference material</td>
<td>Ulianas et al, 2014</td>
</tr>
<tr>
<td>PAT</td>
<td>GCE</td>
<td>voltammetry using Au nanoparticles</td>
<td>Synthetic DNA</td>
<td>Jiang et al, 2011</td>
</tr>
<tr>
<td>PEP gene</td>
<td>GCE</td>
<td>EIS (label-free)</td>
<td>Synthetic DNA</td>
<td>Yang et al, 2012</td>
</tr>
<tr>
<td>sslb, ivrp, cryla/b event, MON 810</td>
<td>Au</td>
<td>SWV with [OsO4(bipy)] indicator</td>
<td>Real samples, PCR amplified</td>
<td>Mix et al, 2012</td>
</tr>
<tr>
<td></td>
<td>CPE</td>
<td>DPV with MB indicator</td>
<td>Synthetic DNA &amp; GM maize</td>
<td>Sun et al, 2014</td>
</tr>
<tr>
<td>35S, PAT, g1b1, sslb, A2704-12 soybean, lectin</td>
<td>Au array</td>
<td>chronoamperometry with enzymatic amplification</td>
<td>Synthetic DNA &amp; GM soybean</td>
<td>Liao et al, 2014</td>
</tr>
</tbody>
</table>
3.1.1.2. Genosensors for Food Pathogens: The detection of food pathogens require a rapid method that gives instant or real-time results. This is important for food safety which usually require off-site studies. Conventional methods mostly rely on bacteria enumeration in specific media. The limitations of this approach has already been discussed. Other challenges include the heterogeneity of food matrices, the non-uniform distribution of bacteria in food, and present risk-free indigenous microbes that can serve as interference. Fast, efficient, and sensitive methods such as genosensors have thus been explored for the detection of these food pathogens. Genosensors are applied in detecting foodborne pathogenic microorganisms such as *Salmonella*, *E. coli*, *Listeria*, and *Staphylococcus*. These genosensors also act by their nucleic acid hybridization, and offer fast, reliable, and cost effective diagnostics.

Manscini and coworkers\(^2^4\) developed a multiplex DNA sensor for detection of *Salmonella* and *Listeria* food pathogens. A streptavidin-alkaline phosphatase was used as capture for the hybridized (PCR amplified) target sequence, such that a biotin-labelled probe can produce the detection signal in the sandwich genosensor employed. A miniaturized genosensor was reported by Berganza *et al*\(^2^5\) for the detection of *E. coli* in food. The design involved a voltammetry transducer in a K\(_4\)[Fe(CN)\(_6\)] redox mediator solution, and probes were immobilized by SAM technique on the electrodes where they hybridized with the *E. coli* strands.

Nanoparticles are widely applied in genosensors for signal amplification. Such designs have to applied for food pathogen detection such as the gold nanomaterial developed by Marques *et al*\(^2^6\) for the amperometric detection of *Salmonella*. They employed an anti-DIG HRP
electroactive label to detect the hybridization of the immobilized DNA and target \textit{Salmonella} sequence. Electrochemical genosensors are in fact the most studied and reported biosensor approach for detection food pathogens. This is because this approach is highly specific, very fast allowing real-time assays, can be miniaturized into small, portable designs such that they can be used in field tests, and off-sites studies. A useful study that highlights these advantages is the aptamer based detection of \textit{Salmonella} in a gold particle based lateral flow biosensor. The specificity of this approach over others, and it’s very low detection limits was exploited in a DNA extraction-free protocol\cite{27}. This allowed for the design of a simple device that can be used for point-of-care testing. The specificity of aptamers allows for very sensitive detections, and different aptamers has been used in the detection of several other bacteria strains. This approach has attracted considerable research interests and novel aptamers for various bacteria and food pathogens are been studied\cite{28}.

Ligaj \textit{et al}\cite{29} recently reported two electrochemical sensors for the detection of Aeromonas hydrophilia in fish and fresh water species. The biosensors used a carbon paste electrode (CPE) with MWCNTs containing covalently immobilized probe, while the other used a gold electrode with SAM of mercaptohexanol and thiolated DNA probe. Indicators such as daunomycin and Hoechst 33258 were used for visual detection. Electrochemical DNA biosensor has also been reported for the detection of the microorganism, \textit{Enterobacteriaceae}, in milk samples\cite{30}. This sensor integrated an Exonuclease-III for target amplification, and this approach was shown to produce very high sensitivities. There are several other biosensor designs that have been used for the detection of foodborne pathogens but are not discussed here.

Biosensors that uses aptamers as biorecognition agents are very useful in assays for food pathogens. This allows for a rapid, sensitive, and simple approach that requires very little of the
target material for detection to occur. Aptasensors can incorporate several transduction means such as colorimetric, fluorescence or electrochemical detections of food pathogens.\textsuperscript{31-33} This is also made possible by the easy engineering of aptamer sequences, as aptamers for different microbacterial and micro-pathogens in food such as \textit{Salmonella},\textsuperscript{34} \textit{Staphylococcus aureus},\textsuperscript{35} \textit{Listeria monocytogenes},\textsuperscript{36} and \textit{Campylobacter jejuni}\textsuperscript{37} has been discovered and studied. Indeed aptasensors are increasingly applied in the field of food screening and microbial pathogen studies.

Duan \textit{et al}\textsuperscript{38} recently developed two models of fluorescent aptamers – the signal-on and the signal-off, based on AccuBlue dye for the detection of pathogenic bacteria. In the signal-on model, the aptamer partially hybridizes to the target, and addition of a complementary DNA (cDNA) and AccuBlue causes complete hybridization thus intercalating the AccuBlue within the double strand, and this exhibited a significantly increased intensity in fluorescence. The signal-off model first allows the complete binding of the aptamer to the cDNA in the presence of the AccuBlue indicator which turns on its fluorescence. The addition of the target causes the cDNA and the target to fall off giving rise to fluorescence quenching. This model has also been applied to the detection of toxins and metals in food. Wang \textit{et al}\textsuperscript{39} showed that aptasensors can offer a very sensitive and highly specific multiplex sensing of the microorganisms \textit{Salmonella enteriditis}, \textit{Staphylococcus aureus}, and \textit{vibrio parahemolyticus} present in food. A multicolor upconversion nanoparticle (UCNP) label was used for the luminescence detection of these microorganisms.

Nanomaterials such as graphene oxide and gold nanoparticles have also been applied to enhance signal intensities in the design of aptasensors for food pathogens. This approach has been employed by Jia \textit{et al}\textsuperscript{40} for the impedimetric detection of \textit{Staphylococcus aureus} in food samples. A similar hybrid nanomaterial sensor was reported by Vanega \textit{et al}\textsuperscript{41} for the internalin A aptasensor detection of \textit{Listeria spp.} using carbon-metal nanoparticles.
A study done by Wu et al \(^2\) used a graphene oxide (GO) signaling for the multiplex sensing of *Salmonella enteritidis* and *Escherichia coli*. They used GO to effectively quench the fluorescence of a labelled aptamer in a FRET approach; the incorporation and hybridization of a target sequence therefore gave rise to a significant fluorescence recovery. They then used this approach for multiplex sensing of both microorganism, using a FAM-modified S.enteriditis aptamer and a Cy3-modified E. coli aptamer. Over 90% fluorescence recovery was observed (Fig. 3.4), showing the applicability of this approach to multiplex aptasensor sensing of food-borne pathogens.

![Fluorescence spectra showing quenching in E. coli O157 (A) and S. enteritidis (B) aptasensors](image)

![Scheme for the multiplex detection of E. coli & S. enteriditis](image)

**Fig 3.4.** Aptsensors for multiplex detection of *E. coli* and *S. enteriditis* in a multiplex assay. Wu et al, 2014
Reviews on the diverse biosensor protocols applied for the detection of food pathogens are available for the interested reader.\textsuperscript{43-48}

3.1.2. Other Biosensors Approaches for Food Pathogen Detection: Though the mostly used biosensor for food microorganisms and pathogen screening and detection employs a DNA based recognition agent, several other types of recognition agents and signal transduction methods have also been applied. Antibodies-based biosensors, also called immunosensors, can also afford a highly specific and sensitive detection of food pathogens and microorganisms. Immunosensors based on electrochemical transduction are also studied extensively for food screening and pathogen detection. Several target-specific immunosensors have been reported. Viswanathan \textit{et al}\textsuperscript{49} reported an electrochemical immunosensor that allowed for the multiplexed detection of food-borne pathogens using a sandwich assay with nanocrystal bioconjugates, and a multi-walled carbon nanotubes (MWCNT) screen-printed electrodes. This approach was used for the detection of \textit{Salmonella}, \textit{Campylobacter} and \textit{E. coli} present in food, meat, and dairy products. This sensor design involved immobilizing anti-\textit{E. coli}, anti-\textit{Salmonella}, and anti-\textit{Campylobacter} on the MWCNT surface, and the specific target was captured during sample treatment. The other sandwich antibody was conjugated with a specific releasable nanometal (\(\alpha\)-\textit{E. coli}-CdS, \(\alpha\)-\textit{Salmonella}-CuS, and \(\alpha\)-\textit{Campylobacter}-PbS), and this attachment and sandwich formation led to the released the attached metal ion from the bound targets, and this step accounted for electroactive detection.

Irudayaraj \textit{et al}\textsuperscript{50} have also described an immunosensor based on gold nanoparticles for in-situ pathogen detection. This design used a network-based ELISA biosensor, and a sample concentration step based on immune-magnetic separation. This biosensor set-up was used in the detection of \textit{E. coli} and \textit{Salmonella typhimurium}. Cho \textit{et al}\textsuperscript{51} also reported a similar
immunomagnetic biosensor, but in this case, it followed an in-situ fluorescence detection protocol. This immunosensor was designed for the multiplex detection of *S. typhimurium, E. coli, and Listeria monocytogenes* in spinach, meat, and milk. It can be seen from these studies that biosensors for the detection of microorganisms and food pathogens mostly involves an electrochemical or optical transduction means. Other approaches to food pathogen detection include SPR, bioluminescence, colorimetric, cell or tissue based assays.52

Bacteriaphages are another approach used as recognition agents for the detection of pathogens in food. Phages are viruses that can inhibit cell activity in a host bacteria, and kill the host by cell lysis. The use of phages in biosensor designs for food pathogens usually employs their peptide and protein structures for recognition of the bacteria, and they are used mostly with the biotin-streptavidin recognition. The metabolic activity of the bacteria is measured mostly with an electrochemical transduction by monitoring a decreasing intensity due to the action of the bacteria-specific phage. An extensive review on the use of phages in biosensor designs for food pathogen detection is available in this text.53

3.1.3. Biosensors for Detecting Pesticides in Foods: Food safety and quality assurance is required to guarantee a risk-free, satisfactory final product before it is sold to consumers. The use of pesticide to enhance the protection and yield of crops in agriculture has placed the extra burden of an added contaminant in food. Because pesticides are persistent and can be extremely lethal even in small concentrations, their monitoring and elimination from food substances is highly important. Biosensors thus represent an important approach to the detection of pesticides in crops and in food products.

Enzyme based biosensors have been reported for the detection of organophosphate pesticides. Single enzyme systems or multi-enzyme assays have been developed. Most single
enzyme biosensors use either acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) as the bioreceptor, and thiocholine production is measured amperometrically, or acid production may be measured potentiometrically. Multi-enzyme systems measure hydrogen peroxide production from the activity of cholinesterase and choline oxidase. An example of a multi-enzyme biosensor was reported by Thierry et al. who incorporated MIPs for a sensitive and selective detection of OPs in olive oil. Their system was based on the immobilization of AChE on a screen-print electrode (SPE) by bioencapsulation in a sol-gel matrix. This gave high performance in operability, reproducibility, and stability.

Most biosensor design for pesticide detection, just as for food pathogens and other microorganisms mostly involve an electrochemical transduction means. This means is versatile as most biological activity involves redox reactions, and electrochemical transduction systems can be applied to almost all recognition events. More recently, Puzari and Dutta described an amperometric biosensor that utilized a polypyrrole entrapped AChE for the detection of organocarbamate and organophosphate pesticides in agricultural storage facilities. The sensor design involves an electro-immobilization of AChE on a polypyrrole surface using gelatin and glutaraldehyde as cross-linking agents. This provided good stability and gave a highly reproducible result, and as such this design was applied to pesticide detection in both wet and dry storage systems.

Single- and multi-enzyme biosensors in pesticide detection have also employed nanomaterials for signal amplification. Wang et al. described a nanohybrid gold nanoparticles and a chemically inreduced graphene oxide nanosheet (GNS) to develop an AChE-AuNP-cr-GNS
electrochemical biosensor for the ultrasensitive detection of an insecticide active agent, paraoxon.

Paraoxon is an organophosphate oxon present in the insecticide parathion and acts as an AChE inhibitor. This sensor proved very sensitive to paraoxons presence in food samples with afforded detection limits in sub-pM levels. This approach was improved more recently by Zhao et al.\(^5^7\) who used a similar biosensor design and added the functionality of a β-cyclodextrin (β-CD) for efficient fixation of the AChE and a Prussian blue-chitosan nanocomposite (PB-CS) in a super-improved detection of organophosphate pesticides. The PB-CS catalyzed the oxidation of thiocholine and shifted its oxidation potential from 0.68 to 0.2 V, thus increasing the sensor sensitivity.

Nanomaterial based enzyme biosensors have been reviewed\(^5^8\) in terms of their design, targets, and comparative sensitivities. Table 3.3 shows some of these biosensor design and applications.\(^5^9\)-\(^6^3\)
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Target</th>
<th>Substrate</th>
<th>Nanomaterial</th>
<th>Sample</th>
<th>Detection Limits</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE</td>
<td>Monocrotophos</td>
<td>SPE</td>
<td>PBNCs/rGO</td>
<td>Cucumber</td>
<td>0.1 nM¹</td>
<td>Zhang et al, 2012</td>
</tr>
<tr>
<td>AChE</td>
<td>Melathion, Chlorpyrifos, Monocrotophos, Endosulfan</td>
<td>Au Electrode</td>
<td>Fe₃O₄/MWCNT</td>
<td>Milk &amp; Water</td>
<td>0.1 nM, 0.1 nM, 0.1 nM, 20 nM</td>
<td>Chauhan &amp; Pundir, 2011</td>
</tr>
<tr>
<td>AChE+CHO</td>
<td>Chichlorvos</td>
<td>Liquid phase</td>
<td>CdTe–QDs</td>
<td>Apple</td>
<td>4.49 nM</td>
<td>Meng et al, 2013</td>
</tr>
<tr>
<td>OPH</td>
<td>Methyl Parathion</td>
<td>GCE</td>
<td>Graphene–ZrO₂</td>
<td>Garlic</td>
<td>0.1 nM</td>
<td>Du et al, 2011</td>
</tr>
<tr>
<td>AChE</td>
<td>Chlorpyrifos, Carbofuran</td>
<td>Au Electrode</td>
<td>Graphene–ZnO</td>
<td>Water, Field crops</td>
<td>1.0 pM, 0.01 pM</td>
<td>Zhou et al, 2014</td>
</tr>
</tbody>
</table>

Table 3.3: Enzyme based biosensors using nanomaterials for pesticide detection

Other biosensor designs other than the ones already described have also been reported for pesticide monitoring and detection in food products. An example of such is the colorimetric detection of carbendazim fungicide in agricultural crops, food samples and environmental water using a 4-aminobenzenthiol functionalized silver nanoparticles (ABT-Ag NPs) as SPR probes.⁶⁴ The ABT-Ag NPs binds with the carbendazim through a strong ion pair and π–π interactions. This interaction causes a change in the SPR properties of the probe, and a red-shifted absorbance from 397 nm to 510 nm (yellow – orange) which is detectable by visual inspection. This biosensor design gave a detection limit of 1.04 µM, and a result reproducibility of 100%. Kim et al.⁶⁵ has described a miniaturized gold/metal chip electrode for the fast (2 – 3 mins), real-time, in-situ detection of several pesticides using a wireless communication sensor system. A visual screening card has been described by Guo et al.⁶⁶ which incorporated a Hybond N+ nylon membrane immobilized enzyme for the detection of pesticides in food samples. They showed their biosensor to offer great specificity, detectable visual changes, and had detection limits in the range of 0.001 – 0.1 M for different pesticides. Further reviews⁶⁷-⁶⁹ are available that describes in more details
the several available/studied biosensor designs used in pesticide detection and applied in food safety, agricultural studies, and environmental screening.

3.1.4. Biosensors for Detection of Natural Toxins: Toxins are a significant health risk to both plants and animals, and their presence in food is one of the biggest known harmful threats to human health. Some toxins are natural defence mechanisms, like those secreted by plants for protection against insect attacks, or those in fruits and vegetables for resistance to some strains of diseases. Most toxins occur as a result of plant damage such as the growth of molds on plants. A third class of toxins are those produced by sea or freshwater microalgae e.g. microcystins are cyanotoxins produced by cyanobacteria in sea bodies, and are known to be hepatotoxic. Some microorganisms also produce harmful toxins such as botulinum neurotoxin which is the most potent toxin discovered and a cause of botulism induced paralysis, enterotoxins which are harmful to intestinal cell walls, and cholera toxins which gives rise to cholera infections.

Toxins are ubiquitous in nature, and their adverse health effects on exposed organisms is a major source of concern. Although humans have detoxifying pathways such as metabolism and excretion processes, and can be exposed at a low concentration where there is expected to be no adverse effects, maximum permitted levels are encouraged by regulatory bodies. Health risk assessors also consider mycotoxins from all natural toxins to be the most significant dietary risk factor. Some mycotoxins such as genotoxic aflatoxins, are so toxic that there is no safe exposure limit and can cause adverse health effects even at very low concentrations. Phycotoxins are also known to have some serious adverse health effects, and they are produced by marine microalgae. Mycotoxins and phycotoxins represents the major classes of natural toxins that poses health risks for exposed human population. Table 3.4 gives a list of the types of these toxins and their known adverse effects.
<table>
<thead>
<tr>
<th>Toxin</th>
<th>Source Organism</th>
<th>Contaminated Food</th>
<th>Health Effects on Humans (IARC* Classification)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mycotoxins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxins (B&lt;sub&gt;1&lt;/sub&gt;, B&lt;sub&gt;2&lt;/sub&gt;, G&lt;sub&gt;1&lt;/sub&gt;, G&lt;sub&gt;2&lt;/sub&gt;, M&lt;sub&gt;1&lt;/sub&gt;, M&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>Aspergillus genus <em>(falvus, nomius &amp; parasiticus)</em></td>
<td>Groundnuts, other edible nuts, peanut butter, figs, spices, corn, rice, cassava, tobacco, seeds, milk, yogurt, cheese.</td>
<td>Hepatomegaly, jaundice, hepatitis, cirrhosis, liver cancer, Reye’s syndrome, immunosuppression. (Group 1: Carcinogenic)</td>
</tr>
<tr>
<td></td>
<td>Aspergillus genus <em>(alliaceus, auricomon, carbonarius, glaucus, melleus, niger, ochraceus)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Penicillium genus <em>(cyclopium, verrucosum, viridicatum)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td></td>
<td>Cereals and derived products, rice, dried vine fruits, coffee, cocoa, wine, beer, spices, pork, cheese.</td>
<td>Kidney damage (i.e. Balkan endemic neuropathy) (Group 2B: Possible carcinogenic)</td>
</tr>
<tr>
<td>Patulin</td>
<td>Aspergillus spp.</td>
<td>Fruits, vegetables, cereals, pies, jam, apple juice, animal silage</td>
<td>Stomach disturbances, nausea, vomiting, gastrointestinal hyperemia, ulceration, hemorrhages. (Group 3: Carcinogenic properties not classifiable)</td>
</tr>
<tr>
<td></td>
<td>Byssochlamys spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Penicillium <em>(expansum and griseofulvum)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumonisins (B&lt;sub&gt;1&lt;/sub&gt;, B&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>Aspergillus alternata, and Fusarium genus</td>
<td>Cereals and derived products, rice, beans, asparagus, beer</td>
<td>Esophageal cancer (Group 2B: Possible carcinogen)</td>
</tr>
<tr>
<td>Zearalenone, deoxynivalenol, nivalenol, fusarenone X</td>
<td>Fusarium genus <em>(culmorum, crookwellense, graminearum)</em></td>
<td>Cereals and derived products, rice, walnuts, milk, beer, meat, animal-feed products</td>
<td>Acutely toxic in humans, gastrointestinal and abdominal pains, vomiting, diarrhea, cervical cancer (Group 3: Carcinogenic properties not classifiable)</td>
</tr>
</tbody>
</table>

**Phycotoxins**
Diarrhea shellfish poisoning (DSP) toxins

- *Dinophysis* genus,
  - *Gonyaulax polyhedral*,
  - *Phalacroma rotundatum*,
  - *Protoceratium reticulatum*,
  - *Protoperidium* genus,
  - *Prorocentrum* genus

- Clams, mussels, oysters, scallops

- Gastrointestinal disorders, nausea, abdominal pain, vomiting, diarrhea, tumor promoters, immunotoxic agents, cardiac muscle damage, liver damage

Paralytic shellfish poisoning (PSP) toxins

- *Alexandrium* genus,
  - *Aphanazomenon flos-aquae*,
  - *Spondylus butler*,
  - *Gymnodinium catenatum.*

- Clams, crabs, gastropods, lobsters, mackerels, mussels, oysters, puffer fish, salmon, starfish, whales, whelks.

- Neurological and gastrointestinal (nausea, vomiting, diarrhea), muscular incoordination, dysmetria, respiratory distress, death

Amnesic shellfish poisoning (ASP) toxins

- *Alsidiun corallinum*,
  - *Amphora coffeiformis*,
  - *Chondria armata*,
  - *Pseudo-nitzschia* genus.

- Clams, crabs, gastropods, lobsters, mackerels, mussels, oysters, scallops

- Neurotoxic and neurological effects (memory loss, disorientation), gastrointestinal (abdominal cramps, nausea, vomiting, diarrhea) optical problems (disconjugate gaze, diplopia, and ophthalmoplegia), neurological deficits (confusion, mutism, seizures, autonomic dysfunction, lack of response to painful stimuli, uncontrolled emotions and aggressiveness), coma, death.

*IARC = International Agency for Research on Cancer

Table 3.4: List of Important Mycotoxins and Phycotoxins

Hence the need for reliable detection means is important for food safety and human health. There is a need for rapid, robust, sensitive, and specific analytical methods that can be integrated into small, portable devices for off-site and field tests. Electrochemical sensors, just as in detection of food-borne pathogens and pesticides, are also the major transduction means for biosensors applied to toxin detection. Ben Rejeb et al.\(^{73}\) used an AChE-based biosensor for the determination of AFB\(_1\) in olive oil. Their design used an immobilized choline oxidase (ChOx) as recognition agent.
which detected H$_2$O$_2$ at a low potential of -0.05 V, when the AChE present in solution acted on the target in the olive oil sample. ChOx was immobilized via a cross-link on screen-printed electrodes which had been modified with Prussian Blue for signal amplification. Immunosensors are also well studied for their detection of toxins. An example is the monoclonal antibodies developed by Kfir et al$^{74}$ for the detection of microcystins, which was the first reported case of an immunosensor for toxins. Several improvements have been applied to this biosensor approach, and several immunosensors against microcystins have also been developed since then. Some have used fluorescent immunosensors,$^{75}$ chemiluminescence,$^{76}$ SPR,$^{77}$ colorimetric,$^{78}$ piezoelectric,$^{79}$ and of course electrochemical transduction means.$^{80,81}$ Volland et al$^{82}$ described a competitive enzyme immunoassay that provided broad specificity in the quantification of microcystins (MC-LR, MC-LA, and MC-YR) in water samples. Monoclonal antibody, mAb MC159, was used as recognition agent, and this could detect an amino functionalized MC-LR coupled to bovine serum albumin (BSA). The broad specificity of this sensor for different MCs has allowed its applicability in the determination of total MCs present in desired samples. Immunosensors and other recognition agents for the detection of microcystins haas been reviewed by Weller et al.$^{83}$

Aptamers are also vital recognition tools in the detection of MCs. Most aptasensor designs for MCs are incorporated with electrochemical detection such as voltammetry. Zuorob et al$^{84}$ designed a square wave voltammetry (SWV) aptasensor for the specific detection of MC-LR on a graphene-modified screen printed carbon electrode in a [Fe(CN)$_6$]$^{4-/3-}$ redox solution system. Graphene oxide as discussed earlier is an effective quencher of aptamer fluorescence, and labelled aptamers can give rise to increased fluorescence when they bind to targets and are detached from the GO surface.
This is especially important for large molecule detection, as they can cause a significant change in the aptamer conformation upon binding. Several electrochemical aptasensors relies on this approach. For this aptasensor, a label-free 60-nucleotide DNA aptamer (5'-GGC GCC AAA CAG GAC CAC CAT GAC AAT TAC CCA TAC CAC CTC ATT ATG CCC CAT CTC CGC-3') caused a significant drop in SWV signal of the Fe redox system, and the presence of MC-LR increased the signal intensities, which can now be used for detection. Hence their work represented the use of aptasensors for the specific detection of the small molecule, MC-LR, in water samples.

Aptamers are an interesting candidate for the specific recognition of several target toxins and are therefore extensively applied in toxin detection. This is due to their high sensitivities and low detection limits, the simplicity in their design and ease of miniaturization, and their application versatility to almost all transduction means. The use of aptamers have been recorded for several toxins and some new studies showing interesting advantages of aptasensors for natural toxins such as OTA, AFB₁, Okadaic acid, MC-LR, fumonisins, and zearalenones have been reported.
Hairpin DNA are also been reported as probes for the detection of naturally occurring toxins in food samples. Ha et al\textsuperscript{92} studied a colorimetric hairpin DNA aptamer which provided an optimized sequence and allowed the covalent conjugation of a hemin cofactor. This approach was used in a simple optical detection of ochratoxin A. In their design, the hemin cofactor was covalently attached to the hairpin sequence, and the hairpin stem was loosened to form a G-quadruplex which expresses HRP activity as depicted in Fig. 3.7.

Cell-based detection is another noble pursuit towards the design of biosensors for toxin detection. This approach relies on the known activity of these toxins on their target cells, and these cells are employed as the recognition agents. An example of this is the cardiomyocyte-based impedance biosensor developed by Wang et al\textsuperscript{93} for use in the detection of toxins that target cardiac muscle cells. This approach provided a portable, label-free, real time toxin detection system, by monitoring cardiomyocyte cell index, beating rate, and growth status.

![Fig. 3.7: Design of colorimetric aptasensor for OTA detection. Ha et al, 2014.](image)
Microfluidics are also an emerging area in bioanalysis. Microfluidics mini systems uses a small volume of fluid (µL, nL, pL, fL) which is mixed, moved, separated, or processed, as these systems exploit the theory of geometrically constrained fluids during this movement. Microfluidic devices were first introduced in the 1970s, and is now used in several fields such as in biological, chemical, and diagnostic analysis. The advantages of these systems is in the small amounts of sample and reagent required, portability, versatility, rapid response, high sensitivities, and a high throughput analysis. Microfluidics have been greatly applied to biosensors and have employed optical and electrochemical transduction means. These microfluidic sensors have been applied to detection of cardiac biomarkers,$^{94,95}$ cancer biomarkers,$^{94,96,97}$ mycotoxins,$^{98}$ and bacterial toxins.$^{99}$

Baeumner et al$^{100}$ recently reported a microfluidic immunosensor that immobilized antibodies specific to Cholera toxin subunit B (CTB) on a supermagnetic bead for the detection of cholera toxin. Two different transduction means i.e. electrochemical and fluorescence was used for detection, and both showed good sensitivities for the detection as carried out in CTB-spiked stool sample.

Further review on these designs and their related advantages is found in these texts.$^{101-104}$

3.2. Biosensors in Environmental Health:

Due to the rise in global chemical and synthetic compounds production, humans have become exposed to many chemicals that may pose serious adverse health effects. The detection and determination of these chemical pollutants has become an advancing area of preventive medicine, and a useful estimate of determining human risk from toxic environmental pollution. Environmental pollution affects a host of factors such as agriculture and crops, soil, water and aquatic bodies, and air.
3.2.1. Biosensors in Soil and Water Applications: Several toxicants find their way into soil and water from various natural or anthropogenic processes such as agricultural activities, industrial spills, and water run-offs. These chemical toxicants may be trapped in soil particles or dissolved in water bodies, and thus can affect the nutrient availability for crops and growth and reproduction of sea animals, which destabilizes the ecosystem. Biosensors are an effective way of detecting the presence of, and monitoring levels of chemical toxicants present in soil and water bodies.

3.2.1.1. Biosensors for Detecting Soil Contaminants: The characterization of main pollutants in soils is very challenging due to the complicated matrix of soils. Whole cell biosensors are an important class of biosensor for soil toxicity studies. Bacteria whole cell sensors can provide for rapid, sensitive, easy-to-use, quantitative measure of contaminants in soils. Wang et al\textsuperscript{105} described a bacteria whole cell biosensor using \textit{acinetobacter baylyi} ADPI and bioluminescence transduction in the detection of soil and water contaminants such as toluene, xylene, crude oil, and salicylate. The same bacteria was used by Song \textit{et al}\textsuperscript{106} in a whole-cell bioreporter gene approach to study toxic compounds present in contaminated Chinese soils. This approach measured the effect of present toxins against the specific \textit{acinetobacter baylyi} strain ADPL\_recA\_lux as determined from changes in its bioluminescence. They showed that this sensor could effectively detect the presence of mitomycin C, Benzo[\(\alpha\)]pyrene, cadmium (VI), and lead (II) without any form of soil pretreatment.\textsuperscript{106}

Soil toxicity is usually monitored by the presence of heavy metals as their presence in large quantities is toxic to plants and may lead to ecosystem destabilization. A whole cell biosensor based on the bacteria \textit{Anabaena torulosa} was designed by Surif \textit{et al}\textsuperscript{107} for the detection of toxic metals such as copper, lead, and cadmium; and other toxicants such as the pesticide, 2,4-
dichlorophenoxyacetate (2,4-D), and the toxin chlorpyrifos. The cyanobacteria was immobilized on a cellulose membrane for attachment to an optical probe making it possible for target detection by fluorescence.

Biosensors have been widely employed for heavy metal detection in both soil and water bodies, and they can be categorized into two broad parts: Enzymatic biosensors, and DNA-affinity biosensors. Enzyme families that are majorly applied to heavy metal detection include oxidases, phosphatases, dehydrogenases, and ureases. Since these metals are toxic to enzymes, the activity of enzymes decreases when heavy metals are sufficiently present, and thus most biosensors for heavy metal detection depend on enzyme-inhibition studies. Glucose oxidase (GOx) enzymes are popular in electrochemical biosensors, and they have also been used for enzyme inhibition assays of heavy metal cations. A study by Ghica et al\textsuperscript{108} in their electrochemical enzyme biosensor used GOx immobilized by crosslinking with BSA and glutaraldehyde to the surface of a carbon film electron that has been modified with copper hexacyanoferrate (the redox mediator). They applied this set-up for the detection of heavy metals in soil and aquatic bodies. The inhibition of GOx when in contact with heavy metals like copper, cadmium, nickel, and cobalt resulted in reduced enzyme activity which was measured with an electrochemical impedance spectra. In another study, the same authors have shown the advantages of the redox mediator, hexacyanoferrate, over other redox mediator such as poly(neutral red) as it provides a high sensitive and rapid assay.\textsuperscript{109}

Some studies have advocated multi-enzyme use in these biosensors for a broader detection of heavy metals and environmental pollutants. The challenge this approach faces is that different enzymes have different optimal conditions, and as such both cannot be immobilized on the same surface without losing or reducing the optimal activity of one. Enzyme electrodes have been proposed as a solution, but this has its own challenges in terms of cost and simplicity of use.
Whole cell biosensors (discussed above) is also another solution proposed. Lagarde *et al*\textsuperscript{110} have achieved the use of a two-enzyme biosensor system in the detection of heavy metals and pesticides. They used a microorganism, *Spirulina*, which is a Gram negative bacteria known as *Arthropira platensis*. This bacteria has the activity of phosphatase and esterase enzymes, and this was exploited in a dual-environmental screening; as the phosphatase enzyme activity is hindered by heavy metals, and pesticides inhibit the activity of the esterase enzyme. The *Spirulina* cells were immobilized on a gold interdigitated working electrode, while BSA was immobilized on a reference electrode. Their design allowed for the conductometric determination of the kinetics for both enzymes using gold nanoparticles for signal amplification.

A similar study was reported earlier by Soldatkin *et al*\textsuperscript{111} using a three-enzyme biosensor approach for a sensitive detection of silver and mercury cations. The enzymes invertase, GOx, and mutarotase were immobilized on a gold interdigitated electrode, and their inhibition studies from the action of these heavy metals was determined by a conductometric means. This same group recently reported a GOx/Urease bi-enzyme biosensor\textsuperscript{112} for the detection of heavy metals, urea, and glucose using an electrochemically etched nanoporous silicon for detection. The detection protocol relied on quantum yield changes in a porous silicon photoilumiscent material at varying pH ranges, and the photoiluminescence of the material increased when glucose was introduced, and decreased when toxic metals (Cu\textsuperscript{2+}, Cd\textsuperscript{2+}, and Pb\textsuperscript{2+}) was introduced. Thus heavy metals quenched the luminescence of the material (Fig. 3.8), and this property was employed for detection.

A similar approach has been reported recently by Shtenberg *et al*\textsuperscript{113} using horseradish peroxidase (HRP) immobilized on a nanoporous silicon and Fabry-Perot thin film to monitor heavy metal ions in water samples. Fourier transform spectroscopy and optical studies reveal the high
specificity and sensitivity of this approach in detecting Ag\textsuperscript{+}, Pb\textsuperscript{2+}, and Cu\textsuperscript{2+}. They also showed that the enzyme laccase, rather than HRP, showed a specific detection of Cu\textsuperscript{2+}, and both approaches gave detection limits of 60 – 120 nM, which is in the environmental relevant concentration.

DNA based biosensors are extensively applied to heavy metal detection in soil and water samples. Although spectroscopic approaches such as atomic absorption spectroscopy (AAS), and inductively coupled plasma atomic emission spectroscopy (cp-AES) provide high sensitivity for heavy metal cation detection such as Pb\textsuperscript{2+},\textsuperscript{114} they do not provide accurate, real-time and on-site measurements as are now needed for the detection of heavy metal in the environment. DNAzymes, also called ribozymes, are a very promising class of recognition agents now employed for biosensor designs as they require these metal cations as cofactors for their numerous catalytic actions. The advantages they offer are stability to hydrolysis, cheap and easy production, and
versatility in mode of action with uses as antiviral agents, biosensors, and nanodesign applications.\textsuperscript{115-118}

The 8-17 DNazyme is a DNA metalloenzyme that can catalyze RNA transesterification process in the presence of divalent ions with orders of activity as Pb\textsuperscript{2+} $\gg$ Zn\textsuperscript{2+} $\gg$ Mg\textsuperscript{2+}. A study by Kim \textit{et al}\textsuperscript{119} showed by FRET studies that Zn\textsuperscript{2+} and Mg\textsuperscript{2+} induced global folding in the 8-17 DNazyme into a compact structure, with stem III approaching a stem I and II defined configuration without altering the stem angle. Pb\textsuperscript{2+} does not induce this folding, and this may account for its high activity in DNazyme catalytic RNA transesterification. An internal probe such as a FRET pair Cy3/Cy5 was placed at 5’ and 3’ end of either 17E (enzymatic DNA strand with all-DNA oligonucleotide sequence) and 17S (substrate strand that contains a single riboadenosine [rA] which is the cleavage site) between stems I and III (Fig. 3.9C), I and II (Fig. 3.9D) and II and III (Fig. 3.9E). This labelling positions is shown not to disturb the metal ion activity, and detection can be easily measured from metal-ion induced cleavage. This approach has been applied to metal cation detection in most water sample assays.

The detection of the heavy metal cation induced DNazyme activity can be measured by effects other than fluorescence. Tsekenis \textit{et al}\textsuperscript{120} recently reported the use of the 8”-17” DNazyme in a capacitive microchemical sensor array which was used for the detection of Pb\textsuperscript{2+}. The DNA ribozyme was immobilized to the sensor surface by laser printing, where it hybridizes with a DNA substrate, and cleaves the substrate DNA strand. This is achieved in the presence of Pb\textsuperscript{2+}, and substrate strand cleavage leads to surface stress changes in the sensor, which can be detected by monitoring the capacitance changes in the device. The sensor was able to detect as low as 10 µM of Pb\textsuperscript{2+}. 
Another similar approach as reported by Tang et al using a simple portable personal glucometer (PGM)-type detection. The DNAzyme here was immobilized to a streptavidin-modified microplate, and a signal transduction tag made of GNP labeled ssDNA and functionalized with the enzyme invertase (Enz-GNP-DNA) was also immobilized to the microplate. The introduction of the Pb$^{2+}$ produced cleavage of the of the ssDNA substrate attached to the DNAzyme, and this cleaved ssDNA can now hybridize to Enz-GNP-DNA. The new hybridization induces the activity of the enzyme invertase, which produces glucose, and the produced glucose can be monitored by the PGM for an in situ signal readout.

Fig. 3.9: 8-17 DNAzyme and constructs for FRET studies on heavy metal detection based on cation induced folding. Kim et al, 2007
In a different approach, Li et al.\textsuperscript{121} developed a DNA molecular beacon probe immobilized on the surface of digital video discs (DVDs) for the detection and quantification of Hg\textsuperscript{2+} and Pb\textsuperscript{2+}. The hairpin DNA sequence used here had T-rich or G-rich loop sequences, and detection was based on opening of hairpin DNA loop structures either by G-quadruplex formation as induced by Pb\textsuperscript{2+}, or by a T-Hg\textsuperscript{2+}-T coordination chemistry.

A recent report by Juewen Liu\textsuperscript{122} showed the ability of DNA-stabilized metal nanoclusters as fluorescent biosensors for the detection of heavy metals and related environmental targets. The detection in these biosensors mostly involves fluorescence quenching or emission of the DNA sensor depending on the design. Yuan et al.\textsuperscript{123} described the detection of Hg\textsuperscript{2+} and Ag\textsuperscript{+}, using DNA-derived bio-dots with a graphite carbon layer. The bio-dots exhibited high fluorescent properties, and could easily capture the target metal cations due to the residual nucleobases that favour interaction, which also leads to fluorescence quenching. Several other detection means are possible aside from those already discussed. Some studies on DNA-based enzyme detection of metals use graphene for fluorescence control,\textsuperscript{124} nanomaterials assembled by DNA or aptamers using Resonance Rayleigh Scattering (RRS) spectral assay,\textsuperscript{125} aptamer functionalized monolithic hydrogel microparticles, with detection occurring in a fluorescent microarray bead.\textsuperscript{126} The field of heavy metal detection is thus highly explored. Some reviews are also available on extensive discussion of heavy metal detection using whole cell detection,\textsuperscript{127} enzymatic-based sensors, macro-, micro-, and nano- materials,\textsuperscript{128} and a more extensive review of several detection protocols.\textsuperscript{129}

3.2.2. Biosensors for Detecting Endocrine Disruptors and Other Environmental Pollutants: The last 50 years has witnessed a rise in the release of toxic chemicals and pollutants into the
environment mostly from anthropogenic sources. This has necessitated the need for new systems for accurate real-time monitoring and environmental screening. Conventional techniques such as GC, MS, and HPLC offer high sensitivities for detecting several pollutants, but also suffer from the drawback of high costs, lab based analysis, and the need for trained staff or personnel for the sample pretreatment process before it is assayed. Biosensors however have offered alternative screening procedures, with the advantage of ease of miniaturization, inexpensive analysis, easy to perform assays, and a sensitive detection of a broad range of environmental pollutants.

**3.2.2.1: Biosensors for Endocrine Disruptor Chemicals (EDCs):** EDCs are synthetic chemical compounds that can mimic (agonists) or block (antagonists) the action of hormones, thus disrupting normal biological or physiological functions. The chemical structure of most EDCs closely resemble the hormone which they mimic, and as such can fit into the hormone receptor, thus activation or suppressing their hormonal functions. EDCs can be highly harmful even at very low doses, and they affect the younger population more. EDCs are found in household product ingredients, personal care products, food additives, flame retardants, plastic and rubber, pesticides, pharmaceuticals, antimicrobial, and biogenics. Table 5 gives a list of some very important EDCs, their hormonal mimic, and their health effects.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>EDCs</th>
<th>Mode of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>Dioxins &amp; Furans</td>
<td>Anti-estrogenic</td>
</tr>
<tr>
<td></td>
<td>PBBs &amp; PCBs</td>
<td>Anti-estrogenic</td>
</tr>
<tr>
<td>Aldrin</td>
<td></td>
<td>Competetive estradiol antagonist</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td></td>
<td>Estrogenic</td>
</tr>
<tr>
<td>DDT</td>
<td></td>
<td>Estrogen agonists and antagonists</td>
</tr>
<tr>
<td>Phtalates</td>
<td></td>
<td>Binds to estrogen receptor</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td></td>
<td>Binds to estrogen receptor</td>
</tr>
<tr>
<td>Cadmium</td>
<td></td>
<td>Activates estrogen receptor</td>
</tr>
<tr>
<td>Thyroid</td>
<td>PBDEs</td>
<td>Interferes with throxine (T4) binding</td>
</tr>
<tr>
<td>EDCs</td>
<td>Effect</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>PBBs, PCBs, &amp; PCP</td>
<td>Binds thyroid hormone binding protein, but not thyroid hormone receptor</td>
<td></td>
</tr>
<tr>
<td>Acetochlor &amp; Alachlor</td>
<td>Decrease thyroid hormone levels</td>
<td></td>
</tr>
<tr>
<td>Melathion</td>
<td>Decrease thyroid hormone in serum</td>
<td></td>
</tr>
<tr>
<td>Androgen</td>
<td>Mirex, Vinclozolin</td>
<td>Anti-androgen</td>
</tr>
<tr>
<td>Phtalates</td>
<td>Anti-androgen</td>
<td></td>
</tr>
<tr>
<td>Benzo[α]pyrene</td>
<td>Androgenic</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>Chlordane</td>
<td>Induce progesterone</td>
</tr>
<tr>
<td></td>
<td>Carbaryl</td>
<td>Progesteronic</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Atrazine</td>
<td>Depress LH, reduce testosterone</td>
</tr>
<tr>
<td></td>
<td>Chlordane</td>
<td>Induce testosterone</td>
</tr>
</tbody>
</table>

**Table 3.5: Some environmentally relevant EDCs**

Electrochemical biosensors are widely used in the detection of EDCs present in water, food, and biological or environmental samples. In most cases, these biosensors require an enzyme as recognition agents, or as the biological catalyst in a signal generating redox process. Zhang *et al.* designed an enzyme-based electrochemical biosensor for the detection of bisphenol A in a simple, sensitive, and rapid approach. This amperometric biosensor was based on an oxidoreductase enzyme, but a laccase or tyrosinase can also be used. The enzyme catalyzed the σ-hydroxylation of the phenol to catechol, and a subsequent oxidation of the catechol to an σ-quinone. The electrochemical reduction of the σ-quinone is used to generate an electrical signal, and this approach was shown to be a sensitive to the detection of phenolic compounds. This is a standard template (Fig. 3.10) for electrochemical enzymes used in detecting most phenolic compounds.
Several methods have been used to improve signals in electrochemical biosensors used for bisphenol A detection. One of this approach involves the use of a functionalized graphene oxide (GO) hybrid with gold nanoparticles as designed by Liu et al.\textsuperscript{130} In this approach, the GO was first prepared by covalently grafting an electron mediator, (4-ferrocenylethyne) phenylamine (Fc-NH\textsubscript{2}), to the GO surface. The GO-Fc-NH\textsubscript{2} was then coupled to GNPs, and electrodeposited to a glassy carbon electrode (GCE). This GO/Fc-NH\textsubscript{2}/GNPs film can act as electron film, thus preventing leakage and improving sensitivities. This method achieved detection as low as 2 nM.

A functionalized GO-GNP electrochemical is widely used for signal amplification in biosensors for bisphenol A. Some approaches involve the use of tyrosinase enzyme with a GO-GNP functionalized electrode,\textsuperscript{131,132} Wang \textit{et al}\textsuperscript{133} used an antibody as recognition agent rather than the enzyme based detection, while Zhou \textit{et al}\textsuperscript{134} used an electrochemical biosensor with a GO-GNP system for detecting bisphenol A in milk samples. Other variations in signal transduction

\textbf{Fig. 3.10: Schematic representation of a tyrosinase-based biosensor for detection of bisphenol A in a polluted aqueous solution} \textit{Zhang \textit{et al}, 2015}

\begin{align*}
(a) \quad \text{Phenol} + O_2 & \xrightarrow{\text{oxidoreductase}} \text{Catecol} \\
(b) \quad \text{Catecol} + O_2 & \xrightarrow{\text{oxidoreductase}} \text{o-Quinone} \\
(c) \quad \text{o-Quinone} + H^+ + 2e^- & \rightarrow \text{Catecol}
\end{align*}
have used a 3D copper-metal organic framework (Cu-MOF),\textsuperscript{135} surface reorganization of carbon nanotubes,\textsuperscript{136} or the nitrogen doped graphene sheets as reported by Fan \textit{et al}\textsuperscript{137}.

Several other transduction means are applied to biosensors used in the detection of bisphenol A. Optical means using aptasensors is highly studied, such as the portable optic fiber sensor with immobilized aptamers as described by Gu \textit{et al}\textsuperscript{138} or the label-free aptasensor immobilized on GNP reported by Mei \textit{et al}\textsuperscript{139} which afforded the visual detection of bisphenol A in water samples. Other approaches that have been recently reported include the use of MIPs,\textsuperscript{140} Fullerene C-60,\textsuperscript{141} QCM,\textsuperscript{142} amongst many others.

Biosensors are applied to the detection of several other endocrine disruptors. Biosensors for pesticides, as in the case of chlordane,\textsuperscript{143} DDT,\textsuperscript{144} mirex,\textsuperscript{145} aldrin, and atrazine\textsuperscript{146,147} follow a similar approach as discussed earlier under section 3.1.3. Biosensors as used in detection of heavy metals such as cadmium have also been discussed. Several biosensor design has also been studied and described for the detection of other EDCs such as Benzo[\(\alpha\)]pyrene,\textsuperscript{148,149} 17\(\beta\)-estradiol,\textsuperscript{138,150,151} and phtalates.\textsuperscript{152}

3.2.2.2. \textbf{Biosensors for Other Environmental Pollutants:} Benzo[\(\alpha\)]pyrene is a member of the class of environmental pollutants known as polycyclic aromatic hydrocarbons (PAHs). PAHs are of great environmental concern as they cause severe adverse health effects such as cancer and tumor formation, mental impairment, muscoskeletal diseases, teratogenic effects, and of course most are endocrine disruptors. Some non-severe health effects of PAHs include gastrointestinal, pulmonary, dermatologic, and renal disorders. Anthropogenic sources plays the most significant role in the increase of PAHs in the environment. Some of these sources include tars and pitch products, oil spills and fossil fuels, automobile exhausts, coal burning, tobacco smokes, and forest
fires. 16 PAHs have been classified by the US environmental protection agency (EPA) has priority PAHs, the European Union (EU) has its 15+1 (15 PAHs was first identified, with an additional one added in 2005) priority PAHs, while the US Agency for Toxic Substances and Disease Registry (ATSDR) has a list of 17 priority PAHs.153 (Table. 3.6)

![PAH structures]

Table 3.6: Chemical structures of environmentally relevant PAHs. (See Table 3.6)

[K-region is a region of high electron density, bay region is a relatively hindered 'inner core' region, while fjord region is an even more hindered 'inner core' region than the bay region]

<table>
<thead>
<tr>
<th>PAH</th>
<th>EPA Priority*</th>
<th>EU 15+1</th>
<th>ATSDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenaphthylene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Anthracene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>PAH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Pyrene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[a]fluorene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cyclopenta[cd]pyrene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Triphenylene</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Chrysene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>6-methylchrysene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[j]fluoranthene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[e]pyrene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Perylene</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Indeno[1,2,3-cd]pyrene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[ghi]perylene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[ah]anthracene</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[al]pyrene</td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[al]pyrene</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Benzo[ai]pyrene</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Benzo[ae]pyrene</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

*Original 16 US EPA priority PAHs included napthalenee, which is not technically a PAH

Table 3.6: Important PAHs in environmental health

Because the genotoxic ability of Benzo[α]pyrene has been well studied, it has become the basis for classifying all other PAHs, and biosensor designs for PAHs use Benzo[α]pyrene as a detection model. Antibodies and aptamers are applied in most biosensor designs that detect PAHs. Because most PAHs are small molecules with M.W less than 500 a.m.u, their actual structure can be recognized by antibodies. However to create an immuno response for the specific antibody creation, the small PAH molecule must be part of a larger structure such as a protein. (Fig. 3.12)
Electrochemical and optical transduction can be easily applied to immunosensors used in the detection of PAHs. Wei et al.\textsuperscript{154} designed an amperometric biosensor that allowed nM detection limits for Benzo[α]pyrene. In their approach, the Benzo[α]pyrene was immobilized on gold electrodes, and the specific antibody was allowed to bind to the electrode when immersed in a solution containing a ferricyanide redox probe $[\text{Fe(CN)}_6^{3-/4-}]$. The recognition of the antibody to the Benzo[α]pyrene increased the hydrophobic layer around the electrode, thus decreasing redox current, and this effect was translated for signal generation. This approach is incorporated into most electrochemical biosensors that uses antibodies for the specific detection of PAHs, as the recognition process leads to changes in the redox process, hence a measurable signal is generated.

Fig. 3.12: Schematic for the generation of antibody specific to PAHs using benzo[α]pyrene as example. Mancini et al, 2008
Variations to this approach has seen the use of dendritic-nanosilica to increase the working electrodes surface area, thus improving sensitivites and providing for a more rapid detection. Lux et al\textsuperscript{148} used an electro-switchable biosurface (ESB) to detect Benzo[\(\alpha\)]pyrene in water samples. The ESB was functionalized with a DNA nanolever probe to which the Benzo[\(\alpha\)]pyrene was attached, and they were made to switch orientions based on association kinetics with the antibody. This biosensor configuration gave detection limits in concentrations as low as 1 pM. Other reported transduction means that immuosensors have applid for detecting PAHs are QCM techniques with GNPs,\textsuperscript{156} fluorescence using biotinylated reporter DNA probes,\textsuperscript{157} piezoelectric,\textsuperscript{158} and SPR approaches.\textsuperscript{159}

Electrochemical DNA detection of Benzo[\(\alpha\)]pyrene and other PAHs is also widely studied, and offers sensitivities and detection limits that equals that of immuosensors. Del Carlo et al\textsuperscript{160} reported the use of ssDNA using graphite SPE for the detection of Benzo[\(\alpha\)]anthracene and phenanthrene. Other studies of a DNA biosensor for the environmental detection of PAHs have been reported such as the sol-gel derived dsDNA array biosensors used by Lee et al\textsuperscript{161} which employed an ethydium bromide dye for a multiplex detection of some PAHs, and the DNA-hemin/nafion-graphene/GCE electrochemical biosensor reported by Ni et al\textsuperscript{162} for the detection and determination of Benzo[\(\alpha\)]pyrene.

Biosensors have become a widely accepted tool in the screening and detection of environmental pollutants. They offer sensitivities as high as conventional methods, give rapid results, and are easy to use. The demand for small, portable devices that can be used for field and off-site tests makes biosensors a very attractive choice for the screening of environmental pollutants. The advancements in design, and the compability of several recognition sensor and transduction means has also increased the number of available biosensors for various detection
models. Nanomaterials is also another contributing factor that has improved the sensitivities of these biosensors, as they help signal amplification. Studies also continue to abound, researching more efficient approaches, thus biosensor designs will continue to improve and has the potential to be a strong analytical tool for a long time to come.

References


41. D.C., V.; Rong, Y.; Schwalb, N.; Hills, K. D.; Gomes, C.; McLamore, E. S. Smart *Biomedical and Physiological Sensor Technology XII*, Baltimore Maryland, USA, **2015**.


43. Palchetti, I.; Mascini, M. *Analytical and Bioanalytical Chemistry* **2008**, *391*, 455-471.


70. Svircev, Z.; Tokodi, N.; Drobac, D.; Codd, G. *Systematics and Biodiversity* 2014, 12, 261-270.


79. Ding, Y.; Mutharasan, R. *Environmental Science & Technology* 2011, 45, 1490-1496.


112. Syshchyk, O.; Skryshevsky, V.; Soldatkin, O.; Soldatkin, A. Biosensors & Bioelectronics 2015, 66, 89-94.
113. Shtenberg, G.; Massad-Ivanir, N.; Segal, E. Analyst 2015, 140, 4507-4514.
120. Tsekenis, G.; Filippidou, M.; Chatzipetrou, M.; Tsouti, V.; Zergioti, I.; Chatzandroulis, S. Sensors and Actuators B-Chemical 2015, 208, 628-635.


Chapter Four: Aptasensors as Bioanalytical Tools

Over the last 30 years, synthetic oligonucleotides have played several roles in biology and medicine. This approach relies on the recognition and molecular interactions mediated by these nucleic acids. The *antisense* approach was one of the earliest oligonucleotide technology to be developed, and it involved specific mRNA target binding of a complementary sequence. This technique was proposed for disease therapy and was extensively validated with the available and burgeoning field of entire genome sequencing.\(^1\) It was soon realized that conventional DNA and RNA oligomers are not ideal for use in biological media, and this finding shifted focus on chemically modified oligonucleotides. Thus, the *antisense* approach was not very encouraging, and only one *antisense* oligonucleotide has reached the market in the form of the antiviral drug, fomivirsen,\(^2\) used in the treatment of cytomegalovirus retinopathy. Small interfering RNAs (siRNAs) have also been proposed as an improvement to the *antisense* approach, as siRNA shows higher efficiency when compared to the homologous *antisense* sequence. Both approaches still suffer from similar limitations such as target accessibility, cellular uptake, and interaction specificity.\(^3\)

Combinatory approaches have allowed the selection of oligonucleotide molecules with a selected specific property, from a pool made up of an oligonucleotide family of potential candidates. In this protocol, the property of the selected sequence is not predetermined, and it’s binding or interactions with its target has not been studied. However in 1990, the procedure now known as SELEX was reported.\(^4\) SELEX is the Systemic Evolution of Ligands by EXponential enrichment. In this approach, nucleic acid sequences (called aptamers) of a predetermined property and strong selective affinity for a specific target is identified from a pool of randomly synthesized sequences.
4.1. **SELEX and its Recent Optimizations:**

Chemical synthesis can afford the production of complex libraries of oligonucleotides sequences, which can now be screened for a more specific properties such as binding affinity to a target such as in aptamers, or catalytic activity such as in DNAzymes. The *in vitro* selection and amplification process is termed SELEX. Tuerk and Gold\(^4\) were the first to describe this process, and they used it for the isolation of a RNA sequence, from a pool of random-sequence RNA, which can bind to small organic dyes. This RNA sequence was termed as an aptamer, derived from the Latin word aptus, which means ‘to fit’. ssDNA was isolated by the same process two years after by Ellington and Szostak\(^5\) from a pool of synthesized random-sequence DNAs.

**4.1.1. Chemistry of SELEX:** The SELEX technology is widely applied to molecular biology, medical research, pharmaceutic, and bioanalysis. It has been applied to various target class such as small organic and inorganic molecules, proteins and peptides, carbohydrates, antibiotics, and even complex targets such as whole cells or organisms.\(^6\) The SELEX process has iterative cycles in a process driving towards the selection of the strongest affinity binding. In the first step, a DNA oligonucleotide library of about $10^{13}$ to $10^{15}$ different random sequences is generated by chemical synthesis (In the selection of an RNA aptamer, the starting pool is made up of random RNA sequences produced by transcribing the DNA library). This process of synthesizing a pool of unbiased DNA sequences is relatively easy, as the nucleobases A, C, T, G phosphoramidite coupling efficiency is similar. Hall *et al*\(^7\) suggested that a balanced mixture of DNA sequences can be produced in a phosphoramidite mixture ratio of A, 1.5 : C, 1.5 : G, 1.0 : U/T, 1.2. Each DNA sequence in the pool has a random region flanked by fixed sequences which acts as the primer binding sites in PCR. The library diversity is defined by the length of the random region. One
usually generates $4^n$ different DNA sequences which are $n$ nucleotides long. Hence the experimental library size of $10^{15}$ usually generates sequences with their random region 25 nucleotides long.

![Diagram of SELEX process](image)

**Fig. 4.1:** Scheme for the in vitro target-specific aptamer selection using SELEX
*Mascini Marco, 2009*

The first step involves target incubation where binding occurs, which is followed by partitioning of the strongly bound oligonucleotides from the weakly bound or unbound ones by a stringent washing step. This step is very crucial to the final aptamer binding features, and this is usually accomplished by a variety of methods such as magnetic separation, centrifugation, filtration or affinity chromatography. The bound oligonucleotides are eluted and PCR-amplified.
in DNA SELEX or RT-PCR amplified in RNA SELEX. This new enriched pool of oligonucleotides goes through the cycle again with more stringent conditions imposed, and by these iterative cycles of several selection and amplification steps, a final pool of very few oligonucleotide sequence which has the strongest binding and affinity for the target is obtained. The number of cycles depends on certain parameters specific to the aptamer studied, but it is usually between 6 – 20 SELEX rounds. Negative selection is a step that most SELEX process use to minimize the enrichment of non-specific oligonucleotide binding, or for the concentration of candidate selection to a specific epitope of the target.\textsuperscript{8}

The SELEX process ends when the strongest affinity sequence is obtained, and the PCR products are further cloned to provide individual aptamer clones. These clones can then be identified for a particular property such as a highly conserved region or a special sequence pattern required for the intended aptamer use. Different aptamer clones are then assayed in separate binding studies to further characterize their binding properties, and identify their relative specificities and affinities. The selected aptamers may be subjected to post SELEX modifications such as capping or modified nucleotide incorporation for enhanced stabilities, or attachment of linker molecules, functional or reporter groups for use in analytical detection assays.\textsuperscript{9}

Chemically modified DNA aptamers are important to address the intrinsic limitations of regular DNA and RNA oligomers.\textsuperscript{10} From the antisense technology, it has been observed that unmodified nucleic acids have very short lifetime in biological media. Nuclease, which is present in serum, rapidly digests natural oligonucleotides. Chemical modification thus affords stability against nuclease, and this has been achieved by several methods such as 2’ substitutions, phosphate modifications such as phosphoarlimidite, phosphorothioate, morpholino), and nucleoside modifications such as the α-anomer.\textsuperscript{10-12} These modifications cannot be done pre-selection, as
modified nucleotides cannot act as substrates for polymerases, and hence are not suitable for generating initial DNA library or for amplification of the selected oligomer.

![Modified Nucleosides and Nucleotides](image)

Fig. 4.2: Modified nucleosides and nucleotides (A1 - A6 are nucleotides for nuclease-resistant aptamers, B1 - B3 are phosphosensitive residues, C1 is the amino-imino equilibrium as used in 2D-SELEX. Mascini Marco, 2009)

2'-Fluoro- or 2'-aminopyrine substitution can be used for aptamer selection where unmodified purines are used. The resulting aptamers show resistance to nucleases in biological samples. The substitution of the non-bridging oxygen with a sulfur in the triphosphate has been used in PCR amplification steps. The polymerase, T7 RNA polymerase, has been shown to be able to polymerize transcripts containing boranophosphates or 4'-thiopyrimidines, a modification that can give a 50-fold increase in stabilities compared to unmodified RNA. Y639F and
Y639F/H784A which are mutated T7 RNA polymerases, can efficiently incorporate all the four possible 2’-O-methyl DNA nucleotides.\textsuperscript{12,15} The unmodified positions can be modified post-selection for any further desired properties. Aptamers thus can give very interesting properties and advantages after their pre-selection modifications. An example is the anti-TAR aptamer whose several variants, 64 in number, was studied and led to the discovery of a locked DNA/2’-O-methyl chimeras that showed anti-HIV-1 properties and was resistant to nuclease in cell culture assays.\textsuperscript{16}

\textit{L}-DNA or \textit{L}-RNA is a mirror image of the natural \textit{D}-DNA. \textit{L}-nucleic acids cannot be processed by polymerases but they are highly resistant to nucleases. Thus to achieve these nuclease resistant nucleic acid enantiomers, the \textit{D}-aptamer is first raised against the intended targets mirror image. Once this is achieved, the mirror image of the \textit{D}-aptamer sequence selected, which is called the spiegelmer, can be chemically synthesized. This strategy however can only be applied in detecting small molecules, as it becomes harder to synthesize \textit{L}-aptamers for larger or complex targets. This approach has been used against targets such as peptides, amino acids, and nucleosides.\textsuperscript{17,18}

A new methodology in the chemistry of aptamer selection, called the 2D-SELEX, is based on dynamic combinatorial chemistry and simultaneous SELEX. This concept has been developed using 2’-aminopyrimidine and unmodified purine nucleosides which can be amplified by the SELEX process.\textsuperscript{19} The 2’-amino group can undergo reversible combination reaction with aldehydes producing imines (Fig 4.2). This reaction is allowed to take place before target introduction, generating a pool of both the oligonucleotide sequence and the 2’-imino substituent, and any selected 2’-imino substituted sequence is hydrolyzed to regenerate an amino oligonucleotide. The regenerated amino oligonucleotide is PCR-amplified and passed through another round of 2D-SELEX. At the end of the process, the selected candidate is cloned and
optimized. This approach has been used for aptamer selection against the target HIV-1 TAR, which gave different sequences other than those achieved in the absence of the aldehyde.

4.1.2. Modifications of SELEX: There are five main steps (binding, partition, elution, amplification, and conditioning) involved in the SELEX process which has been adapted to suit different requirements. In choosing a library of DNA or RNA, size of the pool may be considered. Smaller libraries are cost-effective and more manageable, while shorter aptamer sequences are preferred in many applications. Longer sequences however give greater structural complexity which may be required for some processes. Chemically modified oligonucleotide sequences are sometimes used in SELEX studies, with an aim to increase the library complexity. This can provide new interaction possibilities, stabilize oligonucleotide conformations, and increase resistance to nucleases, all of which are required for many aptamer applications. Common modifications concern the 2’ sugar position in RNA libraries (2’-NH₂, 2’-fluoro, and 2’-O-methyl), nucleobase modifications in C-8 purine positions or in C-5 pyrimidine position. 5-Bromouracil and 5-iodouracil have been used in DNA library for generating photo-cross-linkable aptamers, which binds to its target by a covalent linkage when activated by UV irradiation. This method is known as Photo-SELEX. Different SELEX modifications that have been reported are shown in Table 4.1.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative SELEX</td>
<td>Eliminates co-selection of unneeded nucleic acids e.g. for immobilization matrix, removal of unwanted DNA structures from the pool.</td>
<td>Vater et al, 2003; Vanbrabant et al, 2014</td>
</tr>
<tr>
<td>Counter SELEX</td>
<td>Production of aptamers capable of discriminating closely related structures. A target specific selection step introduced to eliminate aptamers</td>
<td>Wang et al, 2003; Dwivedi et al, 2010</td>
</tr>
</tbody>
</table>
from the pool that are unable to detect between the related structures

**Blended SELEX**

Confers additional properties on the aptamer beyond its binding capability. Enlarging nucleic acid molecules with a unique non-nucleic acid component

Aquino-Jarquin *et al.*, **2011**

**Chimeric SELEX**

Aptamer optimization for gene therapy applications

Kanwar *et al.*, **2011**

**Multistage SELEX**

Special chimeric form where after fusion of the preselected aptamer sequence, there is a reselection to the entirety of targets.

Wu and Curran, **1999**

**Deconvolution SELEX**

Aptamer generation for complex targets.Discriminates between relevant aptamers and irrelevant oligonucleotides by binding to distinct target structures within the very complex mixture.

Daniels *et al.*, **2003**

**Covalent or Cross-Linking SELEX**

Functionalized aptamers capable of covalent linkages to a target protein

Kopylov and Spiridonova, **2000**

**Spiegelmer SELEX**

Aptamer selection for enantiomeric targets with D-nucleic acids, and the synthesis of selected aptamers as L-aptamers which can bind specifically to the original unmirrored target

Kong *et al.*, **2013**

**Photo SELEX**

Aptamers functionalized with photoreactive groups such that they can be UV-activated or form UV initiated cross-links

Golden *et al.*, **2000**; Tolle *et al.*, **2013**

**Tailored SELEX**

For short aptamers and speigelmers. Identifies aptamers with a fixed 10 nucleotide sequence by primer binding sites removal and ligation

Vater *et al.*, **2003**

**Signaling Aptamers or Molecular Beacon**

For aptamers that can report target binding by conformational changes which gives a fluorescent signal

Chen *et al.*, **2012**; Stoltenburg *et al.*, **2012**

**Toggle SELEX**

Target switching or toggling during the alternating selection rounds

Birch *et al.*, **2015**

**Indirect SELEX**

Using a target that is not the final intended target, but whose binding must occur for the intended target to also bind e.g. metal ions

Wu *et al.*, **2014**
Cell SELEX: Cell-based selection and counter-selection method for DNA sequence generation that interacts with a specific target cell. Ye et al., 2012; Dwivedi et al., 2013

Tissue SELEX: Aptamer generation for specific binding to complex tissues such as in the collection of cells in diseased tissue. Daniels et al., 2003

FluMag-SELEX: Fluorescein modified DNA oligonucleotide aptamer generation with target immobilized on a magnetic bead. Stoltenburg et al., 2005; Robbins-Welty et al., 2014

Non-SELEX: Involves repetitive steps of partitioning with no amplification. Non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEEM) used for partitioning. Berezovski et al., 2006; Ashley et al., 2012

MonoLEX: One-step process for aptamer selection by physical segmentation of the affinity resin and affinity chromatography; a single final PCR amplification step of the bound aptamers. Nitsche et al., 2007

On-chip SELEX: Selection, done in combination with a method for point mutations, and analysis of the DNA aptamers all done on chips. Lin et al., 2015

Genomic or cDNA SELEX: Library of organisms genome e.g. organisms cDNA fragments is constructed. Target metabolites or proteins from the same organisms is used. Allows protein targets to be identified directly from mRNA pools. Zimmermann et al., 2010; Wu et al., 2015

In vivo SELEX: A method of selecting RNA-processing signals. This approach uses transient transfections in cultured vertebrate cells using an iterative procedure. Cheng et al., 2013; Van Bel et al., 2014

Table 4.1: Modifications of the SELEX Procedure for Generating Aptamers

4.1.3. Advantages and Limitations of Aptamers: The widespread use of aptamers in bioanalysis has been described extensively in literature. The interest in this field is immense and has shown huge potential in medicine, pharmacy, environmental analysis, and biosensor design. The three
dimensional nature of aptamers enhances their functionality. Typical aptamer motifs are stems, bulges, hairpin, internal loops, tetra loops, G-quadruplexes, kissing complexes, and pseudoknots. Aptamers undergo conformational changes when in contact with their targets, and their 3-dimensional folding gives rise to specific target binding. An advantage of aptamer-based biosensors is their ability to bind specifically and very tightly to their targets, with affinities comparable to that of antibodies. The binding constants $K_d$ of most aptamers are usually reported in the low nM to pM range. Aptamers are also isolated in an in vitro selection process, which does not involve the use of cell lines, animals or any living organism, which makes this process applicable under non-physiological conditions. This also makes the selection of aptamers for non- or very low-immunogenic toxic substances possible. As shown in Table 4.1, aptamer selection can be modified to suit intended aptamer application. The properties that may affect aptamer binding to target such as temperature, buffer composition, and pH can therefore be effectively controlled in the selection process.

Aptamers after their selection process can be chemically synthesized in large scale with high reproducibility and accuracy. Regeneration of denatured aptamers is possible within minutes, an important feature for several applications. Several modifications of the aptamer post-selection are also possible. There is however a limited use of aptamers as drugs. This is because unmodified nucleic acids such as RNA are unstable in biological fluids. A way proposed to overcome this challenge involved chemical modifications at the nucleotide level, or capping. A more successful approach is the spiegelmer technology for selection of aptamers resistant to nuclease, which can identify specific oligonucleotide enantiomer that selectively binds natural biological targets such as peptides and amino acids. Other possible post-selection aptamer modification include molecular attachments such as functional groups, reporter molecules, PEG, lipid or cholesterol
tags. which are used for better aptamer quantification and immobilization, or increasing the pharmacokinetic half-life of potential therapeutic aptamers. Truncation and reselection processes are also useful for generating more specific aptamers with improved affinities.\textsuperscript{47}

SELEX can be used on a wide variety of targets. Complex mixtures or structures are possible targets that can be incorporated in an aptamer selection process. These targets must however have some features that can make aptamer selection possible. These features may include the presence of positively charged groups such as amino acids, the presence of a planarity structure such as aromatic compounds, or hydrogen-bond acceptors and donors.\textsuperscript{48} Aptamer selection for very hydrophobic or highly negatively charged molecules as in phosphate groups may prove difficult. Target requirements necessary for aptamer selection is thus mostly based on the essential intermolecular interactions of the target-aptamer complex.

SELEX, despite its several optimization and the increasing number of publications about it, is still a relatively complicated and slow process. There can be no standard SELEX protocol as each process has to be adapted to fit the current circumstances. The entire iterative procedure is also very time consuming. Manual aptamer selection has been used in most of the published work so far, and an automated selection system can help achieve faster process times.\textsuperscript{49} Automated SELEX has been described using a robotic workstation\textsuperscript{41} or a micro-fabricated chip-based SELEX protocol\textsuperscript{50} for a faster and more accessible aptamer selection process. Several biotech companies now develop specialized SELEX protocols that can afford an high-throughput aptamer production and selection procedure reducing process time from several months down to just a few days.
4.1.4. Applications of Commercial Aptamers: Aptamers have been applied in several fields such as in clinical diagnostics, therapeutics and pharmaceuticals, and molecular recognition agents in several analytical systems. The first approved therapeutic aptamer is the anti-human vascular endothelial growth factor (VEGF). This pharmaceutical product called Macugen (active ingredient pegaptanib, which is a PEG modified form of the aptamer, is used to treat age related macular degeneration) was licensed by Pfizer Inc. and approved by the US EPA in December 2004, and in Canada and Europe in January 2006. Pegaptanib has also been shown to be an active therapy for diabetic macular degeneration.

Development of therapeutic aptamers is a very active research field. Aptamers for applications in medicine has greatly advanced for applications such as tumor therapy and imaging, clinical diagnostics, and virus detection. Clinical aptamer drugs however suffer from the drawback of limited intracellular penetration in some cases. Non-viral delivery systems are used to enhance cellular penetration and proffer long-term delivery. This technique allows direct oligonucleotide drug delivery, or entrapment in cationic polymers or lipids for slow-release delivery systems, as this can be adapted depending on the therapy purpose or intended organ target.

Aptamers are widely employed as detection kits in bioanalysis. Aptamers can be effectively incorporated with several transduction methods for sensitive analyte detections. Efficient aptamer immobilization is important for their use on surface sensitive sensors. Studies have shown the advantages of a linker modified aptamer which is immobilized by biotin-streptavidin for surface sensitive sensors like the quartz crystal sensor. This was also noticed for gold surfaces, giving improved sensitivity and reproducibility. Examples of some commercial clinical aptamers are compiled in Table 4.2.
<table>
<thead>
<tr>
<th>Target</th>
<th>Aptamer</th>
<th>Application</th>
<th>Product State</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>RNA, chemically modified</td>
<td>Therapy, age related macular degeneration</td>
<td>Macugen: Pfizer Inc.</td>
<td>Earnshaw et al; 2007</td>
</tr>
<tr>
<td>Thrombin</td>
<td>DNA, thrombin inhibitor</td>
<td>Therapy, anticoagulant</td>
<td>Phase 1 studies in 2004, Archemix Corp.</td>
<td>Li et al, 2014</td>
</tr>
<tr>
<td>Neoclonin</td>
<td>AS1411</td>
<td>Therapy, cancer</td>
<td>Completed Phase 2, Antisoma Research</td>
<td>Rosenberg et al, 2014</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>E-10030 (Fovista™)</td>
<td>Therapy, neovascular age-related macular degeneration</td>
<td>Phase 3, Ophotech Corporation</td>
<td>Kaiser et al, 2013</td>
</tr>
<tr>
<td>C5 Complement Cascade</td>
<td>ARCH 1905</td>
<td>Therapy, neovascular age-related macular degeneration</td>
<td>Phase 3, Ophotech Corporation</td>
<td>Sundaram et al, 2013</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>L-RNA, NOX-F37, L-RNA</td>
<td>Lymphoma and multiple myeloma</td>
<td>Phase 2, NOXXON Pharma AG</td>
<td>Ni et al, 2011</td>
</tr>
<tr>
<td>CXCL 12/ SDF-1</td>
<td>Nox-A12, spiegelmer</td>
<td>Tumor</td>
<td>Phase 2, NOXXON Pharma AG</td>
<td>Esposito et al, 2011</td>
</tr>
<tr>
<td>Thrombin</td>
<td>NU 172</td>
<td>Anticoagulation in heart disease treatments</td>
<td>Phase 2, ARCA Biopharma</td>
<td>Ni et al, 2012</td>
</tr>
<tr>
<td>Chemokine</td>
<td>NOX E-36, L-RNA spiegelmer</td>
<td>Type 2 Diabetes Mellitus</td>
<td>Clinical trial phase 1</td>
<td>Sundaram et al, 2013</td>
</tr>
<tr>
<td>Glycoprotein Ib</td>
<td>ARC-194</td>
<td>Hemophilia</td>
<td>Phase IIb, Archimex</td>
<td>Kanwar et al, 2011</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>AL6 DNA</td>
<td>Medicine: disease diagnosis and prognosis</td>
<td>Research</td>
<td>Chen et al, 2015</td>
</tr>
<tr>
<td>Compound</td>
<td>Aptamer/Spiegelmer</td>
<td>Application</td>
<td>Product Development</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------</td>
<td>--------------------------------------------------</td>
<td>---------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>NOX-B11, L-RNA</td>
<td>Therapy, obesity treatment</td>
<td>Product development</td>
<td>Vater et al., 2015</td>
</tr>
<tr>
<td></td>
<td>spiegelmer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amylin</td>
<td>NOX-A42, spiegelmer</td>
<td>Therapy, type II diabetes</td>
<td>Product development</td>
<td>Vater et al., 2015</td>
</tr>
<tr>
<td>Influenza virus HA:91-261</td>
<td>DNA</td>
<td>Medicine: Viral infection inhibition</td>
<td>Product development</td>
<td>Arnon et al, 2015</td>
</tr>
<tr>
<td>peptide region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Examples of Commercial Clinical Aptamers

4.2. Aptamer-Based Biosensors:

Nucleic acid aptamers have attracted massive interest and have been applied in several field of study. Aptamer-based biosensors, also called aptasensors, is a rapidly evolving field of research. Aptasensors have the advantage of using aptamers as recognition agents, which affords superior detection properties over several other recognition agents. Aptasensors have been incorporated with several transduction means, mostly electrochemical, optical and mass-sensitive transductions such as in piezoelectric-based detections. The recent advancements in nanomaterials has improved aptamer sensitivities in biosensor designs. Antibodies are the mostly applied recognition agents, but aptamers offer some advantages over antibodies such as small size, ease of device incorporation, more chemically stable and cost effective approaches. Aptamers are sometimes referred to as chemical antibodies, and can be regarded as very suitable alternatives. The pre- and post-modifications of aptamers also suit them for a variety of purposes. The major transduction means of aptasensors are discussed below, plus the advantages of nanomaterials in aptasensor designs.
4.2.1. **Electrochemical Apsensors:** Electrochemical transduction is the mostly used detection method in bioanalysis and biosensor designs, and it accounts for over 70% of aptasensor designs reported in literature. Nucleic acid conformational changes upon hybridization with a complementary sequence is the basis of the design of different electrochemical DNA sensors. For example, hairpin DNA such as the stem-loop DNA structure with a redox-active reporter label, and immobilized to an electrode surface will give rise to a signal upon hybridization due to its conformational changes and the close proximity of its reporter labels with the electrode surface.

![Fig 4.3: Increase in aptamer biosensor publications in the last 8 years. Distribution by country. Liu et al, 2014](image)

Here, the hybridization of the stem loop DNA structure to a complementary sequence gives rise to a duplex structure that can bring the reporter redox species close to the electrode for electrochemical detection. Several different conformational changes can occur leading to signal generation, based on the detection method employed. These approaches are been extended to ssDNA aptamers used as aptasensors for proteins or small molecules detections, as they hybridize with their host to give three-dimensional structures that results in teetering of the redox species to the electrode surface and signal can be measured by probing the electrical contact between the redox probe and the electrode surface.
Thrombin aptamers form G-quadruplexes when they bind with thrombin. Thrombin aptasensors are widely studied and understood. An approach is to tether a methylene blue reporter probe which is an electroactive species to the aptamer, and then immobilized the aptamer on a gold electrode. On immobilization of the aptamer, the methylene blue is in electronic contact with the electrode resulting in voltammetric signal. However on introduction of the thrombin, the thrombin aptamer wraps around the target and forms a G-quadruplex thus cutting off the methylene blue probes contact with the electrode and allowed for detecting using a voltammetric signal quenching approach. A voltametric turn on signal could be achieved the other way, where the methylene blue electroactive probe is not in contact with the gold electrode before the introduction of thrombin, as the aptamer was hybridized to a complementary strand bearing this electroactive species. This method was described by Xiao et al.\textsuperscript{66} On introduction of the thrombin target, the aptamer dissociated from the bound sequence, and wraps around the thrombin, releasing the sequence and thus the methylene blue can now make contact with the gold surface resulting in signal detection.

![Diagram](image.png)

4.2.1.1. Labeled Approaches to Electrochemical Aptasensors: Enzymes are widely used as catalytic labels to amplify signals in electrochemical aptasensors. Most enzymes are redox active
species, and these can be effectively coupled with nucleic acids, thereby acting as biocatalytic conjugates to generate an intensified signal readout. Mir et al.\textsuperscript{67} employed two different aptasensor configurations for an amplified thrombin analysis using the biocatalytic features of enzymes. In one of the design, a thiol-functionalized aptamer was immobilized on a gold electrode, forming a G-quadruplex on interacting with thrombin. The bounded thrombin then acted as a protease hydrolyzing a peptide which has been functionalized with nitroaniline to afford the redox-active \textit{p}-nitroaniline. In the second approach, the double binding sites of thrombin was exploited to bind and form G-quadruplex structures with two of its aptamers in a sandwich aptasensor design. The second bound unimmobilized aptamer acted as a reporter label through an attached horseradish peroxidase, which catalyzed the reduction of hydrogen peroxide. This redox process was thus used as detection protocol.

A similar approach is the use of platinum nanoparticles (Pt-NP) labels rather than the enzyme for signal amplification. An example of this aptasensor design was reported by Polsky et al.\textsuperscript{68} for the detection of DNA and thrombin (Fig 4.5).\textsuperscript{68} This was employed in a sandwich aptasensor design such as discussed earlier, and a Pt-NP functionalized aptamer was made to bind to thrombin after the thrombin was already hybridized with the immobilized aptamer, with the formation of G-quadruplex structures by both thrombin-specific aptamers. The Pt-NP catalyzed the reduction of hydrogen peroxide resulting in measurable electrode current, and allowed detection of the thrombin with an 80-fold sensitivity over a similar enzymatic approach.

Another interesting approach is the use of a ferrocene-functionalized cationic poly-(3-alkoxy-4-methylthiophene) as a redox labeled polyelectrolyte in an electrochemical aptasensor design. This approach was reported by Floch et al.\textsuperscript{69} This redox labeled polyelectrolyte allowed for the detection of the thrombin by its aptamer using a voltammetric transduction means. In one
design, the interaction of the immobilized aptamer and the polyelectrolyte afforded a voltammetric signal recording. On addition of a thrombin target, the aptamer binds to thrombin and loses its interaction

(a) Pt-NP based aptasensor for thrombin detection

(b) Pt-NP based aptasensor for DNA detection

Fig 4.5: Platinum nanoparticles as signal amplifiers in electrochemical aptasensors

Polsky et al, 2006

with the polyelectrolyte resulting in a signal off detection method. These designs described so far are examples of a labeled electrochemical aptasensor designs. Table 4.3 gives a list of other labels used in electrochemical aptasensor designs.  

<table>
<thead>
<tr>
<th>Target</th>
<th>Aptamer Label</th>
<th>Detection Limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>Horseradish Peroxidase</td>
<td>80 nM</td>
<td>Ikebukoro et al, 2005</td>
</tr>
<tr>
<td>PDGF</td>
<td>Au Nanoparticle</td>
<td>0.01 pM</td>
<td>Wang et al, 2009</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Pt-Nanonparticle</td>
<td>1.0 nM</td>
<td>Polsky et al, 2006</td>
</tr>
<tr>
<td>Substance</td>
<td>Label</td>
<td>Concentration</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------</td>
<td>---------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Cocaine</td>
<td>Methylene Blue</td>
<td>10 nM</td>
<td>Zuo et al, 2009</td>
</tr>
<tr>
<td>ATP</td>
<td>Anthraquinone</td>
<td>1 nM</td>
<td>Thomas et al, 2014</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Ferrocene</td>
<td>20 nM</td>
<td>Wang et al, 2010</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Ferrocene</td>
<td>200 nM</td>
<td>Song et al, 2012</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Ferrocene</td>
<td>0.5 nM</td>
<td>Zhou et al, 2015</td>
</tr>
<tr>
<td>Lysozome</td>
<td>(Ru[NH$_3$]$_6$)$^{3+}$</td>
<td>0.5 µg</td>
<td>Xiao et al, 2013</td>
</tr>
<tr>
<td>AMP</td>
<td>(Ru[NH$_3$]$_6$)$^{3+}$</td>
<td>500 nM</td>
<td>Liu et al, 2013</td>
</tr>
</tbody>
</table>

Fig. 4.3: Labels Employed in APTASensor Designs

4.2.1.2. Label-Free Electrochemical APTASensors: Label-free electrochemical APTASensors have also found application in several fields. Label-free APTASensor techniques using Faradaic impedance spectroscopy (FIS) can probe recognition events of biomolecules based on electron-transfer resistance changes at the electrodes. FIS approach stems from the discovery that biomolecule interactions such as antibody–antigen or protein–receptor can increase the electrical contact barrier between redox active species and electrodes leading to decrease in the electrical signals of electrochemical cells. Similarly, the activity of nucleic acids with dsDNA can increase the negative charge around an electrode thus inhibiting electron transfer from redox species at the electrode. Alina et al$^{77}$ recently reported the use of a label-free FIS for the aptameric detection of food allergens such as tropomyosin in shrimps, Ara h1 in peanuts, gliadin in grains (wheat, barley and rye), and lysosome in egg white. In their approach, a partial complementary DNA sequence (pc-DNA) was hybridized to the lysozome binding aptamer on a gold electrode and functionalized with 6-mercapto-1-hexanol (MCH) as the redox active probe. The introduction of lysosome decreased the electron transfer of the electroactive probe by displacing the bound LBA from the...
duplex structure into the electrochemical solution. This brought about a measurable change for target detection.

Queiros et al\textsuperscript{78} also used this exact approach for the detection of E. coli, while Anjum et al\textsuperscript{79} used a similar approach based on capacitance detection of protein cancer biomarkers HER2/ErbB2.

Field effect transistor (FET) is also another approach to label free electrochemical aptasensor design. This process uses an ion-selective approach which is modulated by the bio-recognition event occurring at the surface gate. The device set up usually involves the deposition of a gate surface on a semiconductor such as silicon such that it separates an electron source and drain electrode. This imposes a gate potential (source–drain potential, V\textsubscript{ds}), which maintains a certain

\begin{center}
\textbf{Fig 4.6: Schematic of FIS aptasensor for the detection of lysozyme.}
\textit{Alina et al, 2014}
\end{center}

\begin{itemize}
\item Functionalized graphene,\textsuperscript{80,81} single-walled carbon nanotubes (SWCNT),\textsuperscript{82} and nanomaterials,\textsuperscript{83} have all been employed as gate surface.
\end{itemize}
Graphene-based electrochemical transductions have offered improved advantages such as a wide electrochemical window, rapid kinetics of their electron transfer, large signal to noise ratio, and a feasible and very easy incorporation making them very attractive platforms.\textsuperscript{84}

![Diagram](image)

**Fig 4.7: A reusable graphene-based electrochemical aptasensor for detecting cancer cells. Feng et al, 2011**

Graphene-based aptasensors without additional signaling device, can also serve as a label-free detection model for biomolecules. Feng et al\textsuperscript{85} described a reusable graphene based electrochemical aptansensor that affords the label-free detection of cancer cells. The aptamer AS1411 (a 26-mer DNA currently in clinical trials, see Table 4.2), was employed exploiting its high specificity and affinity to nucleolin cell surface in cancer cells. This approach used a water soluble perylene derivative [3,4,9,10-perylene tetracarboxylic acid (PTCA)], which was adsorbed via $\pi - \pi$ hydrophobic interactions for a carboxylic group based immobilization of the NH$_2$-modified aptamer. This aptamer in its quadruplex conformation hybridizes to the nucleolin cell, a
binding which disrupts its quadruplex structure. This approach thus relies on the target-binding induced folding in an electrode-immobilized DNA sensor, and changes in the electrochemistry of the cell from this binding serves as detection. Detection of nucleolin as described depends on the conformational changes of the aptamer as shown in Fig. 4.7. Further review on the various designs of electrochemical aptasensors based on graphene and other approaches has also been reported.

4.2.1.3. Applications of Electrochemical Aptasensors: Aptasensors are applied in the detection of important biological samples such as proteins and nucleic acids. Since the elucidation of aptamer binding to thrombin, several aptamers are being designed for the detection of biological samples especially in the area of food pathogens, toxins, viral strains, whole bacteria cells, and microorganisms. Electrochemical aptasensors have also been used extensively especially in food safety, environmental screening, medicine and diagnostics. The following examples present some reports of electrochemical aptasensors designed for pathogens, viruses, whole bacterial cells and bacterial toxins.

It has been shown that aptamers are capable of self-assembly on carbon nanotubes (CNTs) based on their puric and pyrimidine π – π stacking with CNTs. So et al. have used this principle in an electrosensor employing single-walled CNTs functionalized with an E. coli-specific RNA aptamer to form an hybrid material used as a screening tool for E. coli. The aptamer binding to the target E. coli resulted in a drastic drop in the conductance of the cell in less than 20 min, giving a negative signal detection criteria. Hernandez et al. reported the use of a potentiometric aptasensor that uses a chemically modified graphene layer based either on a graphene oxide (GO) or a reduced graphene oxide (RGO). The aptamer used could recognize surface epitopes of S. aureus in less
than 1 min with a detection limit of a single CFU/ml (colony forming unit per ml). In the design set up as shown in Fig. 4.8, the aptamer was immobilized on both GO and RGO through the effective \( \pi - \pi \) interactions. Introduction of the \( S. \) aureus target dissociated the aptamer from the graphene surface, thus releasing its negatively charged phosphodiester linkage. This charge separation induces a recordable change in cell potential which is used for the detection signal.

Fig. 4.8: Graphene based electrochemical aptasensor for the detection of \( S. \) aureus. Hernandez et al, 2014
An electrochemical aptasensor for the detection of *S. aureus* has also been reported. This approach used a dual aptamer system, with an immobilized aptamer acting as the capture probe, while a second aptamer immobilized on a carbon electrode modified with silver nanoparticles (AgNP) acted as the signaling probe. This sandwich system was made in contact with nitric acid (HNO$_3$), which stripped the AgNP and resulted in a differential pulse-stripping voltammetric AgNP signal that was used for the analytical detection of the target.

Ding *et al* described the use of potentiometric aptasensing for the detection of *Listeria monocytogenes* with the use of a protamine indicator. In this approach, the binding of the *L. monocytogenes* aptamer to its target prevents any electrostatic interaction of the aptamer with the protamine indicator when introduced, and this is detected in a polycation-sensitive membrane electrode. This approach enabled accurate and efficient target detection to a limit of 10 CFU/mL. Some further reviews are available for the electrochemical aptasensor detection of whole cells.

Another major use of electrochemical aptasensors is in the detection of toxins, especially those present in food substances. Several approaches have also been used to achieve this.

Nguyen *et al* described the use of a magnetite (Fe$_3$O$_4$) incorporated into a polyaniline film, Fe$_3$O$_4$-PA, and polymerized on an interdigitated electrode (IDE). This served as a very sensitive film for the electrochemical aptasensor used in the label-free detection of aflatoxins in cow milk. The aflatoxin-specific aptamer was immobilized on the Fe$_3$O$_4$-PA-modified electrode, and its conformational change on hybridization to the aflatoxin target was detected efficiently by square and cyclic wave voltammetry, which afforded detection limits of 1.98 ng/mL.

Castillo *et al* reported an electrochemical aptasensor immobilized on a dendritic gold electrode surface for the detection of aflatoxin B1 in peanuts and food samples. The aptasensor
used an electrochemical impedance spectroscopy (EIS) based signal detection, and they immobilized the aptamer to a cystamine covered and polyamidoimine dendrimer functionalized gold surface. The same authors had earlier reported with a similar approach, but with the use of the electroactive ferrocyanate redox probe, \([\text{Fe(CN)}_6]^{3-/4-}\), the detection of ochratoxin A (OTA) in food samples.

There have been several approaches in the design of electrochemical aptasensors for the detection of OTA. Hayat et al.\(^9\) reported a label-free, signal-quenching detection of OTA by immobilizing a carboxylated OTA aptamer to a hexamethyldiamine linker on a screen-printed carbon electrode (SPCE). The aptamer acted as a gate for the macromolecular clusters, which maintained maximum signal readout before target introduction. However, on the introduction of the aptamer and subsequent binding, the imposed conformational change in the aptamer closed the gate and thus decreased the electrochemical signal recorded. Rhouati et al.\(^9\) described a similar SPCE-based aptasensor, but in an automated flow-based system for the detection of OTA in beer samples. Their approach involved the use of magnetic beads to immobilize the aptamer on a SPCE, and employed two detection formats. In the direct method, free OTA and a biotin labeled OTA competed for binding with the immobilized aptamer, while in the indirect approach, free OTA and an immobilized OTA were the competing species. An avidin–alkaline phosphatase conjugate was used for enzymatic detection, with the enzymatic rates inversely proportional to OTA concentration.

Rolling chain amplification is an approach recently described by Chen et al.\(^9\) and used in an electrochemical aptasensor for the determination of OTA in food samples. In their design, the RCA primer was designed to incorporate two halves, with one part serving as an OTA aptamer sequence, while the other part as complementary sequence to the capture probe. On introduction
of the OTA target, the primer which is hybridized to a RCA padlock unbinds and hybridizes the OTA thus generating the aptasensor signal. Merkoci et al\textsuperscript{100} also reported a label-free impedimetric aptasensor that uses an iridium oxide (IrO-NP) nanoparticles on an SPE for signal amplification. EIS using (Fe[CN]$_6$)$^{3-/4-}$ as the redox probe was employed to obtain very low detection limits 14 pM.

Chrouda et al\textsuperscript{101} designed an electrochemical aptasensor based on PEG grafted to the surface of a boron-doped diamond (BDD) microcell. The PEG was immobilized on a BDD microcell and served as a spacer chain which held the amino-functionalized aptamer, through covalent attachment to its carboxyl end, to the electrode surface. Conformational changes of the aptamer hybridization of the target to the introduced substrate decreases the electron transfer rate of the ferrocyanate probe, and this accounted for signal detection.

![Diagram](image.png)

Fig. 4.9: Detection of OTA using PEG on a boron-doped diamond microcell. Jia et al, 2014
Jia et al.\textsuperscript{102} reported an EIS-based aptasensor for the detection of \textit{S. aureus}. They immobilized a thiolated ssDNA, which is the probe aptamer, to a glassy carbon electrode via its functionalized gold nanoparticles-reduced graphene oxide (AuNP-RGO) nanohybrid. Their set-up allowed for a label-free EIS detection of the target on binding with the probe aptamer, and this method gave a \textit{S. aureus} detection of 10 cfu/mL. Abbaspour \textit{et al.}\textsuperscript{92} in their own approach as earlier described used a biotinylated aptamer probe immobilized to magnetic beads coated with streptavidin as capture probe and a second aptamer conjugated to silver nanoparticles as the signaling agent in a sandwich aptasensor approach. This sensor was used to detect \textit{S. aureus} in water samples and gave a detection limit of 1.0 cfu/mL. Good detection limits have thus been achieved using electrochemical aptasensors for toxin detection in real samples.

Several other detection means has been applied in electrochemical aptasensors for the detection of food toxins. Wu \textit{et al.}\textsuperscript{103} used a methylene blue dual labeled aptamer whose binding-induced folding was used for the detection of OTA in on-site complex food systems an showed detection limit of 0.095 pg/mL, Tong \textit{et al.}\textsuperscript{104} used an exonuclease catalyzed target recycling to detect OTA in wheat samples show good detection limits of 1.0 pg/mL, Evtugyn \textit{et al.}\textsuperscript{105} reported the use of GCE modified with an electropolymerized polycarboxylated ligand for the EIS-based aptasensor detection of AFB1 in peanuts, cashew nuts, soy sauce and wine, and obtained a detection limit of 0.05 nM. Reviews on the applications of electrochemical aptasensors is covered in these texts.\textsuperscript{81,88,106}

4.2.2. 	extbf{Optical-Based Aptasensors:} Though electrochemical transduction is the most used transduction means in biosensor designs, optical detection is also receiving increasing attention due to its simple procedures, faster responses and higher sensitivities.\textsuperscript{107} Optical aptasensors is also a well-studied aspect of aptasensor designs, and incorporates detection models such as colorimetry,
fluorescence, and chemiluminescence. There have been several small biomolecule targets that optical aptasensors have been used to detect such as ATP, adenosine, bisphenol A, dopamine, cocaine, OTA, AFB1, glucose, and tetracycline. Table 4.4 gives a list of some optical aptasensor design and their specific targets.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Target</th>
<th>Detection Limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence Based Labels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRET</td>
<td>VEGF</td>
<td>875 pM</td>
<td>Freeman et al, 2012</td>
</tr>
<tr>
<td></td>
<td>17β-estradiol</td>
<td>0.22 nM</td>
<td>Long et al, 2014</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>18 pM</td>
<td>Li et al, 2014</td>
</tr>
<tr>
<td>Nanoparticle-Based</td>
<td>OTA</td>
<td>2 pM</td>
<td>Duan et al, 2012</td>
</tr>
<tr>
<td></td>
<td>Mycotoxins</td>
<td>0.1 nM</td>
<td>Wu et al, 2012</td>
</tr>
<tr>
<td></td>
<td>Salmonella typhimurium,</td>
<td>10 cfu/mL</td>
<td>Wu et al, 2014</td>
</tr>
<tr>
<td></td>
<td>Vibrio parahemolyticus</td>
<td>15 cfu/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence Based Label-Free</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescent Intercalators</td>
<td>Proteins</td>
<td>aM range</td>
<td>Yong et al, 2015</td>
</tr>
<tr>
<td>Fluorescent Aptamers</td>
<td>OTA</td>
<td>20 pM</td>
<td>Zhang et al, 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 nM</td>
<td>Lv et al, 2014</td>
</tr>
<tr>
<td></td>
<td>AFB1</td>
<td>0.3 nM</td>
<td>Shim et al, 2014</td>
</tr>
<tr>
<td></td>
<td>Heavy Metals</td>
<td>0.92 nM</td>
<td>Li et al, 2013</td>
</tr>
<tr>
<td>Silver Nanoparticle</td>
<td>Adenosine</td>
<td>48 nM</td>
<td>Wang et al, 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetry based</td>
<td>DNA-functionalized Au-NP</td>
<td>Adenosine</td>
<td>Ma et al, 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.6 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plasmodium falciparum</td>
<td>80 cfu/mL</td>
<td>Jeon et al, 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Label-Free AuNP</td>
<td>Adenosine deaminase</td>
<td>0.16 HAU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16 HAU</td>
<td>Cheng et al, 2015</td>
</tr>
<tr>
<td></td>
<td>HRP-mimicking DNAzyme</td>
<td>Proteins</td>
<td>Huang et al, 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 pM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chemiluminescence | PDGF | 0.68 pM | Bi et al, 2014  
Mercury (Hg II) | 1 pM | Liu et al, 2014  
Surface Plasmon Resonance | VEGF | 6 nM | Cennamo et al, 2015  
Avian influenza virus (H5N1) | 0.128 HAU | Bai et al, 2012  
Resonance Scattering Spectral | Tetracycline | 11.6 nM | Luo et al, 2014  
Raman Scattering | Bisphenol A | 10 fM | Chung et al, 2015  
Ellipsometry | Ricin B | 1.0 fM | Zengin et al, 2015

Table 4.4: Optical Aptasensors for detection of small biomolecules

4.2.2.1. Fluorescence-based Aptasensors: Structural and conformational changes of aptamers before and after target binding is the most widely applied recognition process in aptasensor designs. When aptamers directly interact with dyes or fluorophores used as optical labels, they can change the fluorescence of these labels or the Forster resonance energy transfer (FRET) between two labels. When fluorescence increases due to aptamer interaction, this is known as the signal-on approach, while signal-off approach involves decrease in fluorescence on aptamer binding.

In their unmodified states, very few aptamers can exhibit auto-fluorescence, and even when they do, intensities are so low that they are not useful analytically. Fluorescent labels, or aptamer structural modifications can lead to sensitive and efficient fluorescence-based aptasensor designs. FRET-based aptasensors incorporates two fluorescent labels, the fluorophore and the quencher (the quencher can also be another fluorophore), with the fluorescence of one of the labels quenched by the other through FRET. FRET-based aptasensors can be signal-off detection-based, where aptamer binding to target brings the fluorophore in close proximity with the quencher such that its fluorescence is effectively quenched, while the signal-on approach is when aptamer conformational changes releases both labels such that the fluorescence is detectable.
Common FRET pairs include σ-aminobenzoic acid (ABZ)/2, 4-dinitrophenyl (DNP), dimethylamino-1-sulfonyl (DANSYL)/fluorescein (FAM) or tyr-NO₂, FAM/tetramethylrhodamine (TAMRA), FAM/Cy3, [N-(aminoethy)-5-naphthylamine-1-sulfonic acid (EDANS)/[4-((4-dimethylamino)phenyl)azo]benzoic acid] (DABCYL). Other similar or more different fluorescent dyes also used in bioanalytical studies include the chlorinated FAMs such as hexachloro-FAM (HEX) and tetrachloro-FAM (TET). Others include the cyanine dyes such as Cy3 and its derivatives which are resistant to photobleaching, cover a broad range of absorption and emission spectrum in the visible wavelength, and have high quantum yields; or the bodipy dyes which are stable under physiological conditions, are insensitive to pH variations, and usually have high quantum yields. Bodipy dyes, though insoluble in water, can be effectively applied in aptamer labeling, as aptamer functionalization is carried out in non-aqueous medium and the bodipy-labeled aptamer is usually soluble in water. These pairs are selected such that the emission wavelength range of the fluorophore is the absorption wavelength range for the quencher giving rise to an effective quenching.

<table>
<thead>
<tr>
<th>Name</th>
<th>$\lambda_{\text{max}}$ nm⁻¹ (absorption)</th>
<th>$\lambda_{\text{max}}$ nm⁻¹ (emission)</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>495</td>
<td>520</td>
<td>Green/yellow</td>
</tr>
<tr>
<td>TET</td>
<td>525</td>
<td>550</td>
<td>Orange/yellow</td>
</tr>
<tr>
<td>HEX</td>
<td>535</td>
<td>565</td>
<td>Pink</td>
</tr>
<tr>
<td>ROX</td>
<td>580</td>
<td>605</td>
<td>Purple</td>
</tr>
<tr>
<td>DABCYL</td>
<td>428</td>
<td>453</td>
<td>-</td>
</tr>
<tr>
<td>TAMRA</td>
<td>550</td>
<td>575</td>
<td>Red</td>
</tr>
<tr>
<td>ABZ</td>
<td>320</td>
<td>420</td>
<td>White/Yellow</td>
</tr>
<tr>
<td>DANSYL</td>
<td>334</td>
<td>519</td>
<td>Yellow/orange</td>
</tr>
<tr>
<td>DNP</td>
<td>365</td>
<td>400</td>
<td>Yellow</td>
</tr>
<tr>
<td>Fluorophore</td>
<td>Absorption</td>
<td>Emission</td>
<td>Color</td>
</tr>
<tr>
<td>------------------</td>
<td>------------</td>
<td>----------</td>
<td>----------------</td>
</tr>
<tr>
<td>EDANS</td>
<td>340</td>
<td>490</td>
<td>Light yellow</td>
</tr>
<tr>
<td>Cy3</td>
<td>550</td>
<td>570</td>
<td>Dark pink</td>
</tr>
<tr>
<td>Cy3.5</td>
<td>591</td>
<td>604</td>
<td>Purple/blue</td>
</tr>
<tr>
<td>Cy3b</td>
<td>558</td>
<td>572</td>
<td>-</td>
</tr>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
<td>Blue</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>675</td>
<td>695</td>
<td>Blue</td>
</tr>
<tr>
<td>BODIPY® 493/503</td>
<td>500</td>
<td>506</td>
<td>Green</td>
</tr>
<tr>
<td>BODIPY® FL-X</td>
<td>504</td>
<td>510</td>
<td>Green</td>
</tr>
<tr>
<td>BODIPY® FL</td>
<td>505</td>
<td>513</td>
<td>Green</td>
</tr>
<tr>
<td>BODIPY® R6G</td>
<td>528</td>
<td>550</td>
<td>Green/Yellow</td>
</tr>
<tr>
<td>BODIPY® 530/550</td>
<td>534</td>
<td>554</td>
<td>Green/Yellow</td>
</tr>
<tr>
<td>BODIPY® TMR-X</td>
<td>542</td>
<td>574</td>
<td>Yellow</td>
</tr>
<tr>
<td>BODIPY 558/568</td>
<td>558</td>
<td>569</td>
<td>Yellow</td>
</tr>
<tr>
<td>BODIPY® 564/570</td>
<td>565</td>
<td>571</td>
<td>Yellow</td>
</tr>
<tr>
<td>BODIPY® 576/589</td>
<td>576</td>
<td>590</td>
<td>Orange</td>
</tr>
<tr>
<td>BODIPY® 581/591</td>
<td>584</td>
<td>592</td>
<td>Orange</td>
</tr>
<tr>
<td>BODIPY® TR-X</td>
<td>589</td>
<td>617</td>
<td>Orange/Red</td>
</tr>
<tr>
<td>BODIPY® 630/650-X</td>
<td>625</td>
<td>640</td>
<td>Red</td>
</tr>
<tr>
<td>BODIPY® 650/665-X</td>
<td>646</td>
<td>660</td>
<td>Red</td>
</tr>
</tbody>
</table>

Table 4.5: Characteristic absorption and emission wavelengths of common fluorophores
(a) fluorescein (FAM) phosphoramidite monomer and its chlorinated derivatives

(b) Cyanine (Cy) dyes. R = SO$_3^-$ for aqueous solubility, R = H (or alkyl) for organic solubility
However recently, the use of nanoparticles and quantum dots other than the conventional protein dye pairs in FRET aptasensor designs has received huge attention. Li et al.\textsuperscript{110} reported the use of upconversion nanoparticles (UCNPs) and a graphene FRET pair in the design of an aptasensor for the detection of kanamycin. In their design, the kanamycin specific aptamer was
tagged with UCNP, and immobilization of the aptamer to the graphene brought the UCNP in close contact to the graphene via the aptamer – graphene π-π interactions quenching UCNPs fluorescence. Introduction of the kanamycin however altered the aptamer conformation to a hairpin structure, which effective blocked the energy transfer and led to fluorescence recovery. Other examples of labeled FRET aptasensor designs include the quantum dots functionalized aptamers and Cy5 dye as receptor for signal off detection of VEGF reported by Freeman et al., the detection of β-estradiol described by Long et al. also used the quantum dot nanoprobe in a FRET based detection.

The use of nanoparticle-based aptasensors is also widely reported for fluorescence based detection of small biomolecules such as mycotoxins, whole cell bacteria, and other food pathogens. Duan et al. reported the use of the FAM/Au-NP FRET pair where the Au-NP acted as the fluorescence – quenching species. In this approach, a FAM-tagged ssDNA was made to

Fig 4.11: Schematic of the FRET-based kanamycin aptasensor. Li et al, 2014
hybridize with an Au-NP labeled OTA aptamer bringing both pairs in close proximity thus effectively quenching the fluorescence of the FAM tag. However on introduction of OTA, the aptamer detaches from the Au-NP and binds selectively to the OTA thus bringing about a recovery in fluorescence, which corresponds to the detection signal. This aptasensor was successfully applied to detect the presence of OTA in naturally contaminated crops such as maize samples.

Label-free fluorescent detection of small biomolecules using aptasensors is also possible and has been reported severally in literature. These include the use of an aptamer functionalized with a fluorescent functional group and used in the detection of food pathogens and toxins such as AFB1,\textsuperscript{117} and OTA,\textsuperscript{118} and also for the detection of heavy metals,\textsuperscript{119} or the use of nanoparticles as aptamer labels for a fluorescence modulating effect, and this was used in the detection of adenosine.\textsuperscript{120}
Another approach to the label-free fluorescent detection of biomolecules using aptasensors is through the use of fluorescent intercalators. Yong et al\textsuperscript{121} recently reported the use of an hairpin aptamer probe labeled with a fluorescent dye, a polystyrene nanoparticle PS-NP functionalized dsDNA probe (trigger probe), and DNA polymerase for the detection of protein cancer biomarkers. Both probes were shown to be stable in the absence of the protein target, with the dye displaying very low fluorescence polymerization (FP). Signal detection is based on continuous strand-displacement amplification (CSDA) by the present DNA polymerase whose activity is triggered when the aptamer binds to the target on introduction, which opens the hairpin stem structure, and this opened stem sequence hybridizes the single strand portion of the dsDNA probe. This effectively activates the CSDA process, and numerous dyes can be assembled to the PS-NP by this process leading to fluorescent signal detection. This aptasensor gave remarkable aM (10\textsuperscript{-18} M) detection limits. The Scheme for this aptasensor detection is given in Fig 4.12.

4.2.2.2. Colorimetric Aptasensors: Colorimetry-based detection is also another widely used signal detection approach in aptasensor designs. In most of these cases, the aptasensors often use Au-NPs or the HRP-mimicking DNAzyme. Ma et al\textsuperscript{122} used a double Au-NP functionalized aptamer probe which hybridizes at different sequence sections of a complementary sequence (the linker) and the presence of adenosine, which is the target, leads to the disassembly of the Au-NPs due to aptamer structural switching activity. Signal amplification is achieved by the exonuclease III, (Exo III), which digests the aptamer enzymatically from the linker, and releases the adenosine for a new round of detection and digestion. Other approaches include the use of a plasmodium specific aptasensor functionalized with Au-NPs and cationic polymers for the detection of the malaria parasite by a noticeable color change from red to blue\textsuperscript{123} and the detection of adenosine
using adenosine deaminase and a label-free Au-NP aptasensor.\textsuperscript{124} This approach is based on the Au-NP color change from blue, when locked in the aptamer binding structure to the adenosine, to red when the adenosine deaminase disrupts this binding this dispersing the Au-NP.

Huang \textit{et al}\textsuperscript{125} used an HRP-mimicking DNAzyme for the detection of protein biomarkers. Their approach consisted of the protein-specific hairpin aptamer probe, G-rich DNA whose sequence has a recognition and a cleavage site, the nicking enzyme and a blocker DNA. The hairpin aptamer probe opens its stem structure on hybridization with the protein target, and the opened stem sequence can further hybridize to the recognition sequence of the G-rich DNA. This interaction initiates the selective cleavage of the cleavable DNA sequence by the nicking enzyme, such that the aptamer-target hybridized structure is released, and the G-rich DNA is free for another round of binding-cleavage process which continues into a repeating cycle. The free G-rich DNA has a recognition sequence that interacts with hemin, and this interaction allows for generation of a colored oxidized dye, which gives the detection signal.

\textbf{4.2.2.3. Chemiluminiscent Approach to Aptasensor Designs:} Several other approaches apply to aptasensor design for detection of molecules. These includes chemiluminescence reported by Bi \textit{et al}\textsuperscript{126} and Liu \textit{et al} for the detection of platelet-derived growth factor (PDGF) and heavy metals respectively. These approaches are usually similar in design to fluorescent aptasensors. For the detection of PDGF, Bi and coworkers used an Exo III assisted cleavage for a continuous detection cycle such as was discussed earlier. In this approach, they used a blocker DNA, two hairpin aptamers (MB\textsubscript{1} and MB\textsubscript{2}), and the nicking enzyme Exo III. MB\textsubscript{1} and MB\textsubscript{2} self-hybridize to stem-loop structures which has protruding terminus resistant to Exo III, effectively caging the HRP-mimicking DNAzyme. However on introduction of the PDGF target and the subsequent
hybridization with the aptamer, the Exo III-resistant terminus is lost, hence Exo III can now digest the aptamer from its 3’ end. This process frees the target for subsequent rounds of binding and cleaving which ensures signal amplification.

4.2.3. Other Transduction Methods in Aptsensor Design: Mechanical transduction is also used in aptasensor designs, and can offer the advantage of label free detections where structural changes from binding interaction results to changes in mass, which changes the properties of a bound surface, and this gives rise to a detectable signal. Surface plasmon resonance (SPR), is also used for aptasensor designs, and has been reported in the detection of vascular endothelial growth factor (VEGF) by Cennamo et al.\textsuperscript{127} Their design used a functional layer that is responsible for signal transduction based on the conformational changes in the DNA aptamer, and a plastic optical fiber (POF)-based light guiding structure with a planar gold layer used as the sensing region. Bai et al\textsuperscript{128} in a similar method immobilized their recognition biotin functionalized-aptamer to the surface of a gold sensor coated with streptavidin, and on aptamer binding with the target, avian influenza virus (H5N1), brought about a change in the surface refractive index of the sensor, and this afforded the detection signal.

Other mechanical approaches that have been applied to signal transduction in aptasensor designs include Raman scattering as reported by Chung et al\textsuperscript{129} for the detection of bisphenol-A, and by Luo et al\textsuperscript{130} for the detection of tetracycline in milk samples, or the ellipsometry method used by Zengin et al\textsuperscript{131} for the detection of the toxin, ricin B.
4.3. **Future Perspectives:** Although there is increasing interests in the use of aptasensors for detection and analysis, their use is still limited to small molecule targets, proteins, metabolites, and metal ions. Only very few of them have however been applied to biological samples as most studies have been conducted in model systems such as aqueous buffers. Although there is promise that aptamers could be diagnostic tools in disease diagnosis, very few clinically relevant targets has so far been explored. This is because not many aptamers have been discovered for several biological targets, despite the abundance of variations in the SELEX process for aptamer generation. Antibodies thus dominate the commercial diagnostic market, as very few commercial aptasensors are available.
Future studies however will concentrate on generating specific and very sensitive aptamers for biological targets. The promises for commercial aptasensors also comes from recent innovations in nanotechnology and nanomaterials, as aptasensors can easily be incorporated for a label-free optical sensor. The ability to also modify aptamer structure for enhanced detection and sensitivity without altering their target specificity and interaction is a strong feature for aptamers over antibodies, and which will be largely explored. Thus aptasensors remain a very promising field for bioanalysis and diagnostics.

References


53. Khati, M. *Journal of Clinical Pathology* 2010, 63, 480-487.


84. Ping, J.; Wang, Y.; Fan, K.; Wu, J.; Ying, Y. Biosensors & Bioelectronics 2011, 28, 204-209.


89. Xu, Y.; Cheng, G.; He, P.; Fang, Y. Electroanalysis 2009, 21, 1251-1259.


121. Huang, Y.; Xiaqian Liu; Huakui Huang; Jian Qin; Liang-Liang Zhang; Shulin Zhao; Zhen-Feng Chen; Liang, H. *Analytical Chemistry* **2015**.


Chapter Five: Synthesis and Optical Properties of Fluorescent Aptamer

Nucleic acids are known for their Watson-Crick base pairing and double helical structures. Due to the ability of nucleic acids to form duplex structures, they have been effectively utilized as molecular recognition agents (MREs) in several bioassays such as in the Southern blot or the more complicated microarray technology. The discovery of aptamers has further revolutionized their use, due to aptamer abilities to recognize a broad range of ligands and non-nucleic acid targets.\(^1\) Although aptamers have significantly broadened the use of nucleic acids in bioanalysis, standard DNA or RNA aptamers still lack the intrinsic properties to report multiple target bindings and interactions. The pre-selection modification of aptamers though has afforded several interesting properties and enhanced their suitability for different applications. Of particular interest is the stability of aptamers in biological medium, and nuclease-resistant aptamers have been generated for this purpose.\(^2\) Some modifications however cannot be achieved pre-selection, and these are done post-aptamer selection. These modifications can improve the structural features of aptamers for a more selective and accurate reporting of target interaction and detection. The complex and dynamic nature of biological compositions require an aptasensor detection approach that can use a convenient and universal reporting of all aptamer-target interactions. Fluorescent aptasensors, obtained by post-selection modifications of aptamers, offer such a technique.\(^3\)

Fluorescent techniques are well suitable for aptasensor designs. There are several identified fluorophores that can be used to modify aptamers using post-selection chemistry.\(^4\) The use of a fluorophore-modified aptamer approach eliminates the need for labels and thus it is applicable to several aptamer sensing designs.\(^5\) Fluorescent aptamers offer real-time detection with no need for any signal transduction or conversion. The large number of available fluorophores afford a broad range of detection wavelengths solving the initial aptamer limitation in multiplex assays. Detection
of different targets is now possible using fluorescent aptamers without any interference or cross-reference. These features have made fluorescent aptamers suitable for design in several analytical platforms.

5.1. Fluorescent Aptamers

5.1.1. Principles of Fluorescence: Before a molecule is excited, it is usually in its lowest electronic ground state with its least vibrational energy. However on excitation, i.e. the absorption of light at its characteristic wavelength, the vibrational energy of its electrons increases and it moves to an excited state. A series of radiation less energy deactivation allows the molecule to return to its ground state with the release of a photon. The process of photon release is known as fluorescence. Many de-excitation pathways are possible aside from fluorescence such as internal conversion, intramolecular charge transfer, conformational changes, and intersystem crossing. Other competing interactions may include electron/energy transfer from an excited molecule to other nearby molecules, exciplex or excimer formation.

![Jablonski Diagram](image)

*Fig 5.1: Jablonski diagram of the energy levels of a fluorescent molecule*
Fluorescence characteristics such as spectrum, lifetime and quantum yields are usually affected by excited-state processes when excited molecules interacts with its nearby micro-environment. This makes most fluorescent molecules sensitive to temperature which causes non-radiative processes such as solvent molecule collisions, intermolecular rotations and vibrations. These non-radiated processes becomes more efficient at elevated temperatures leading to decrease in fluorescence intensities.\(^9\) The polarity of solvents is another micro-environment factor that affect the fluorescence of molecules. Effect of solvents on molecular fluorescence is so complex and no single theory accounts for them all.\(^10\) Solvent-fluorophore interactions due to the charge-separation of the excited state can lead to changes in spectra shifts. Highly polar solvents tend to reduce the energy of the excited state, leading to emissions at longer wavelengths and lower energies. This is usually true of polar fluorophores, as non-polar molecules such as unsubstituted aromatic hydrocarbons are not very sensitive to polarity changes of solvents.

5.1.2. Fluorescent Oligonucleotides: The standard bases of nucleic acids, purines and pyrimidines, are not fluorescent under normal conditions. But when modified, their structural analogues can be highly fluorescent and still capable of forming hydrogen base pairing with other standard bases. Most approaches use a fluorescent label attached at the end of a linker to a double helix, ensuring the fluorophore is far from the nucleic acid base as possible. Some of these techniques have been discussed in Chapter four. It is however more advantageous to incorporate the fluorophore closer to the DNA double helix without disrupting the helix. This approach rigidly places the fluorophore within the helix restricting its movement, and allowing for a better prediction of its orientation. This is beneficial for most sensing applications such as in FRET studies and fluorescent anisotropy.\(^11\)
2-aminopurine is the first fluorescent base analogue used. It gives high fluorescence, it is well studied, and readily available. Like most fluorescent base analogues however, it is very sensitive to its micro-environment which changes its quantum yield drastically. The DNA duplex has been shown to effectively quench the fluorescence of 2-aminopurine, a factor which limits its applicability. Tricyclic cytosine analogues are also a strongly fluorescent DNA base analogues that are not very sensitive to their environment. Their quantum yields are largely unaffected by their states, and they do not disturb base pairing interactions. They also bind efficiently with guanine, just like the standard cytosine base. They maintain the B-DNA double helix structure when incorporated into nucleotides, making them effective fluorescent base analogues. These modified nucleobases can be incorporated via solid-phase synthesis into oligonucleotide sequences through their available phosphoramidites.

![Chemical structures](attachment:images.png)

**Fig. 5.2: Modified nucleobase analogues and their base pairing**

**5.1.3. Research Background:** Research in the Manderville lab has focused on the synthesis of modified oligonucleotides and their incorporation into aptamers for a label-free fluorescent...
detection of small biomolecules, mainly food toxins.\textsuperscript{4,14,15} To this end, several modified nucleobases have been studied for their probe properties, and their ability to report changes in their micro-environments. This approach offers significant advantages over other techniques aimed at developing fluorescent nucleic acids such as the tagging of fluorophores at either end of the strands, or by tethers attached at specific 5’ and/or 3’ positions in the nucleotide structure. Tethering of fluorophores allows for a great deal of mobility leading to uncertainties in the location and orientation of the fluorophore. This also imposes some structural hindrance to the nucleobase where it is attached as tethers and their attached fluorophores bring about some bulky strain effect.\textsuperscript{16}

In contrast, fluorescent base analogues are smaller, less disruptive to biological interactions, and properly align to local structure of the DNA. These analogues perfectly stack within the double helix making them directly involved in hybridization interactions such that they can easily report small structural changes.\textsuperscript{15,17} They are also in a more rigid conformation, thus making it easier to predict their location and orientation.\textsuperscript{18} Because they do not disrupt any structural feature, their incorporation retains the biological properties of their sequences. These properties have made modified base analogues a very important tool in many biological studies.

The synthesis and fluorescent properties of 8-aryl guanine analogues has been described and used in the Manderville lab for the monitoring of DNA duplex and quadruplex structural changes.\textsuperscript{19} These internal probes were shown to be able to effectively detect structural folding of the 15-mer thrombin binding aptamer (TBA) to a G-quadruplex structure. The modifications despite their preference for the \textit{syn}-position did not disrupt the aptamer TGGT sequence into which they were incorporated.\textsuperscript{4}
The furan-dG nucleoside was also used to probe conformational changes in dsDNA, and shows high fluorescence in water, but it is efficiently quenched in duplex DNA similar to the aminopurines bases described earlier.\textsuperscript{18} The σ-bond linkage from the furan to the purine is also locked in a \textit{syn}-conformation, as the structure is restricted from rotating to the more preferred \textit{anti}-conformation. This is also true for the cyanophenyl modified nucleobase. The dihedral angle $\chi$ [angle $O^4'-C^1'-N^9-C^4$] is said to be \textit{anti}- when the glycosidic bond orientation is $\chi = 180 \pm 90^\circ$ and \textit{syn}- when $\chi = 0 \pm 90^\circ$ (Fig. 5.3). The degree of twist between the attached C$^8$ modification and the nucleobase is defined by the angle $\theta$. These analogues however had the electron push-pull moiety, stimulated by the ring structures, which allows a donor-acceptor effect. This can allow the probe to act as a fluorescent optical switch in aptasensor designs.\textsuperscript{20}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.3.png}
\caption{Modified furan- and cyanophenyl- dG nucleosides showing syn- and anti- conformations}
\end{figure}

Earlier studies in the Manderville lab had also reported a Benzo[b]thienyl-dG nucleoside as a fluorescent reporter of conformational changes in duplex DNA structures\textsuperscript{21} and an indole-modified deoxyguanosine probe which could report structural interactions such as the Watson-Crick versus the Hoogsteen base pairing in oligonucleosides.\textsuperscript{22} These probes preferably adopt the \textit{syn}-conformation, which does not stabilize the duplex structure of DNA. In an \textit{anti}-conformation, the attached group stays in the major groove, thus allowing the nucleobase to maintain its Watson-
Cricks base pairing. *Syn*-conformations however can change the orientation of the bases such that the pyrimidine pairing the purine may flip outside the groove to a solvent exposed environment.

![Fig 5.4: benzo[\(b\)]thienyl and an indole modified deoxyguanosine nucleobase](image)

**5.1.4. Research Objective:** In this work, a biphenyl modified nucleoside was developed as a possible enhancement of the fluorescence properties of probes earlier developed, and its photophysical properties was evaluated and compared with other modified nucleobases to understand its likely probe characteristics. It is known that biphenyl-based probes can exhibit a high donor-acceptor character, especially with an attached electron withdrawing or donor group. Thus fluorine atom, which is the most electronegative single atom (electronegativity of 4.0) was attached at the para-position of the biphenyl ring.

![Fig 5.5: biphenyl modified dG with a vinyl linker](image)

Their steric hindrance of the double ring can also promote charge-transfer states formation, making them highly sensitive to solvent polarity. Their increased conjugation can promote better electronic interactions, and this is thought to push their fluorescence into longer wavelengths emission, expected to make them red-shifted far in the visible region. This is also expected to enhance their fluorescence intensities. This is an important feature required for label-
free off-site detections, as their fluorescence can be visually detected directly without any need for a signal transduction device. Due to the planarity of a double ring structure, and the rigidity that is imposed by the direct single bond linkage to the nucleobase in other probe designs, it was thought to introduce a vinyl switch as linkage to the nucleobase. This should permit rotation about the double bond, allowing the nucleobase switch orientations in both the syn- and anti- conformations.

The biphenyl ring is also expected to be capable of alternating between its planar structure and a semi-planar twisted states which should make it well adapted into a nucleic acid sequence with minimal disruption of base pairing interactions, and an ability to report any structural change that occurs. These properties were the basis of the design for this nucleobase analogue. The probe properties of this nucleobase analogue was to be investigated by incorporating it into TBA and the OTA aptamer to see how it responds to simple changes in its microenvironment. This would provide an understanding of further structural nucleobase changes that can afford better or more excellent fluorescent aptasensors of biological samples such as OTA and AFB1 mycotoxins.

5.2. Experimental Procedure

5.2.1. General methods: A detailed description of the experimental procedures performed in this chapter are given in Appendix A. Specific details of pertinent procedures are described below.

5.2.2. Synthesis of FBPV-dG: The synthetic pathway used in the preparation of FBPV-dG is outline in Scheme 5.1. The synthetic details are however described below. 8-Bromo-2’-deoxyguanosine was synthesized according to literature procedures and described below.\textsuperscript{23} NMR spectra of the synthetic compounds were recorded on 300 MHz Bruker spectrometer at the NMR laboratory
center, University of Guelph. Mass spectrometry was done at the spectrometry center, University of Guelph.

5.2.2.1: Synthesis of 1,1’-Biphenyl, 4-bromo-4’-fluoro- (3): The synthetic details applied was according to literature procedures. 1,4-dibromobenzene (2) (2.02 g, 8.56 mmol) and 4-fluorophenylboronic ester (1) (1 g, 7.14 mmol) was reacted to give 1,1’-Biphenyl, 4-bromo-4’-fluoro- (3) (1.24 g, 95% yield). 2.02 g (8.56 mmol) of 1,4-dibromobenzene, 1 g (7.14 mmol) of 4-fluorophenyl boronic ester, 60 mg (0.09 mmol) of bis-(triphenylphosphine) palladium(II)dichloride, and 20 mL of 1,4, dioxane was stirred in a 100 ml flask. 2.4 g (28.56 mmol) of sodium bicarbonate was dissolved in 10 ml water and added. The mixture was stirred under reflux at 80°C for 1 hr. Reaction was monitored on thin layer chromatography, and product was isolated using column chromatography to obtain 1.24 g of 3 (95% yield). 1H NMR (300 MHz, CDCl3) δ 7.55 – 7.52 (d, 2H), 7.50 – 7.48 (d, 2H), 7.38 – 7.33 (d, 2H), 7.14 – 7.09 (t, 2H).

5.2.2.2. Synthesis of 1,1’-Biphenyl, 4-ethynyl-4’-fluoro- (4): This was carried out in a 2-step, one pot protocol according to a similar literature procedure with little modifications to allow for reaction optimization. 1,1’-biphenyl, 4-bromo-4’-fluoro- (1.24 g, 4.94 mmol) reacted with trimethylsilyl acetylene (1.2 mL, 7.41 mmol) in the presence of bis-(triphenylphosphine) palladium(II)dichloride (173 mg, 0.247 mmol) to give 1,1’-Biphenyl, 4-trimethylsilyl, 4’-fluoro- (1.22g, 61% yield). 1.24 g (4.94 mmol) of 3 was added with 173 mg (0.247 mmol) of bis-(triphenylphosphine) palladium(II)dichloride and stirred in a 100 ml flask under vacuum. A mixture of 10 mL THF and 5mL TEA was stirred under argon for 30 mins and added. The mixture was stirred for 15 mins under argon. 1.2 mL (7.41 mmol) trimethylsilyl acetylene was added, followed by the addition of 9.4 mg (0.05 mmol) of CuI. The mixture was stirred at 40°C for 8 hours, and for 70°C for a further 8 hours.
The solution was cooled down to room temperature and evaporated to give a dark grey solid. 100.65 mg of K$_2$CO$_3$ was added to the dark grey solid obtained, in 10 mL methanol and 5 ml methylene chloride for a further 3 hours to give 0.88 g of 4 (0.88 g (66% yield). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.542 – 7.512 (d, 6H), 7.524 – 7.492 (d, 2H), 7.502 – 7.469 (d, 2H), 7.140 – 7.082 (t, 2H), 3.1 (s, 1H).

5.2.2.3. **Synthesis of 1,1'-Biphenyl, 4-vinylboronic ester-4'-fluoro- (5):** This was carried out according to literature procedures$^{26}$ of the hydroboration of triple bonds to yield a vinyl boronic ester. The 1,1'-Biphenyl, 4-ethynyl-4'-fluoro- (0.88 g, 4.49 mmol) was reacted with bis-pinacolato diboron (1.25 g, 4.94 mmol) to give 1’,1'-Biphenyl, 4-vinylboronic ester-4'-fluoro- (5) (0.77 g, 76% yield). 32 mg (0.2245 mmol) of CuBr, 72 mg (0.2694) of triphenylphosphine (PPh$_3$) and 48.5 mg (0.898 mmol) of sodium methoxide (NaOMe), was dissolved in 15 mL dry THF and stirred for 30 mins under argon. 1.25 g (4.94 mmol) of bis-pinacolato diboron was added. O.88g (4.49 mmol) of 1,1'-biphenyl, 4-ethynyl-4'-fluoro- was added. 5 mL methanol was added and the mixture was stirred under argon for 3 hours. This gave 0.77 g (76% yield) of the desired product (5). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.532 – 7.483 (m, 6H), 7.438 – 7.377 (d, 1H), 7.130 – 7.072 (t, 2H), 6.214 – 6.152 (d, 1H), 1.306 (s, 12H).

5.2.2.4. **Synthesis of 8-Bromo deoxyguanosine (8-BrdG):** This reaction was carried out according to procedures obtained from literature.$^{27}$ Deoxyguanosine (dG) (16 g, 59.87 mmol) was reacted with N-bromo succinimide (15.3 g, 119.74 mmol) in acetonitrile : water, 4 : 1 mix to give the desired 8-BrdG (17.8g, 91% yield). 16 g (59.87 mmol) of dG and 400 mL of 4:1 CH$_3$CN: H$_2$O was added in a 1 L Erlenmeyer flask with stirring. 15.3 g (119.74 mmol) of N-bromosuccinimide (NBS) was added slowly in three equal parts, waiting for the formed yellow colour on adding the NBS to fade away before adding the next portion, and the reaction was stirred for 45 min at room temperature.
temperature. The resulting suspension was filtered under vacuum, resuspended in 250 mL of cold acetone and stirred at room temperature for 2 h. The suspension was left at 0 °C overnight, filtered and washed with cold acetone. 8-Br-dG was collected as a faint pink solid (17.8 g, 91%); 1H NMR (300 MHz, DMSO-d6) δ 10.80 (s, 1H), 6.47 (s, 2H), 6.16 (t, J = 7.4 Hz, 1H), 5.23 (d, J = 4.5 Hz, 1H), 4.86 (t, J = 6.2 Hz, 1H), 4.39 (s, 1H), 3.80 (m, 1H), 3.60 (m, 1H), 3.51 (m, 1H), 3.15 (m, 1H), 2.11 (m, 1H); 13C NMR (150 MHz, DMSO-d6) δ 155.4, 153.1, 151.9, 120.5, 117.4, 87.8, 85.1, 71.1, 62.0, 36.5.

5.2.2.5. Synthesis of 1’’,1’’-Biphenyl, 4’’’-fluoro, 4’’-vinyl-dG (FBPvG): This reaction was carried out according to literature procedures of the Suzuki-Heck coupling of boronic esters with halides. 1’,1’-Biphenyl, 4-vinylboronic ester-4’-fluoro- (0.77 g, 2.10 mmol) was reacted with 8-Bromo deoxyguanosine (0.54 g, 1.47 mmol) to afford FBPvG (6) (0.61 g, 82% yield). 0.77 g (2.10 mmol) of 5 was added with 94.29 mg (0.42 mmol) of palladium acetate (Pd[OAc]2), 59.69 mg (0.11 mmol) of triphenylphospine 3,3’,3’’ trisulfonyl acid, trisodium salt (TPPTTS), and 0.54 g (1.47 mmol) of 8-BrdG was added in a 100 mL flask and stirred deaerated in 10 mL acetonitrile. 556.5 mg (5.25 mmol) of sodium carbonate (Na2CO3) was dissolved in 5 mL H2O and the mixture was heated to 80° C for 5 hours. The pH of the mixture was brought to 7 by adding dropwise 1.0 M HCl, and monitoring with a pH meter. This was then stored overnight at 0° C. The solution was vacuum filtered and washed in methylene chloride and methanol. A light army green solid obtained was then dried under high vacuum to obtain 0.61 g (82% yield) of the desired product. 1H NMR (300 MHz, DMSO-d6) δ 10.855 (s, 1H), 7.773 – 7.733 (d, 2H), 7.746 – 7.743 (d, 2H), 7.570 – 7.536 (d, 2H), 7.65 – 7.536 (d, 1H), 7.536 (s, 1H), 7.298 – 7.244 (t, 2H), 6.585 (s, 2H), 6.40 – 6.37
(d, 1H), 6.021 – 6.003 (s, 1H), 5.211 – 5.181 (d, 2H), 4.452 – 4.288 (s, 1H), 3.815 – 3.574 (s, 2H),
2.052 (s, 1H), 1.125 – 1.039 (d, 1H).

5.2.3. Fluorescence Studies of FBPV:dG: Stock solutions of FBPV:dG was made in 4 mM DMSO, as
the nucleoside had partial solubility in other solvents. Spectroscopic solutions of the modified
nucleoside was then prepared in 12.5 µM solutions of different solvents (MOPS buffer–pH 7,
acetonitrile, THF, DMSO, methanol, isopropanol, and chloroform) Fluorescence spectra was
obtained from 10 nm above the excitation wavelength to 700 nm. This was done in a 1mL quartz
cuvette, with excitation and emission slit widths maintained at 2.5 nm throughout the studies,
except when stated otherwise. Viscosity studies was done in 12.5 µM of MOPS buffer–pH 7, by
increasing the viscosity with glycerol in orders of 20%, 40%, 60%, and 80%. The fluorescence of
the modified nucleoside was done under this conditions by scanning emission at 10 nm above
excitation wavelengths. The temperature studies was then done in the 80% glycerol – 20% MOPS
pH 7, by heating the mixture in incremental orders of 10° C magnitude from 20° C to 70° C.
The same method as described above was applied for the fluorescent studies of similar base
analogues as have been recently synthesized in the Manderville lab. The fluorescent properties
was compared as an evaluation of the likely probe properties of the 10’’-fluorobiphenyl-vinyl-dG
when incorporated in an oligonucleotide sequence.

5.2.4. MS experiments: This was done on a Bruker AmaZon SL quadrupole ion trap SL
spectrometer to confirm the correct synthesis of FBPV:dG. Spectral data was acquired by negative
ionization mode using an electrospray ionization source. FBPV:dG sample was dissolved in a 90%
Milli-Q filtered water, 10% DMSO, and injected directly into the electrospray source at 20 uL/min.
Ionization was conducted using the ESI settings: nebulizer gas pressure (40 psi); drying gas flow (10 L/min); drying gas temperature (200 °C); spray voltage, (−4000 V). The mass range done was between m/z 70–2000, and the scan rate was 8100 m/z/s.

5.3. Results and Discussion

5.3.1. Synthesis of FBPVdG: The strategy employed in the synthesis of FBPVdG is outlined in Scheme 5-1. 8-Bromo dG was synthesized according to methods outlined in literature. The initial Suzuki coupling reaction of the 4-fluorophenyl boronic acid and 1,4-dibromobenzene is a well-known procedure of coupling halides to boronic esters. This reaction is described in literature to run for 3 hours to achieve reaction completion. Following this approach however gave rise to very poor yields, as the boronic ester can react with the initial product as it is formed, to give rise to triphenyl 4,4''-difluoro side product. In optimizing this process, kinetic control was utilized as the biphenyl product is formed faster than the triphenyl. Hence the reaction was monitored on TLC to ascertain the start of triphenyl side-product formation, estimated to be 1 hr. The reaction was stopped then, and flash column chromatography of the mixture using 100% hexane as the eluent afforded the desired compound. The starting compound still present in the mixture was recollected, which can be reused for the same reaction. Other measures taken to optimize product yield was to reduce the equivalence of the 1,4, dibromobenzene such that it was present only in sufficient amounts for complete conversion of the starting material. The solvent volume was also increased to create a surface distance barrier, thus reducing the unwanted conversion of the desired product to the side products. This measures afforded the 1,1'-diphenyl, 4-fluoro, 4'-bromo compound in good yields.
The next step was the Sonagashira reaction of trimethylsilyl acetylene with halides using procedures as described in literature. However, none of the procedures in the literature could provide for effective yields, as the high temperatures or long reaction times described did not generate the desired product in good yields. It was however found that slowly raising the reaction temperature after allowing it to run at 40°C for 8 hours, to around 70°C for a further 4 hours gave the desired product in good yields. The second step of desilylation was done at room temperature according to literature procedures and gave 100% product yield. The hydroboration step was effective as described in literature, though it was found that starting the reaction at slightly warmer temperatures ≈ 40°C and cooling to room temperature after one hour improved product yield by a factor of around 10%.

Scheme 5-1: Synthesis pathway for 1''''-Biphenyl, 4''''-fluoro, 4''''-vinyl-dG
5.3.2. Fluorescence Studies of $^{FBPV}dG$: Fluorescence studies of the photophysical behavior of the $^{FBPV}dG$ in several solvents of different polarities was conducted to determine the maximum absorption and emission of the nucleoside. Similar studies were also done to determine the behavior of the modified nucleobase under different conditions of temperature and viscosity. As the viscosity of the medium increases, it was expected that the modified nucleobase achieve a more rigid structure due to the inhibition in movement, achieving a more stable planar state, which should increase its fluorescence.\textsuperscript{32} On the other hand, increase in temperature is expected to bring about a decrease in fluorescence and lifetime, as thermal agitation should lead to the occurrence of more non-radiative processes such as intramolecular vibrations, higher degrees of movement and collision with solvent, and also the enhanced rotation all characteristic of elevated temperatures.\textsuperscript{33} The behavior of $^{FBPV}dG$ under these changing conditions will serve as a basis to understand its likely probe properties when incorporated into an oligonucleotide sequence.

The fluorescence properties of this nucleoside was then compared with other similar modified nucleosides that have been synthesized in the Manderville laboratory. This was to enable an assessment of the effect of a second ring for enhanced conjugation as the more conjugated system ($\pi$-electron systems) should provide for more red-shifted absorption and emission spectra to longer wavelengths, and also give increased quantum yields.
The π-electron system conjugation of the FBPV dG is also enhanced through the vinyl linker, which extends the electron flow into the imidazole ring of heterocyclic purine aromatic moiety.

To obtain this spectra, the fluorescence of FBPV dG was compared in solvents of different polarities as shown in Fig 5.4 and Table 5.1. It is observed from the spectra that the modified nucleoside showed strong emission in polar aprotic solvents and in the non-polar chloroform, and much weaker emission in polar aprotic solvents such as water and methanol. However the emission was red-shifted to longer wavelengths of lower energies in the polar solvents. Non-polar solvents cannot stabilize a charge separated excited state in an electron donor – acceptor pair relationship as in FBPV dG. Thus as polarity increases, the planar excited state of the nucleobase analogue becomes energetically unfavourable requiring more energy.\textsuperscript{10} This results in an hyposochromic shifted fluorescence to shorter wavelength and higher energy as noticed in Table 5.1.

The observed bathochromic shifts can also be attributed to solvent relaxation effects.\textsuperscript{34} The solvent cages formed around the fluorescent molecules when excited is relaxed due to changes in dipole moments. Changes in dipole moment of the excited state (\(\mu_e\)) of an aromatic compound is
different to that in its ground state ($\mu_g$). These molecules when excited will therefore experience an intramolecular charge transfer state such that $\mu_e > \mu_g$. The higher the polarity, the lower the solvent relaxation energy, which leads to a more red-shifted emission spectra.$^{35,36}$

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Excitation Wavelength (nm)</th>
<th>Intensity (a.u)</th>
<th>Emission Wavelength (nm)</th>
<th>Intensity (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>362</td>
<td>380</td>
<td>424</td>
<td>335</td>
</tr>
<tr>
<td>Chloroform</td>
<td>362</td>
<td>245</td>
<td>427</td>
<td>195</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>352</td>
<td>124</td>
<td>455</td>
<td>100</td>
</tr>
<tr>
<td>DMSO</td>
<td>367</td>
<td>130</td>
<td>465</td>
<td>93</td>
</tr>
<tr>
<td>DMF</td>
<td>363</td>
<td>59</td>
<td>461</td>
<td>59</td>
</tr>
<tr>
<td>Methanol</td>
<td>352</td>
<td>56</td>
<td>456</td>
<td>48</td>
</tr>
<tr>
<td>Water</td>
<td>349</td>
<td>21</td>
<td>466</td>
<td>19</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>351</td>
<td>9</td>
<td>450</td>
<td>7</td>
</tr>
</tbody>
</table>

**Table 5.1: Absorption and emission maximums of the FBPVdG in various solvents.**

From Table 5.1, it is also observed that the excitation-emission band of FBPVdG is narrowed as polarity decreases. Thus in THF, FBPVdG absorbs at higher wavelengths and emits at lower wavelengths when compared to in more polar solvents like methanol and water where it has a larger difference in absorption and emission wavelengths. Though emissions in polar solvents like water and isopropanol is red-shifted, it also appears to be quenched. However, if the amount of light passing through the sample is increased from 2.5 excitation slit width to 5.0 excitation slit width, there is a visible detection of fluorescence as shown in the fluorescence spectra in water (Fig 5.5.). This thus implies that FBPVdG is also emissive in water.
The viscosity studies show that the intensity of emission increases as the environment becomes more viscous. This is as a result of the imposed stiffness to movement, such that other radiationless excitation deactivation cannot occur leading to improved fluorescence. The structure is also locked in a planar system, limiting twisted conformations or rotations, processes that reduce intensities of emissions. However in the temperature studies, it is observed that the modified nucleoside does not drastically respond to thermal agitation, as small degrees of intensity changes were recorded with gradual increase in temperatures.

These optical properties of the FBPV\textsuperscript{\textregistered}dG analogue seems promising so far, and it would be interesting understanding its probe properties in an oligonucleotide sequence. As it is emissive and bathochromic shifted in water, it would be nice to see how it responds to changes in a probe environment. Some unique properties such as the insensitivity of the nucleobase to temperature may be exciting, as this properties may be maintained in incorporated sequences. This would mean that the fluorescence is not disturbed under conditions of increased temperatures.
The solvatochromic properties of a similar analogue recently synthesized in the Manderville laboratory was also conducted, as a means of comparison. The compound, a 4''-trifluorobenzo-1''-vinyl-dG, CF3PV-dG, is a perfect comparison to understand the effect of the double ring structure as this nucleoside contains only a single ring attachment, and the fluorine
atom as an electron push-pull effect for the aromatic structure as this compound contains three fluorine group attached to the aromatic modification.

Fig 5.10: Structure of $\text{CF}_3\text{PV} \text{dG}$ as synthesized by Xing Li in the Manderville laboratory

### Table 5.2: Absorption and emission maximums of the $\text{CF}_3\text{PV} \text{dG}$ in various solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Absorption Wavelength (nm)</th>
<th>Intensity (a.u)</th>
<th>Emission wavelength (nm)</th>
<th>Intensity (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>365</td>
<td>111</td>
<td>466</td>
<td>116</td>
</tr>
<tr>
<td>Chloroform</td>
<td>348</td>
<td>91</td>
<td>444</td>
<td>92</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>353</td>
<td>93</td>
<td>472</td>
<td>94</td>
</tr>
<tr>
<td>DMSO</td>
<td>369</td>
<td>75</td>
<td>492</td>
<td>69</td>
</tr>
<tr>
<td>DMF</td>
<td>366</td>
<td>14</td>
<td>485</td>
<td>29</td>
</tr>
<tr>
<td>Methanol</td>
<td>353</td>
<td>80</td>
<td>465</td>
<td>84</td>
</tr>
<tr>
<td>Water</td>
<td>343</td>
<td>40</td>
<td>475</td>
<td>39</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>362</td>
<td>4</td>
<td>452</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.2: Absorption and emission maximums of the $\text{CF}_3\text{PV} \text{dG}$ in various solvents.
From the results, both nucleobase analogue show similar characteristics. Their fluorescence are both increased and blue-shifted in less polar solvents. However it is noted that increased conjugation has enhanced the molar absorptivity of \( \text{FBPV} \text{dG} \), as it gives a 3-fold increase in intensities in THF, in which they both exhibit their highest intensities of emission. The presence of the three fluorine atoms might also be responsible for the decreased intensities, based on the heavy atom effect.\(^{37}\) This might suggest that the chances of intersystem crossing increases as the fluorine atom increases, leading to reduced intensities in fluorescence. It can also be noticed that the fluorescence of \( \text{CF3PV} \text{dG} \) is not as blue-shifted as in \( \text{FBPV} \text{dG} \) in solvents of lower polarities. This might also be from the extra ring structure on the biphenyl system, which gives it an increased destabilization of its charge transfer excited states in less polar solvents as opposed to the single ring structure where this effect is not very reduced.\(^{10}\)

Further comparison of the fluorescence spectra of \( \text{FBPV} \text{dG} \) with another nucleobase analog, 4-acetylbenzo-dG (\( \text{AcP} \text{dG} \)). This was chosen to further compare a single ring system with a different attached group other than the fluorine. \( \text{AcP} \text{dG} \) had been synthesized and its fluorescence spectra had also been studied by K. Faddock in the Manderville lab. The excitation and emission table of \( \text{AcP} \text{dG} \) is given in table 5.3.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Absorption Wavelength (nm)</th>
<th>Intensity (a.u)</th>
<th>Emission wavelength (nm)</th>
<th>Intensity (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>339</td>
<td>176</td>
<td>472</td>
<td>172</td>
</tr>
<tr>
<td>Chloroform</td>
<td>338</td>
<td>169</td>
<td>464</td>
<td>168</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>328</td>
<td>23</td>
<td>496</td>
<td>23</td>
</tr>
<tr>
<td>DMSO</td>
<td>347</td>
<td>21</td>
<td>520</td>
<td>20</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>337</td>
<td>9</td>
<td>510</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 5.3: Absorption and emission maximums of the \( \text{CF3PV} \text{dG} \) in various solvents.
Similar analogies can also be drawn from the comparison of AcPdG and FBPVdG as was earlier done for FBPVdG and CF3PVdG. Here, both compounds showed similar fluorescence quenching in isopropanol and highly polar solvents and also had hypsochromic shifts in less polar solvent environments. These sensitivities, as observed in the modified nucleobases, is characteristic of donor – acceptor systems as present in the three samples.\textsuperscript{17} The intensities of emission was also shown to be 2-folds increased in FBPVdG when compared to this analog, similar to what was observed with CF3PVdG. This, as explained earlier, is likely a feature of the extra conjugation present in FBPVdG. AcPdG also had larger bathochromic shifts (red-shifted to longer wavelengths and less energy emissions), as likely caused by destabilization of the excited charge transfer states in FBPVdG.

5.4. Conclusion and Further Studies: Fluorescence studies of FBPVdG provide informative findings on possible optical properties of its probe when incorporated into larger sequences for sensing platforms. As this compound is emissive in water, does not lose its fluorescence under increased temperature, and shows sensitivities to solvent polarities, it shows features of an effective sensor when incorporated as probes for detecting changes in the microenvironment of nucleic acids. It will be interesting to find out how this analogue does in stabilizing DNA structures, and in its sensitivities to small conformational changes of the sequence where it is inserted. From the fluorescence studies done however, the analogue shows promise of an exciting sensing probe for DNA structural changes.

5.5.1. Future Work: The next phase of this project would be to incorporate FBPVdG into an aptamer sequence and ascertain its properties. Suitable aptamers will be the thrombin binding
aptamer (TBA), as it is well studied and suitable for understanding the probe properties of new and prospective sensing analogues. The analogue can also be incorporated into the OTA aptamer if properties in TBA are deemed encouraging. To incorporate this nucleobase into an oligonucleotide, its phosphoramidite monomer has to be synthesized. The process for this synthesis is shown in Scheme 5-2.

![Scheme 5-2: Proposed synthesis of FBPVdG DNA phosphoramidite monomer](image)

The resulting phosphoramidite monomer can then be incorporated into an appropriate oligonucleotide via solid phase DNA synthesis as described earlier in the Manderville laboratory. Based on the interesting features and promising fluorescence properties observed in FBPVdG, the analogue is expected to become a valuable fluorescent probe for its microenvironment when incorporated into an oligonucleotide.

References


Appendix A

General Experimental Procedures

A.1. General Chemical Details: Unless otherwise stated, all commercial compounds were used as received. 1,4-dioxane, methanol, and acetonitrile were purchased from Caledon Labs, Ontario Canada. 1,4-dibromobenzene (98%), N-bromosuccinimide (99%), sodium methoxide (NaOMe), (95%), CuBr (99.99%), and CuI (99.99%) was purchased from Sigma-Aldrich, USA. Sodium hydrogen carbonate (NaHCO₃), potassium carbonate (K₂CO₃), sodium carbonate (Na₂CO₃), and methylene chloride (CH₂Cl₂) was purchased from Fisher Scientific, USA. Palladium bis-triphenylphosphine dichloride (PdCl₂[PPh₃]₂, 98%), bis(pinacolato)diboron ([CH₃]₄C₂O₂B)₂, and triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt hydrate (TPPTTS, 95%) was purchased from AK Scientific Inc. CA, USA. Trimethylsilyl acetylene and palladium acetate (Pd[OAc]₂, 47.5% Pd) was purchased from Acros, NJ USA. 4-fluoro-benzene, 1-boronicacid was purchased from Frontier Scientific. Triphenyl phosphate (PPh₃) was purchased from Alfa Aesar, England. Deoxyguanosine (dG) was purchased from ChemGenes Corp, USA. Dry solvents, tetrathydrofuran (THF) and methanol, was obtained from an LC technology SP-105 solvent purification system at the University of Guelph chemistry department.

A.2 Nuclear Magnetic Resonance (NMR): 1H NMR spectra was recorded on a Bruker Avance DPX 300 MHz spectrometer in either DMSO-D₆ or CDCl₃ at the NMR facility, University of Guelph.
A.3 Mass Spectrometry: Low resolution Mass Spectrometric analysis was obtained on a Bruker AmaZon SL spectrometer equipped with an ion trap through direct injection at the Biological Mass Spectrometry Facility, University of Guelph.
Appendix B.

Mass Spectrometry Data

Mass spectra of $\text{FBP}^\text{v} \text{dG}$
Appendix C

NMR Spectra for Synthetic Compounds

\[1^H \text{ NMR spectra for 1,1'-biphenyl 4-bromo, 4'-fluoro}\]
\[^{1}\text{H NMR spectra of 1,1'-biphenyl 4-ethyne, 4'fluoro}\]
$^1$H NMR spectra of 1,1'-biphenyl 4-vinyl boronic ester, 4'fluoro
$^1$H NMR spectra of FBPydG