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Developing a Triad of DNA Sensors to Analyse Forensically Important Samples - Polypyrrrole, Metal-ion Implantation and Resistive Pulse Sensing

Marsilea Adela Booth, MSc (hons)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Forensic Science, The University of Auckland 2012
Abstract

Many medical, forensic and environmental scientific needs may be satisfied by the existence of suitable DNA sensors. For forensic applications an important DNA sensor is one that is able to selectively and sensitively detect the presence of a specific body fluid, for example blood. A variety of approaches have been adopted to develop DNA sensors including those involving polypyrrole and electrical impedance spectroscopy. Combined, these are able to sense as well as aptly transduce signal. A sensor was subsequently developed. Investigations demonstrated that improvements in stability and sensitivity, as well as reduction in non-specific DNA binding for fabricated sensors, could be achieved through the use of a specific dopant, redox couple and post-growth treatment.

Much DNA sensor research-to-date has focused on short target DNA strands as model genes; therefore, following DNA sensor development, the effect of the length of oligonucleotide probe and target strands was studied as a significant step towards real world applications for DNA detection. Longer target (and probe) DNA strands produced increased response, presumably a feature of associated negative charges. Furthermore, real blood sample interactions with sensor surfaces were examined. Although there was initially non-specific binding, this was reduced through the use of a dialysis membrane.

Furthermore, metal-ion implantation was investigated for its effect on polypyrrole conductivity, stability and other characteristics. Low-energy implantation of platinum and lead ions into synthesised polypyrrole films was performed. Characterisation experiments were undertaken before and following implantation. Results displayed optimal fluences, where polypyrrole films implanted with $2 \times 10^{16}$ Pt at. cm$^{-2}$ and $(2$ and $20) \times 10^{14}$ Pb at. cm$^{-2}$ display and retain enhanced conductivity compared with non-implanted samples. A simple DNA sensor was constructed with Pt-implanted film that displayed successful detection of complementary DNA.

In a third DNA sensing system, probe oligonucleotide-grafted particles were used to detect target DNA through resistive pulse nanopore detection. Particle-by-particle analysis together with a statistical intercept model and a variable pressure method were used to distinguish particles before and following target hybridisation. Hybridisation was selectively detected at micromolar concentrations and confirmed by complementary techniques.

In conclusion, three sensor systems were developed and were successfully shown to detect target DNA. The strengths and weaknesses of the different sensor systems provide vital information for the development of future DNA sensors.
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Firstly I’d like to thank my supervisor A/Prof Jadranka Travas-Sejdic for the suggestions, opportunities, guidance and support. How she can juggle teaching, supervising, and motherhood is an inspiration.

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Research Outputs

This thesis is comprised of the following publications (papers and a proceeding), alongside additional experiments, details and discussions:


Unpublished submitted paper:


Other publications:

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<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\theta$</td>
<td>Angle</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Extinction coefficient or permittivity</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Angular frequency or parameter frequency</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohm</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Refractive index</td>
</tr>
<tr>
<td>$\rho$</td>
<td>Resistivity</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Conductivity</td>
</tr>
<tr>
<td>$v$</td>
<td>Sweep rate</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$\zeta$</td>
<td>Zeta potential</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>A</td>
<td>Area / Absorbance or CPE coefficient</td>
</tr>
<tr>
<td>$a$</td>
<td>Chemical activity coefficient ($a_{ox}$ and $a_{red}$ for oxidised and reduced)</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>at. cm$^{-2}$</td>
<td>Atoms per centimetre squared</td>
</tr>
<tr>
<td>a.u.</td>
<td>Arbitrary unit</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated total reflection Fourier transform infrared</td>
</tr>
<tr>
<td>BZQ</td>
<td>Benzoquinone</td>
</tr>
<tr>
<td>c</td>
<td>Concentration</td>
</tr>
<tr>
<td>C</td>
<td>Concentration of particles</td>
</tr>
<tr>
<td>$C_{dl}$</td>
<td>Double layer capacitance</td>
</tr>
<tr>
<td>ClO$_4^-$</td>
<td>Perchlorate ion</td>
</tr>
<tr>
<td>CPE</td>
<td>Constant phase element</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammetry</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPV</td>
<td>Differential pulse voltammetry</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide</td>
</tr>
<tr>
<td>$E$</td>
<td>Electric field</td>
</tr>
<tr>
<td>$E^*$</td>
<td>Formal electrode potential</td>
</tr>
<tr>
<td>$E_b$</td>
<td>Binding energy</td>
</tr>
<tr>
<td>$E_k$</td>
<td>Kinetic energy</td>
</tr>
<tr>
<td>$E_p$</td>
<td>Peak position</td>
</tr>
<tr>
<td>EIS</td>
<td>Electrical impedance spectroscopy</td>
</tr>
<tr>
<td>$F$</td>
<td>Faraday’s constant</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster (Fluorescence) resonance energy transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>GCE</td>
<td>Glassy carbon electrode</td>
</tr>
<tr>
<td>GOX</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>H₂Q</td>
<td>Hydroquinone</td>
</tr>
<tr>
<td>I</td>
<td>Current</td>
</tr>
<tr>
<td>ICP</td>
<td>Intrinsically conducting polymers</td>
</tr>
<tr>
<td>Iₚ</td>
<td>Current peak height</td>
</tr>
<tr>
<td>J</td>
<td>Particle flux</td>
</tr>
<tr>
<td>K</td>
<td>Equilibrium constant</td>
</tr>
<tr>
<td>Kₜ</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>m-PEG</td>
<td>Methyl poly(ethylene glycol)</td>
</tr>
<tr>
<td>NFM</td>
<td>Nanofibrous membrane</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxy succinimide</td>
</tr>
<tr>
<td>ON</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>PAA</td>
<td>3-Pyrrolylacrylic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Propylene carbonate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>PSS</td>
<td>poly(4-styrenesulfonic acid)</td>
</tr>
<tr>
<td>PPY</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>P(Py-co-PAA)</td>
<td>Copolymer of pyrrole and PAA monomers</td>
</tr>
<tr>
<td>q</td>
<td>Charge on the particle</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>Qₚ</td>
<td>Volumetric flow rate</td>
</tr>
<tr>
<td>R</td>
<td>Resistance or universal gas constant or particle radius</td>
</tr>
<tr>
<td>Rₜ</td>
<td>Charge transfer resistance</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rs</td>
<td>Solution resistance</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>V</td>
<td>Potential</td>
</tr>
<tr>
<td>VPM</td>
<td>Variable pressure method</td>
</tr>
<tr>
<td>w</td>
<td>Work function</td>
</tr>
<tr>
<td>W</td>
<td>Warburg impedance</td>
</tr>
<tr>
<td>Zₜ</td>
<td>Real impedance</td>
</tr>
<tr>
<td>Zₗₐ</td>
<td>Imaginary impedance</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Marsilea Booth
Chapter 1. General Introduction

Discovering and identifying the presence of body fluids at a forensic scene can play a crucial role for investigation. Identifying a sample involves the determination of the physical or chemical identities with as near absolute certainty as existing techniques will permit.\(^1\) It is the first step in the analysis of an unknown fluid or stain, shedding light on further processes and sample handling. If, for example, the body fluid is considered semen through a presumptive test, analysis may follow with microscopic examination for spermatozoa. Alternatively if the evidence is considered blood based on initial tests, an aliquot of sample may be collected, dried and stored in a particular way to retain maximum recoverable analyte for a future time.\(^2\) In particular with deoxyribonucleic acid (DNA), sample handling can greatly influence analysis success - pertinent for this sample which is often of high evidentiary importance.\(^3\) Identifying body fluids present may aid in establishing or corroborating a possible series of events at a forensic scene. As an example, imagine a stain is found on a suspect and is considered a possible result of blood spatter from a victim’s knife wound. The suspect however, reports the stain originated as aspirated blood. The presence of saliva may support the suspect’s claim.

Common body fluids found at forensic scenes include blood, semen, saliva and vaginal secretions.\(^4,5\) Blood is commonly found at the scene of a homicide, seminal fluid may be present at the scene of an alleged sexual assault, as well as vaginal secretions. Saliva may be found on cigarettes or food substances. Arising from the need to identify these body fluids, detection methods have been developed and established. Each body fluid maintains a unique composition, though with some body fluids sharing common components.\(^5\) Therefore the tests for different body fluids differ depending on the fluid in question, and may be visual, microscopic or chemical techniques. An example of testing for the body fluid blood is typically a presumptive test such as Kastle Meyer, luminol, leucomalachite green and Hemastix®, followed by the use of a test such as the Rapid Stain Identification-Blood (RSID\textsuperscript{TM}-Blood), or other immunological tests.\(^6\) Advantages of current techniques exist for each examination, along with their validation and standard procedures. Meanwhile, performing these tests may be time consuming and labour intensive, and some body fluids do not have a confirmatory technique, such as saliva or vaginal secretions.\(^4,7,8\) Lowered sensitivity due to false signalling from amplified contaminants may also pose a difficulty.\(^9\)
A considerable disadvantage where fluid identification is involved is that the tests are performed in series. This way, until a positive result is found, multiple tests must be performed. Mixed fluids complicate analysis, where one fluid may mask another’s presence, such as a case involving an alleged sexual assault where a mixture may be found of semen and vaginal secretions. Because most tests are designed to detect a specific body fluid, investigators must decide which test to perform, and in cases where a mixture is present, care must be taken that a test for one body fluid does not render the tests for the other body fluid ineffective. Precious sample may be wasted, all the more difficult as samples are often found in small volumes, typically less than 50 µL, or may be found in poor condition.

Hence, there is an urgent need for a rapid, sensitive, selective and simple sensor, which is able to identify body fluids. A DNA sensor targeting a specific nucleotide sequence would fulfil this role. Such a sensor may also be adapted for a range of other applications such as medical diagnostics, food analysis, pathogen analysis, sequence testing and species identification.

1.1 Thesis Objectives

The research herein is aimed as a step towards the development of DNA sensors able to detect body fluids in forensically important samples. Current methods fail to provide simple, reliable, sensitive, selective and robust DNA sensors able to be used with real samples. For this reason the thesis aims to develop a sensor aimed at addressing these issues.

The thesis objectives are as follows:

1) Develop, characterise and optimise a PPY-based DNA sensor based on a previously developed platform.
2) Investigate the effects of metal-ion implantation into PPY films and subsequently develop a second DNA sensor platform.
3) Develop a sensor involving detection by resistive pulse sensing to detect target hybridisation on functionalised particles.
4) Investigate the interactions and effects of real sample testing using whole blood samples with developed electrochemical DNA sensors.
5) Discuss and conclude thesis findings with recommendations for future directions.
1.2 Designating an Appropriate Sensor Target

When choosing a target for the identification of body fluids a number of options are available, for example DNA, ribonucleic acid (RNA), proteins, antibodies etc. Many of the body fluids have common components; hence a target must be chosen that is specific to that body fluid, or at least specific in the amount of the compound found. The inherent complexity of nucleic acids as targets offers an appealing approach to create a selective sensor. Their ability to form duplexes means they exhibit ideal probe qualities for sensing.

1.2.1 Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid (DNA) is present in each cell nucleus in the human body. DNA contains the genetic instructions used in living organisms for their development and functioning. It exists as a long chain of nucleic acids, with two main sections consisting of a negatively charged phosphate backbone and varying bases, Figure 1.1. When complementary DNA converges on a single-stranded DNA (ssDNA), the two strands hybridise forming double-stranded DNA (dsDNA). As discovered through the legendary works of Watson and Crick and Wilkins and Franklin, dsDNA can form a duplex where the two DNA strands run in an anti-parallel manner producing a double helix, Figure 1.1B. For convention DNA is typically written from the 5'- (terminal phosphate group of phosphate backbone) to 3'- end (terminal hydroxyl group of phosphate backbone). In actual fact, relative to one another, the two strands hybridise with one with a 5' to 3' arrangement and the other with 3' to 5'. The bases determine the sequence of DNA and are composed of adenine (A) and guanine (G) (the purines) and thymine (T) and cytosine (C) (the pyrimidines). They pair through hydrogen bonds in the manner shown in Figure 1.1C. Adenine pairs with thymine, while guanine pairs with cytosine due to the complementary positions of the hydrogen bonds. Hybridisation is affected by a number of solution characteristics such as the salt concentration, temperature, viscosity, the presence of accelerating agents, contacting time, base composition and length of the probe sequence. The length of DNA strands are described by the number of base pairs. This can be written with the addition of the suffix ‘mer’, for example a strand with 23 base pairs can be labelled a 23-mer strand.
DNA profiling is extensively used, where any tissue sample or fluid may be used for DNA collection, excepting red blood cells. It has played an increasingly important role as technology advances, awareness increases and the understanding of the importance of this evidence deepens. The DNA collected from an individual’s cells is identical between the cells and unique to the person. For body fluid discrimination, however, using DNA is unsuitable due to identical DNA being present in each cell nucleus.

1.2.2 **Ribonucleic Acid (RNA)**

DNA is used by organisms to translate specific sequences of nucleotide bases called genes. Genes, in turn, contain the information to form proteins, which exhibit and carry out cell function. Prior to protein formation an intermediary nucleic acid strain is formed during transcription, named ribonucleic acid (RNA). RNA is very similar to DNA, with the exceptions that it is formed by a ribose sugar in place of DNA’s deoxyribose sugar, and that RNA contains the nucleotide base uracil rather than thymine exhibited in DNA.\(^\text{14}\)

Messenger RNA, or mRNA, can be thought of as the DNA template which is used to build a protein. Within genomic DNA there are both exons (gene coding regions) and introns (non-coding regions). The non-coding introns are spliced (removed) during the transcription process, while the exons are joined together forming mRNA, Figure 1.2.\(^\text{14,15}\) Because of RNA’s function, particular sequences are solely exhibited in a specific tissue, as only the proteins required for that cell’s functions are translated into mRNA. From this it can be seen

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**Figure 1.1** A) Structural composition of the phosphate backbone in a DNA chain, B) Schematic indicating dsDNA, and C) the pairing of DNA bases.
that RNA is an ideal target for body fluid identification. So long as a target sequence includes an exon-exon boundary, one does not have to consider any signal being generated from DNA or nuclear RNA binding, as both still include the intron sequences. Provided an appropriate mRNA sequence can be discovered this approach may hence be used to identify the body fluid present.

![Gene expression diagram](image)

**Figure 1.2** Figure showing the transition from a gene composed of DNA through to proteins. Initially transcription of DNA into nuclear RNA occurs. Nuclear RNA is then spliced to remove the non-coding introns, giving the coding mRNA which can be translated into proteins. A body fluid specific target may focus on the sequence highlighted by the green line, whereby an exon-exon boundary is incorporated ensuring that DNA sequences will not contribute to signal. Figure is reproduced from reference.15

Advantages to using RNA include the fact that linear amplification may be readily performed, and there is high heteroduplex stability.16 RNA is also known to readily fragment, which may be of use where smaller RNA sections are required as a target.16 The amount of mRNA present in a cell will generally depend on the amount of protein required for that cell’s functions, so, depending on the gene chosen, significant amounts of mRNA may be present.

To use mRNA as a target for body fluid identification, inherent genes are investigated for each of the body fluids, the detection of which indicates the body fluid present. The majority of emerging techniques for the analysis of blood, as well as other body fluids such as vaginal fluids, involve the use of RNA with a focus on mRNA.5 Protein based assays are another avenue pursued for fluid identification, though less so than mRNA due to further progress in technology for mRNA detection than in proteomics.5 The body fluid blood is chosen as a focus due to its prominence in forensic evidence and ubiquitous nature.
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For the analysis of the body fluid blood, a suitable target sequence exists in mRNA sequences such as those which form β-spectrin or Glycophorin A (Glyco A). These blood proteins form part of erythrocytes (red blood cells), and hence have multiple transcription copies within blood. Because erythrocytes are solely present in blood the test is therefore specific for the body fluid blood.

Although not discussed here, sample treatment to liberate RNA targets has been considered.

1.2.3 Sensor Target

An ideal target for the body fluid blood has hence been identified as RNA. Despite targeting an RNA sequence, a DNA sensor can be developed. Research has shown that DNA sensing platforms are transferable to RNA testing, as was the case in work performed by Lubin et al., 2006. Meanwhile there is extensive literature for DNA as a sensor target, and many companies producing synthetic DNA for research purposes. As discussed previously, the base-pairing of dsDNA gives rise to high selectivity. Even a single base pair mismatch will lead to some destabilisation of the dsDNA, making this a very selective pairing. For these reasons, despite an RNA sequence being the intended target, a DNA sensor is developed in this research.

Other biosensors are highly researched including enzyme biosensors, antibody biosensors (immunosensors) and aptamer biosensors, though these are not discussed in detail here.

1.3 Deoxyribonucleic Acid (DNA) Sensors

Biosensors consist of a sensitive biological recognition element or bioreceptor, a transducer and associated electronics. DNA sensors are a subset of biosensors in which the biological recognition element is found in a probe DNA or oligonucleotide strand, Figure 1.3. Oligonucleotides (ONs) exist as short strands of nucleic acid polymer, with the same composition as DNA, however often synthetically formed and of short length (e.g. less than 50 base pairs). As previously discussed, the inherent complexity observed in DNA sequences provides high sensor selectivity. Selectivity is an important property in DNA sensors, where the complex sample mixture may involve a plethora of interferences such as non-complementary DNA strands, proteins, cells and other species.
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Figure 1.3 Figure showing A) the main components of a biosensor and B) an example of a DNA sensor.

An abundance of approaches have been employed to develop DNA sensors. These methods include:

- Optical DNA sensors\textsuperscript{23, 38-42}
- Electrochemical DNA sensors\textsuperscript{8, 43-48}
- And resistive pulse sensors\textsuperscript{34, 49-54}

1.3.1 Optical DNA Sensors

Optical DNA sensors use a range of techniques which include ultraviolet, visible, or infrared absorption, fluorescence, gel-electrophoretic mobility, and surface plasmon resonance (SPR). Although initially optical DNA sensors used radio-labelling\textsuperscript{36} fluorescence has since replaced radio labelling offering a safer, more stable labelling system with increased capabilities.

1.3.1.1 Fluorescent DNA Sensors

Many optical sensors exist with fluorescence techniques, relying on a fluorescent signal indicative of DNA hybridisation. Measurement is based on the amount of light absorbed or emitted as a consequence of hybridisation\textsuperscript{39} The sensitivity achievable with fluorescence is high\textsuperscript{36, 55} and it remains the routine and most commercially successful method for detecting the association between two ON strands\textsuperscript{23, 58}

An example of a fluorescence based DNA hybridisation detection platform involves ethidium bromide, where the ethidium cation behaves as a fluorescent marker by strongly associating
with dsDNA by intercalation into the DNA strand at either the base stacking region or the major groove of the double helix.\textsuperscript{57} The ON recognition event is then evaluated depending on the intensity of the fluorescence emitted.\textsuperscript{23}

Another fluorescence based technique which is commonly used for DNA sensing is Förster (fluorescence) resonance energy transfer (FRET). This method describes the energy transfer between two chromophores, namely a donor chromophore and an acceptor. When the two are within a certain distance, FRET can occur between the donor and acceptor, therefore making this technique extremely sensitive to small distances. An example of a FRET DNA sensor is seen in the research by Gaylord et al.,\textsuperscript{40} whereby a cationic conjugated polymer is used in conjunction with a fluorescein- labelled neutral peptide nucleic acid (PNA) probe strand. PNA is a synthetic oligomer that is similar to DNA and RNA, however is synthesized with a neutral backbone. When complementary target DNA is in solution the probe and target hybridise, and a second electrostatic interaction occurs between the negatively charged target and positively charged polymer. This brings the labelled probe and cationic polymer in close enough proximity to allow FRET to occur, Figure 1.4. Meanwhile, FRET does not occur with non-complementary DNA. Detection of concentrations of 10 pM indicate the sensitivity of such a system.\textsuperscript{40} Within our group research has also progressed towards the development of fluorescence-based DNA sensors.\textsuperscript{41}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fret_schematic.png}
\caption{Schematic of the FRET based sensing system developed by Gaylord et al.. A) When non-complementary DNA is annealed with the polymer and probe no FRET occurs. B) when complementary target DNA is present FRET occurs. Figure is based on reference.\textsuperscript{40}}
\end{figure}
Although high sensitivity is reached with fluorescence based sensors a major disadvantage is the need for a two-step process.\textsuperscript{23} Fluorescence in itself requires labelling with fluorescent tags, which complicates sensor manufacturing. Moreover, inefficient tagging may occur, hazardous waste disposal and the added label may increase costs of the sensor.\textsuperscript{21} Problems may arise with a background signal, due to the illumination of the support bearing the biosensor layer,\textsuperscript{23} as well as potential subjection to a low fluorescence yield and quenching.

### 1.3.1.2 Surface Plasmon Resonance based DNA Sensors

A recent advance in DNA sensing exploits the phenomenon of surface plasmon resonance (SPR). SPR detection can be used to provide real time monitoring to biological interactions.\textsuperscript{23, 38} This surface-sensitive optical technique provides advantages such as label-free recognition, rapid quantification and the ability to perform affinity constant measurements. The method uses SPR imaging and analyses the change in refractive index or surface character of the sensor upon hybridisation based on the SPR or resonance angle.\textsuperscript{38, 58} Metal nano-particles and surface enzyme reactions may be used to enhance the signal achieved from this technique.\textsuperscript{58}

Work by Watts et al. has shown a DNA sensor able to detect target DNA down to a concentration of 9 nM successfully for 40 base pair ON. This uses the resonant mirror system for measurement (an integration of SPR devices and wave guiding devices).\textsuperscript{39} Meanwhile, a different system used the design by Guedon et al.\textsuperscript{38} whereby a series of polypyrrole spots on gold surfaces allowed analysis of parallel DNA hybridisations and denaturations. With their 15-mer probes they were able to selectively detect target DNA with a sensitivity of a few picograms per millimetre squared, as well as being able to regenerate the sensor surface.\textsuperscript{38}

### 1.3.2 Electrochemical DNA Sensors

Electrochemistry as a detection method has found substantial support due to the achievable sensitivity, selectivity, rapid and direct detection, low-power, cost-effectiveness, opportunity for miniaturization and simplicity the method possesses.\textsuperscript{55, 58, 59–62} Mass fabrication of electrochemical sensors is also possible,\textsuperscript{61} and they have aided in the move towards simplified testing.\textsuperscript{58} Electrochemical detection refers to the process involved in transducing the sensing signal. Techniques to measure the signal such as changes in current (amperometric sensors),\textsuperscript{30, 33} voltage (potentiometric sensors),\textsuperscript{33} and impedance
(impedometric sensors) include cyclic voltammetry (CV),\textsuperscript{43,63} differential pulse voltammetry (DPV),\textsuperscript{64} square wave voltammetry\textsuperscript{65} and electrical impedance spectroscopy (EIS).\textsuperscript{44,48} First introduced by Millan and Mikkelsen in 1993,\textsuperscript{27} ‘the electrochemical DNA hybridisation sensor’ received intense attention, and continues to do so.\textsuperscript{61}

Electrochemical detection of DNA hybridisation can be obtained through different pathways: 1) a change in signal from the electroactive DNA bases such as guanine or adenine (direct DNA electrochemistry), 2) monitoring the electrochemical signal with nanoparticle based electrochemical amplification, 3) indirect DNA electrochemistry using electrochemical mediators (DNA-specific redox indicator detection), 4) indirect electrochemistry of DNA as a detection platform, and 5) monitoring the electrochemical signal of the substrate after hybridisation (DNA-mediated charge transport).\textsuperscript{55,61} Benefits exist for pathways 1 and 5 in that no labelling is required. The majority of electrochemical detection systems report sensitivities in the micromolar range.\textsuperscript{66} However, systems can display high sensitivities in the femtomolar range using direct detection, and down to high attomolar or zeptomolar concentrations with enhancements.

Direct DNA electrochemistry as a detection platform was used for the earliest DNA sensing strategy.\textsuperscript{55} The platform by Palecek and co-workers were able to distinguish between single and double stranded DNA through direct DNA-base reduction.\textsuperscript{67} In later work by Palecek et al., the use of commercially available magnetic beads allowed separation of the DNA. Subsequently detection using the purine bases was performed.\textsuperscript{68} In a more recent example, work by Bartošík and Palecek (2011)\textsuperscript{69} used square wave voltammetry to determine the difference between ssDNA and dsDNA. Observed peaks were identified as the adsorption of sugar-phosphate backbones as well as the reduction of base residues. In dsDNA the bases are hidden inside the double-helical structure, while in ssDNA they are more accessible for reduction. Detection down to ~150 pM (1.2 ng mL\textsuperscript{-1}) can be detected under certain conditions. High sensitivity was found with a sensor system developed by Lusi et al. for the detection of microRNAs.\textsuperscript{70} They found a detection limit of 0.1 pmol using 22-mer inosine-modified probes. Despite the high sensitivity achievable with this technique, problems can exist with high background currents or the required separation to remove the sources of background signal.\textsuperscript{55}

Enhancement of DNA sensing using nanoparticles is examined in the research by Peng et al.,\textsuperscript{20} where the use of CdS nanoparticles enabled a linear sensing range of between 3.7 and
370 nM, and a detection limit of ~1 nM ON. The increase in response with CdS particles is due to the combined effects of negative charges on the CdS ligand, as well as the semiconducting nature and size of the CdS nanoparticles. They were also able to display regeneration of the sensor surface. Additionally the sensor system by Wang et al.\(^71\) shows the use of nanoparticles (quantum dots in this case) with a DNA sensor system. A DNA sensor was developed using different inorganic-colloid nanocrystals (quantum dots) in order to detect multiple target DNA strands.\(^71\) They were able to report limits of detection down to 270 pM, and the ability to detect multiple target DNA sequences. Their DNA sensors also exhibit the potential of a dual sensing system whereby fluorescence may also be examined of the quantum dots. Meanwhile, high sensitivity can be observed for nanoparticle amplification strategies such as the system proposed by Mirkin and colleagues, whereby a sensitivity has been demonstrated down to \(~5 \times 10^{-13}\) M.\(^72\) Even higher sensitivity has been reported using a sandwich assay and polystyrene microsphere tags impregnated with ferrocenecarboxaldehyde, where the technique has demonstrated a detection limit of \(5 \times 10^{-21}\) moles.\(^73\)

Indirect methods for DNA detection can occur either through the use of redox indicators or through the indirect electrochemistry of the DNA itself. The latter of these can involve the use of electrochemical mediators such as a complex of polypyridyl with Ru(II) and Os(II) which are able to oxidise guanine. Using model PCR products, sensitivities as low as 550 attomoles have been reported.\(^55\) Meanwhile redox indicators may be either incorporated into the polymer backbone, as is the case with Reisberg et al.,\(^74\) or as a separate redox indicator, for example intercalators such as daunomycin.\(^61\) Intercalators may work in a similar manner to that depicted in Figure 1.5. Other redox-active substrates may cause signal quenching upon hybridisation. Enzyme labelling can also be used, whereby an enzyme tagged 'signalling probe', when in contact with complementary target, is able to create a product which can be monitored as a function of target concentration.\(^61\) Research by Zhang et al. (2002) using an enzyme-amplified amperometric sensor was able to show detection of a 38-mer target at 20 pM in a 30 µL droplet.\(^75\) Indeed, high sensitivities are achievable mainly due to the amplification step provided by the label or enzymatic reaction.\(^61\)
A very popular detection technique for DNA sensing involves monitoring the electrochemical signal of the substrate following target hybridisation. This may involve incorporation of reporting groups within the polymer framework, such as was the case with research by Reisberg and co-workers. The cation-exchange redox group of quinone was used as an immobilised redox-active label. Differential pulse voltammetric (DPV) measurements were then used as a “signal-on” detection technique. The sensor was able to show selectivity and sensitivity to the 0.1 µM complementary target investigated, as well as uncovering interesting findings including that the signal resulting from different positions of shorter targets can be differentiated. Alternatively the hybridisation process can be probed at the electrode surface using a technique such as electrical impedance spectroscopy (EIS).

1.3.2.1 Electrical Impedance Spectroscopy a technique and EIS sensors

Of special interest for electrochemical techniques for detection is electrical impedance spectroscopy (EIS). It should be noted that this technique can be used as a method for the characterisation of electrodes as well as being a detection technique. With careful experimentation EIS may result in appropriate sensor read-out as well as a wealth of other information such as DNA hybridisation kinetics. EIS can either be performed in electrolyte solution with a salt of interest (may be preferred for characterisation), or in the presence of a redox couple (may be preferred for DNA sensing). Examples of the redox couples used include the pairs; ferricyanide and ferrocyanide, hydroquinone and
benzoquinone, and hexaammineruthenium ruthenium (III) chloride and hexaammineruthenium (II) chloride.

EIS allows 1) the ability for high-precision measurements as the response may be indefinitely steady and so can be averaged over long time periods, 2) the response can be theoretically simplified, 3) the measurements can be performed over a large frequency or time range, and 4) only a small potential amplitude is applied therefore causing only minimum perturbation to the system. EIS has found use in studying corrosion, semiconductors, batteries and electro-organic synthesis. In the case of DNA sensing, EIS can be ideal as both the probe immobilisation and the target hybridisation induce changes in both the electrode surface and the various interfacial film properties, for example capacitance and interfacial charge transfer resistance. This allows a label-free measure of the system. The majority of DNA sensors use labelling, a common example being the use of glucose oxidase as an enzyme label as discussed previously. Labelled schemes are able to offer higher sensitivity however require additional steps introducing the potential for more error as well affecting the bioactivity of labelled species.

An example of an electrochemical DNA sensor using EIS as a detection technique can be found in the research by Gębala and Schuhmann. A DNA sensor is developed using thiol-tethered ssDNA co-assembles with short-chain thiol derivatives. In this work they examine the influence of the electrode potential, ssDNA coverage, ionic strength, nature of the thiol derivative and the Debye length on the impedance spectra. Conclusions found a useful combination is the use of thiolated ssDNA, mercaptohexanol and mercaptopropionic acid as the interface design allowing a high efficiency of DNA hybridisation as well as a reliable change in charge transfer resistance upon hybridisation. The increased efficiency of hybridisation is thought to arise from the improved orientation of the capture probe at the surface interface, an important but perhaps somewhat overlooked feature, Figure 1.6. Additionally the mixed film allows modulation of the properties of the film with respect to an increase in the double-layer capacitance, and affects the potential drop into the solution. The concentration used for this experiment was 1 µM complementary target DNA, to which the sensor displayed a good response.
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High sensitivity with EIS can be achieved, as displayed in the sensing platform by Zhang et al. whereby carbon nanotubes form part of the sensing platform. A nanocomposite membrane consisting of nanoshuttle-shaped cerium oxide (CeO$_2$) particles, single-walled carbon nanotubes and 1-butyl-3-methylimidazolium hexafluorophosphate was used for DNA immobilisation and subsequent target hybridisation with detection by EIS. The nanocomposite gave high sensitivity displaying a detection limit of $2.3 \times 10^{-13}$ M. Other examples can be found in the review paper by Katz et al. for detection limits on the order of $10^{-16}$ M following amplification.

A particularly interesting sector of research involves the investigation of DNA sensing with the intrinsically conducting polymer, polypyrrole. Such a polymer substrate can be a very effective substrate on which to immobilise biological entities.

1.4 Intrinsically Conducting Polymers and Polypyrrole

Intrinsically conducting polymers (ICPs) also known as electrochemically active conducting polymers have been widely used since their discovery. The work performed in the 1970s by Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa provided a major breakthrough in the field of ICPs. ICPs consist of organic polymers that, upon oxidation, are able to conduct electricity. Typically they are semi-conducting in nature with conductivities lying over a range of about fifteen orders of magnitude. They are materials of interest based on both their mechanical properties together with their conducting properties. The inclusion of
ICPs within a DNA sensor framework provides both an anchor of the DNA probe strand on the electrode surface, as well as a transducer of the hybridisation event. Additionally the use of ICPs produces a wider variety of starting electrode metals, chemical functionalities and morphologies. Examples of conducting polymers include polyacetylene, polythiophene, polyaniline, polyindole and polypyrrole, Figure 1.7.

Polypyrrole (PPy), in particular is widely researched. In 1968 it was noted that pyrrole could be electrochemically polymerised, and in 1973 chemical polymerisation was reported. Since then, research activity on PPy has brought to light many interesting properties, adding to its allure. PPy is able to offer a multitude of beneficial properties including 1) the versatility of the polymer - shown by redox activity, 2) ability to form nanostructures (e.g. nanowires) the maintained electroactivity in neutral solutions, 4) the ability to functionalise pyrrole through synthetic chemistry to cater for specific attachment chemistry, 5) PPy’s exhibited improved stability over some other polymers and easy polymerisation electrochemically at reasonable (low) potentials and from aqueous solution, 6) the redox potential of PPy is well suited for biosensing, being low at around 0 mV vs. SCE, 7) high conductivity can be achieved, in the region of 100 S cm$^{-1}$ for oxidised PPy, 8) PPy is considered biocompatible and possesses low intrinsic cytotoxicity, with no conformational change observed in proteins in close proximity to the PPy, and lastly 9) the commercially availability.
1.4.1 Synthesis and Properties of Polypyrrole

The formation of PPy can be performed in two ways, either though chemical or electrochemical means. Chemical processing has the advantages of being scalable for processing and for production of powder samples, for example in the design of chromatography columns, where uniform overoxidation of synthesized polymer may be useful. A major obstacle of this growth method is poor adherence to surfaces. Meanwhile, electrochemical synthesis allows tighter control over the film that is grown, the ability to perform polymerisation at room temperature, and a film with better adherence to the electrode surface. The ability to localise the polymerisation reaction allows spatial resolution and miniaturization. Moreover electrochemical synthesis avoids the inclusion of additional chemicals such as the oxidant, within the polymer film.

A proposed mechanism for the growth of PPy films can be seen in Figure 1.8. The first step in electrochemical growth involves the oxidation of pyrrole monomer at the electrode surface, which occurs at a particular potential. This gives rise to radical cations, which in turn can react with one another to form a dimeric species. In the third step, oxidation gives rise to a dimeric radical cation, which can react with another radical cation to give oligomeric species. The radical cations and diradical cations (steps 2 and 4) involve the loss of two protons. The diradical and oligomeric species have lower oxidation potentials than the pyrrole monomer itself, hence this process leads to step 5, chain propagation. Eventually a layer of this polycationic chain will precipitate onto the anode. The chain propagation is not indefinite as radical cations may also react with monomer pyrrole or other nucleophilic species in solution. It should be noted that the simplistic representation and idealised structures discussed here both belie the complexity of the polymerisation process.
Figure 1.8 Proposed mechanism for the formation of PPy.
1. Oxidation of pyrrole monomer at the electrode surface forming a radical cation. 2. Radical cations react to give a dimer. 3. Oxidation of the dimer forming a dimer cation. 4. Reaction with a radical monomer giving higher oligomers. 5. Chain propagation eventually giving polypyrrole films. Figure is based on references.29, 91

The potential chosen for this polymerisation influences the average length of the PPy polymer chains, in turn influencing the final polymers conductivity. Electrolyte composition can affect the polymer grown, including degree of dissociation of the electrolyte salt, solubility and the nucleophilicity of the salt, and chemical reactivity.90 Other important factors include the temperature, the monomer concentration, and the electrode surface.29 All influence the polymer surface morphology and properties.

The way in which the potential is applied also affects the polymer grown. For example polymer grown by cyclic voltammetry (CV) exhibit macroscopic size features92 and can have improved efficiency as partial reduction of the oligomers occurs during the reaction,85 though can exhibit a lack of control over growth,92 while those grown with constant potential can exhibit uneven films (due to substrate roughness), porosity and often overoxidation (particularly with thick films).90 A pulse growth technique may lead to enhancements in electrical conductivity and molecular anisotropy when compared to those grown with constant potential.93 Galvanostatic deposition gives films that are homogeneous layers with both mechanical strength and good adhesion to the electrode layer,90 however can suffer from polymer overoxidation due to the poor control over the applied potential.29
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1.4.2 **Polypyrrole Conductivity**

Although the mechanism of conduction is of a highly complex nature,\textsuperscript{90} it is thought the conductivity of PPy is possible due to the ordered $\pi$-stacking system of the polymer backbone. These $\pi$-bonds ($\pi-\pi^*$) are partially localized due to the Peierls instability and distortion.\textsuperscript{28} This results in a dimerized structure, a series of shorter and longer double bonds rather than a single bond length along the chain length.\textsuperscript{94} The excitation across the $\pi-\pi^*$ band gap creates self-localised excitations in the gap region, named polaron and bipolaron.\textsuperscript{28} The polymers conductivity is dependent on these charge carriers along the polymer chain. Also influential are the conjugation length, the overall chain length and the charge transfer to adjacent molecules.\textsuperscript{37}

The charge carriers in this case are polarons and bipolarons. In the oxidised state, after removal of an electron, a free radical and a positive charge are formed, giving rise to a polaron.\textsuperscript{95} In this case the surrounding monomer pyrrole units undergo a structural rearrangement or lattice deformaity to balance the energy level created by the electron loss.\textsuperscript{85}, \textsuperscript{94} Upon further oxidation, removal of the free radical occurs, giving rise to a new spinless species called a bipolaron, again associated with a localised lattice distortion.\textsuperscript{85} This in turn affects the valence and conducting bands, and as there is no forbidden gap between the highest occupied and lowest unoccupied levels under the application of an electrical field, electrical conductivity arises, Figure 1.9.\textsuperscript{95, 96} The density and mobility of the polarons and bipolarons along the polymer chains influence the polymer conductivity. The mobility of the charge carriers can be influenced by the polymers crystallinity, orientation and the presence of defects.\textsuperscript{94}

![Energy level diagram of an ICP showing band gap and energy levels at different oxidation states. Shown are diagrams for neutral polymer, polarons, bipolarons and a quasi-metallic state.\textsuperscript{95, 96}](image)

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\textsuperscript{90} Reference

\textsuperscript{28} Reference

\textsuperscript{94} Reference

\textsuperscript{37} Reference

\textsuperscript{95} Reference

\textsuperscript{96} Reference
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Following from the terminology applied to inorganic semiconductors, often the oxidation of ICPs is named “doping”. Care should be taken, however as it does not refer to the replacement of atoms in the material’s framework, but instead the redox reaction of oxidation. Using electrochemical techniques, PPy can be reversibly doped and undoped, Figure 1.10A. The rate of oxidation can be influenced by the anion in solution, which can in turn give rise to complicated cyclic voltammograms (CVs) where combined faradaic and capacitive currents contribute to the resulting CVs. An example of a PPy CV is given in Figure 1.10B, along with descriptions for the peaks arising during the process.

As well as biosensing, polypyrrole has been used for conductive textiles and fabrics, mechanical actuators, corrosion protection layers, batteries, supercapacitors, electrochromics, anti-static coatings and drug delivery systems.

![Figure 1.10 A) Oxidation and reduction of PPy, and B) a cyclic voltammogram (CV) of PPy. A) Shown is PPy in the oxidised state and neutral reduced state. B) The CV shows characteristic oxidation and reduction peaks along with the anion movement. Whether it is the anion or the cation which moves is dependent on the size and mobility of the anion used.]

1.4.3 Polypyrrole Stability

Polypyrrole offers many advantages when involved in DNA sensing. A drawback can lie in the stability of PPy. It is subject to overoxidation, which may occur at low positive potentials in water and oxygen-containing environments as well as other slow irreversible reactions. This may lead to partial destruction of the polymeric backbone and generation of oxygen-containing groups such as carboxy, carbonyl and hydroxyl groups, and
ultimately result in a loss of conjugation. For this reason methods able to improve the lifetime of PPy active matrices are highly sought.

An example of a method used to improve PPy stability is the inclusion of metal nanoparticles within the PPy framework, as was performed in the research by Chen et al.. The formation of a PPy and gold nanoparticle composite was able to exhibit high stability as well as good immuno-sensing properties. The gold nanoparticles are suggested to aid in stability through acting as large, negatively charged counterions, preventing polymer degradation to an extent. Detection limits of 10 fg mL\(^{-1}\) in phosphate buffered saline (PBS) solution and 1 pg mL\(^{-1}\) in 1% human serum were reported displaying the capabilities of the developed sensor.

Another method which has shown promise for increases in stability for PPy is ion implantation.

14.4 Ion Implantation in Conducting Polymers

Ion implantation is a materials engineering process allowing the modification of the mechanical, physical and chemical properties of a material by the controlled incorporation of another material. Many existing research directions involve implantation of metals into non-conducting polymers. By utilizing a conductive precursor less metal may be required to enhance conductivity, thereby decreasing the cost of the composite. To this cause ICPs may fulfill the role of the conductive precursor material.

Previous studies on ion implanted and swift heavy irradiated (~MeV/u energy) conducting polymers, targeted for a range of applications such as p or n type junctions, capacitors and actuators, have reported a range of outcomes from decreases to increases in conductivity. The energy, type and fluence (number of ions deposited) of implanted ions affects the changes in conductivity observed. Increases in conductivity are reported and explained by increases in ionic and charge carrier concentrations within the films, cross-linking of polymer chains and facilitated hopping of charge carriers within the polymer, and light reordering of polymer chains. Meanwhile, decreases in conductivity are thought to arise from damage to the conducting polymer rings (as revealed through XPS and XRD data analysis), disruption of the conducting \(\pi\)-system and loss and/or damage to the polymer counterions. Other characteristics affected by ion implantation include surface morphology, crystallinity, mechanical stiffness, electrochemical features, mechanical work outputs and stability.
Improved stability has been observed for ion implanted samples as tested by conductivity, mass change measurements, and cyclic voltammetry. This increase in stability has been justified by the stabilisation of volatile surface species (e.g. OH), formation of protective surface structures, re-ordering of the polymer increasing conductivity as well as a general improvement in the quality of the PPy film after irradiation and stability of formed radicals. This research shows that with the correct conditions, ion implantation can offer beneficial enhancement of PPy properties.

1.5 Polypyrrole-based DNA Sensors

The surface chemistry and character of DNA sensors is of great importance to ensure high sensor success. The correct DNA orientation, receptor accessibility and stability of the surface-bound probe are all highly influential. Research by Watterson et al. has shown the density of immobilised DNA probes can influence the thermodynamics of hybridisation, and therefore the sensitivity and selectivity of DNA biosensors. PPy is able to provide a high surface area with desired characteristics from which to graft probe-ON.

The main techniques used for probe-ON attachment are as follows: 1) binding or entrapment of the ON probe within the transducer, 2) adsorption on to the surface, and 3) covalent bonding to the transducer surface. The latter of these offers the advantages of not requiring harsh environments or conditions, forming stronger bonds, structural flexibility of the biological entity can be retained, and the final surface is subject to a lower possibility of DNA desorbing and leaking into the solution. Additionally, if the transducer is an ICP, the sequential steps of firstly growing the film and secondly incubating for DNA attachment means the DNA is not exposed to growth conditions. This allows optimised polymerisation conditions to be used instead of only aqueous solutions. By no longer requiring DNA to be present during polymer growth the potential issue of DNA damage during polymerisation can also be avoided. As radical cations are formed during the monomer polymerisation, they can oxidatively damage the DNA leading to partial degradation. Furthermore covalent binding can produce better ON orientation (depending on chemistry of the attachment), and therefore may overcome some of the steric hindrance and kinetic barriers experienced which can limit target hybridisation capability.

Generally probe ONs are functionalised with –NH₂ or –COOH groups which can then react with the transducer surface. PPy offers a good example of a transducer surface able to
covalently bind the ON probes. The binding of probes to functionalised pyrrole or PPy can happen either before \cite{124} or following polymerisation.\cite{48, 99, 120, 125} The type of chemistry for binding is commonly peptide bond formation,\cite{10, 48, 63, 99, 125} but may also be through metal-complexation.\cite{120, 126} streptavidin (or avidin) and biotin binding\cite{38, 127} or other methods.\cite{124, 43}

An interesting, innovative method for ON attachment involving a polythiophene based conducting polymer (poly(3-methylthiophene)) is displayed in the research by Ferreira et al.\cite{43} The strong interactions between particles of the noble metal platinum and thiol sulphur groups are exploited to give platinum particles attached to the polymer, as well as thiol modified probe ON attached to the particles, Figure 1.11. Electrochemical quartz crystal microbalance measurements and cyclic voltammetry then allow micromolar target DNA detection.

![Schematic representation of the developed DNA sensor](image)

**Figure 1.11** Schematic representation of the developed DNA sensor, figure reproduced from reference.\cite{43} A) Thiophene based polymer with platinum nanoparticles deposited by immersion, B) thiolated ON self-assembles on particle surfaces, C) complementary target ON in solution hybridise.

A summary table of several polypyrrole based DNA sensors is given in Table 1.1.
Table 1.1 Characteristics of polypyrrole based DNA sensors.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Immobilisation technique</th>
<th>Detection Method</th>
<th>Sensitivity, Selectivity and limit of detection (LOD)(^a)</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| Functionalised Polypyrrole          | Amide bond formation after copolymerisation     | Cyclic voltammetry                   | 14-mer target ON, LOD = 10\(^{11}\) mol
Selectivity with NC ON                                                                                                 | 63   |
| Polypyrrole                         | Entrapment of ON dopant                         | Amperometric                         | Sensitivity = 0.52 nA \(\mu\)g\(^{-1}\)                                                                                   | 121  |
| Polypyrrole                         | Entrapment of ON dopant                         | Potential pulse amperometry          | LOD = 0.37 ng mL\(^{-1}\), Measured 10 ng mL\(^{-1}\)
Small signal (8x less) for NC ON                                                                                         | 128  |
| Polypyrrole                         | Entrapment of ON dopant                         | Chronoamperometry                    | 20-mer target ON, Measured 1.6 fmol (10 pg)
concentration = 16 pM
Selectivity with NC ON                                                                                                   | 66   |
| Composite of PPy and multiwalled carbon nanotubes (MWCNT) | Amide bond formation with COOH functionalised MWCNT | Electrical impedance spectroscopy    | Initial work, LOD = 10 nM, 1 basepair mismatch = 51 % of full
Later, LOD = 5 pM
Selectivity with NC ON                                                                                                   | 129  |
|                                    |                                                 |                                      | Later, LOD = 5 pM
Selectivity with NC ON                                                                                                   | 130  |
| Au-Ag/Polypyrrole nanocomposite     | Covalent probe ON attachment                    | Electrical impedance spectroscopy     | 21-mer target ON, LOD = 0.5 nM
3 base pair mismatch = 8 % of full                                                                                            | 131  |
| Polypyrrole                         | Metal complexation                               | Cyclic voltammetry                   | LOD = 58 pM
Some signal with NC ON                                                                                                    | 126  |
| PPy nanowire                        | Linker molecule                                 | Gating effect and work function       | 19-mer target ON, LOD \(\sim\) 10\(^{16}\) M
Selectivity with NC ON                                                                                                     | 132  |

\(^a\)Selectivity with NC ON indicates no significant sensor response observed with non-complementary ON.
The quantity of PPy-based sensors is a tribute to its sensing potential. Despite alternate platforms, such as thiol probe-ON attachment on a gold surface, PPy is able to provide many advantages such as surface chemistry, grafted-probe orientation, surface area and solid scaffolding. As described earlier (section 1.3.2.1), EIS is a highly sensitive technique able to provide an appropriate detection technique for DNA sensors. The combination of PPy and EIS allows the detection of small perturbations in the surface chemistry of the sensor such as those with target hybridisation, as well as a means to transduce this signal giving sensitive electrochemical read-out.\textsuperscript{28}

1.5.1 \textbf{Electrical Impedance Spectroscopy Polypyrrole-based DNA Sensors}

A number of successful PPy-based DNA sensing platforms have been developed involving EIS as the detection technique. One example involves the use of 500 nm sized nanotubes comprising of composites between polypyrrole and polyaniline (PANI), found in the research by Wilson et al.\textsuperscript{133} Onto the nanotubes were electrodeposited gold nanoparticles, giving a PPy-PANI-Au composite film. Thiolated ssDNA can subsequently be immobilised on the film surface, allowing sensing of the 15-mer target ON. Examinations into the composite film showed efficient interactions, echoed in the sensing ability with EIS whereby complementary target DNA (signal of 7742 $\Omega$ cm$^{-2}$) can be distinguished from non-complementary DNA (signal of 1216 $\Omega$ cm$^{-2}$) and target DNA with one and two mismatched bases (signal of 3512 $\Omega$ cm$^{-2}$ and 1355 $\Omega$ cm$^{-2}$, respectively). A detection limit of 100 fM was reported.\textsuperscript{133}

Meanwhile, research by Tlili et al. also used label-free EIS detection to detect target hybridisation in solution.\textsuperscript{134} A functionalised PPy matrix was used to covalently attach probe ON. Subsequently, EIS measurements in PBS buffer at a potential of -1.4 V were performed prior to and post target incubation. At this potential the PPy is in a semi-conducting state, chosen to minimise Warburg impedance and emphasize the contribution of the impedance of the PPy-DNA:electrolyte interface. Results showed that incubation with the 25-mer target ON produced an increase in the electrode charge transfer resistance, suggested to occur through modification of the (morphological) structure of the polymer backbone upon hybridisation. A concentration minimum of 50 nM was examined, and a limit of detection reported of 1 pmol. Incubation of the sensor platform with non-complementary DNA demonstrated the method used was selective.
Another example is seen in the research by Baur and Cosnier et al. An ultrahigh performance sensor is developed using a PPy based polymer. Electropolymerisation of a copolymer of poly(11-pyrrol-1-yl-undecanoic acid N\textsubscript{α},N\textsubscript{α}-bis(carboxy methyl)-L-lysine amide) film (abbreviated as polypyrrole-NTA) was performed. Subsequent coordination with Cu\textsuperscript{2+} ions was performed along with coordination of a polyhistidine tagged ssDNA thereby giving a surface with immobilised probe ON, Figure 1.12. Hybridisation of target DNA (48-mer) was confirmed by fluorescence microscopy (with fluorescently-labelled target) and quartz crystal microbalance experiments. A labelled platform using glucose oxidase and amperometric detection corroborated the duplex formation. EIS measurements were then used for an un-labelled DNA detection technique. With the redox reporter hydroquinone (10\textsuperscript{-3} M), the sensor was able to exhibit selectivity and sensitivity. Non-complementary DNA gave a greatly reduced signal (~10 % of the signal for complementary DNA of the same concentration), while a limit of detection of 10\textsuperscript{-15} M was reported.

![Schematic of the system described in reference by Baur et al.](image)

**Figure 1.12** Schematic of the system described in reference by Baur et al. A) Polypyrrole-NTA film with immobilised probe ON, and B) following target hybridisation. Also shown is the copper ion complex coordinating with the probe ON and the polymer surface.

Within our research, functionalised PPy is generally bound to probe ONs through amide bonds. Research by Peng et al. within our group has previously focused on an investigation into the ‘linker’ group connecting the PPy backbone and the peptide bond and probe ONs. To this purpose, two functionalised pyrrole monomers were examined, both with carboxylic acid

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end groups and one with a saturated side chain and the other with an unsaturated side chain, Figure 1.13. Copolymerisation of these monomers with pyrrole allowed formation of a copolymer film, onto which probe ON was immobilised. The sensing ability of the two films was investigated with different complementary target DNA concentrations, and the results compared. EIS was used as the detection technique, with Fe(CN)$_6^{3/-4}$ as a redox couple. Results revealed copolymers possessing an unsaturated side-chain displayed superior DNA sensing abilities as compared to those with saturated side chains, Figure 1.13. The superior sensing with unsaturated side chains is explained through extension of the polymer backbone conjugation via π-electron overlap, causing the electronic structure of the polymer to be highly receptive to the perturbations caused by ON hybridisation. Additionally, the unsaturated side chain displayed good selectivity, with one-base pair mismatch, two-base pair mismatches, and non-complementary ON giving rise to signals of 27, 38 and 3 % of the fully complementary target signal, respectively. The limit of detection displayed is 0.5 nM.

![Figure 1.13](image.png)

Figure 1.13 Figure describing the sensing platform used by Peng et al. in their investigations of monomer side chain functionality. A) Monomer used with unsaturated side chain, 5-(3-pyrrolyl)-2,4-pentadienoic acid (PPDA), and B) Monomer with saturated side chain for comparison, 3-pyrrolylpentanoic acid (PPA).

### 1.5.2 Polypyrrole-based Biosensors with Nanostructures

Nanostructures in PPy are of growing interest as the size dependent changes (chemical and physical) these nanomaterials experience separate them from their bulk counterparts. The large surface to volume ratio provides substantial changes which may be exploited for
sensing enhancements. Other factors affected by sensor size include response time, selectivity and sensitivity. A schematic comparison for the methods of PPy nanostructure incorporation can be seen in Figure 1.14. A recent review by Xia et al. discusses conducting polymer nanostructures and their application in biosensing devices. The term nanostructure refers to formations such as nanotubes, nanowires and nanoparticles.

Figure 1.14 Schematic examples of the different incorporation of nanostructures in PPy based DNA sensors. A) A DNA sensor whereby the nanostructured PPy is present in a layer. B) DNA sensing based on a single PPy nanowire. Figure adapted from reference.

An example of nanoparticle incorporated PPy sensors can be seen in the research by Ramanavičius et al.. The redox enzyme glucose oxidase (GOX) was self-encapsulated within PPy, allowing monitoring of the catalytic activity of the GOX, and hence glucose concentrations, through amperometric testing. A working biosensor was developed which exhibited improved stability when compared to electrodes modified with adsorbed GOX (cross-linked through glutaraldehyde). Nanowires, meanwhile, have received considerable attention. An avidin (or streptavidin) functionalised PPy nanowire was fabricated by Ramanathan et al. and subsequently used for biotin labelled DNA detection, Figure 1.15. Resistance was used as a measure for target sensing, with which concentrations as low as 1 nM could be detected. Further work from the same group has shown the development of label-free DNA sensors exhibiting a detection limit of ~10^{-16} M. Within our group, work by Kannan et al. has also progressed towards DNA sensing with ICP nanowires.
Figure 1.15 Schematic of the nanowire sensor developed by Ramanathan et al. A) Avidin functionalised PPy nanowire in the absence of target, B) Following introduction of the biotin-DNA target, affinity attraction causes a change in resistance. Figure is reproduced from reference.127

1.6 Resistive Pulse Sensors

As discussed previously, a sensing platform able to selectively and sensitively detect sequences of DNA holds the potential to fulfil many asks of modern medical research and clinical diagnostics. Meanwhile, there have been many advancements in characterisation techniques for nanometre sized species, including nanometre-sized pores.138 In particular, there has been growing interest for using nanopore based resistive pulse sensors for characterisation.34, 50, 51, 139-141 Many such sensing methods use approaches similar to the well-known Coulter counter. This can be employed as a means for label-free characterisation of individual particles within solution, fine-tuned to allow precise discrimination between particle populations.51 Another emerging avenue of resistive pulse sensing exists in the detection of isolated DNA strands holding capacity for DNA sequencing and identification.49, 142-144

Currently there are sensor platforms which utilise biological protein membranes such as α-hemolysin to analyse DNA, nitroaromatic compounds, metal ions, proteins and other species.49, 50, 139 The size of the biological nanopores are on the same size scale as the biological compounds being investigated, ensuring signal current drops are observable. Other advantages include the reproducibility of such pores, where biology dictates the synthesis, as well as the selectivity inherent in the engineering of the pores.50 Despite the sensitivity and
Chapter 1

selectivity these nanopores are able to provide, disadvantages are present in the fragility of the lipid bilayer membrane used as a support for the nanopore. Because of this fragility, numerous research groups have focused on artificial nanopores. Artificial nanopores can be limited by the minimum size achievable for reproducibly creating the nanopores. To combat this problem, larger colloid particles can be used with a coating of the probe and subsequently target analyte in place of directly analysing probe and target. Recent technology using synthetic nanopores includes portable resistive pulse sensor instruments such as the qNano (IZON Science). The flexible, tunable pore employed allows simple precision and sensitive characterisation of particles at relatively low cost. 

Resistive pulse sensing of DNA-coated particles holds great promise for DNA detection. Steinbock and Keyser et al. demonstrated the ability to detect a few tens of DNA strands bound to colloids as compared to bare colloid particles, through changes to the surface charge using micro- and nanocapillaries and optical tweezers. Additionally, similar changes to signal duration were observed by Roberts et al. between carboxy-functionalised particles, bare colloids (streptavidin coated) and those with grafted-DNA, Figure 1.16. In both these cases the DNA used were large double-stranded DNA fragments (4, 6 and 48 kbp DNA).

![Figure 1.16 Biofunctionalised nanoparticles as reproduced from Roberts et al.](image)

Schematic illustration of A) silica nanoparticles, B) particles with streptavidin-coating, and C) particles with DNA-coating.

Furthermore, research by Carbonaro and Sohn demonstrated the ability to detect different antigens in solution after binding to modified colloid particles, Figure 1.17. They were able to demonstrate an integrated multiple pore chip, on which they are able to detect simultaneously two antigens. Detection was performed based on the increase in size of the particles following the antigen binding. Concentrations examined were 80-90 ng mL$^{-1}$ and have a predicted limit of detection of 15 ngmL$^{-1}$. This work beautifully describes the potential
for single chip synthesis for the resistive nanopore sensing of multiple targets. Furthermore, in later work Carbonaro and Sohn et al. were able to characterise cell type as they passed through a protein-functionalised pore.

These developed resistive pulse sensors show high potential for sensing. However, it is of interest to selectively detect smaller species such as target DNA sequences (1000 > bp) in-place of the larger antigens, cells and long DNA strands (kilobase pair range). These ssDNA strands may be more pertinent to the DNA diagnostics targeted.

An interesting example of a DNA sensor focusing on smaller strands of DNA which uses resistive pulse sensing coupled with laser-based techniques and electron microscopy can be seen in the work by Low et al., Figure 1.18. Two related sensing techniques are described, one as a PNA aggregation diagnostic assay for detecting PNA (Figure 1.18A), the second a DNA sensor for detecting target DNA sequences (Figure 1.18B).53 In both sensing systems a 20 base pair ON is used, however the probe used is PNA. This charge neutral probe is attached to citrate capped gold nanoparticles (50 nm). This causes the particles to aggregate and exhibit a change in size which can be detected using all three techniques. Following

Figure 1.17 Description of the research by Carbonaro et al. A) to C) show the sandwich assay performed on the surface of colloids. D) Side-view schematic of the single pore developed by Carbonaro et al. Figure is reproduced from reference.
incubation with target DNA, aggregation is retarded allowing quantification of the DNA. The PNA aggregation diagnostic assay exhibited sensitivities as follows, sensitivity measurement of 5 nM for resistive pulse sensing, >50 nM for light scattering techniques, and >400 nM for UV-vis spectroscopy measurements. The limit of detection for the DNA sensor is reported to range from sub-nM to tens of nM, dependant on the characterisation technique used.\textsuperscript{53}

The surface properties of the particles and pore membrane,\textsuperscript{51, 52, 54, 145} interactions of particles with pore wall surfaces\textsuperscript{49, 144, 146} and ion concentration,\textsuperscript{51, 143} all affect resistive pulse sensor response. An example of a pore modified sensing system can be seen in Figure 1.19, where research by Kececi and Martin et al. used modified nanopores for the detection of short double-stranded DNAs (50 and 100 bp) The modification allowed movement of the DNA through the nanopore where previously electrostatic rejection of the negatively charged DNAs had occurred. Distinction between the 50 and 100 bp DNAs was achieved through current-pulse amplitude differences. Such nanopore modifications have shown interesting promise for enhanced sensing detection, allowing tailored control over the interactions between solution components and the nanopore walls.
Sensors aimed at detecting changes in particle surface properties can be desirable due to the ease of particle modification, the commercial availability of suitable particles, and the lack of labelling and sample processing required for analysis. Parameters used for distinguishing between particle populations can be size and blockade magnitude, signal duration and particle mobility and frequency translocation. Another recently published method uses a variable pressure method (VPM) to characterise surface charge. Using size to distinguish particles may not be applicable where only small changes in size are expected, or where aggregation is common. Meanwhile signal duration may carry with it high distribution due to the complexity involved in both entropic and electrostatic particle-nanopore interaction contributions. Consequently, analysing changes in the parameter duration will be improved and simplified by the inclusion of a relevant statistical model. Additionally, VPM is a frequency-based approach able to measure the electrokinetic surface charge of particles as well as being able to calculate zeta potentials. The range of characteristic information able to be measured using resistive pulse sensing is testament to its capabilities.

1.7 Realistic Target Samples

Although previous approaches to DNA sensing using ICPs have achieved high sensitivity and single-base pair mismatch discrimination, the practicality of such sensors with real samples can be lacking. Real world samples are complex with many variables under less control than laboratory testing, such as the presence of contaminants, interferants and the length of target DNA present. In such cases it is unlikely that the length of the target exactly matches the length of the probe, with most cases exhibiting targets that are longer. Shorter target lengths have been shown by Reisberg et al. (2005) to complementarily bind to
longer probe lengths.\textsuperscript{65} Longer target lengths, however, have not been extensively researched.\textsuperscript{45}

Lubin et al. (2009) used probe and target variations with a developed E-DNA sensor, investigating relatively short targets (17 and 34 base pairs). They found that longer probes exhibited greater signal changes than their shorter probe counterparts; however this was at the cost of sensor selectivity.\textsuperscript{152} Kalantari et al. (2010) used targets of 38, 44, 107, 1788 and 2907 base pairs coupled with their probes of 16 and 22 base pairs. Their results indicated that target length had only a small effect on the normalized signals.\textsuperscript{126} Korri-Youssoufi et al. found that longer target DNA (25 base pairs vs. 14) gave increased signal by CV.\textsuperscript{63} Meanwhile Komarova et al. (2005), when using probe DNA as a dopant to PPy, discovered that shorter probes (15 base pairs vs. 30) produced higher amperometric signals, thought to be an artefact of the difference in molar concentration of the two dopants.\textsuperscript{66} Work by Watts et al. (1995) found that shorter strands of DNA probe had higher hybridisation efficiency, believed to be due to steric factors.\textsuperscript{39} The difference in the effect of altered DNA length illustrates how the results depend on the sensing platform used.

An in-depth study by Shamsi and Kraatz (2011) investigated both overhangs for probe and target DNA strands using electrical impedance, Figure 1.20.\textsuperscript{45} They observed that overhangs at the electrode surface end cause decreased charge transfer resistance as compared to fully complementary (size and sequence) hybridisation, presumably due to lower hybridisation efficiencies from steric congestion and film non-homogeneities (Figure 1.20B). Conversely, overhangs at the end furthest from the film gave rise to increases in charge transfer resistance (Figure 1.20C). Meanwhile, when both overhangs were present a similar effect to bottom overhang was observed, namely a decrease in the change in charge transfer resistance.\textsuperscript{45}
Figure 1.20 Schematic representation showing surface-bound probe ON, and subsequent hybridisation with overhang target ON strands. Figure reproduced from reference Shamsi et al.\textsuperscript{45}

Hybridisation of 51-mer probe with A) 51-mer target, B) 54-mer target with bottom overhang, C) 60-mer target with top overhang, and D) 63-mer target with bottom and top overhangs.

The effect of target length in particular is of high importance in bringing researched DNA sensors closer to real sample testing, as typically the real DNA or RNA sequences being targeted are present as much longer sequences. The mRNA sequence specific for Glyco A, Figure 1.21, for example is longer than 1000 basepairs.\textsuperscript{17}

Figure 1.21 Image of the structure of the dimeric transmembrane domain of human glycophorin A determined by solution under NMR spectroscopy. Image originates from the primary citation reference by MacKenzie et al.\textsuperscript{153} and the protein data bank (PDB), PDB ID: 1afo/pdb

Another important step is the testing of developed sensors in synthetic mimics of the real sample matrices, or the actual matrices themselves. Often DNA sensors are designed to be examined in complex, contaminant-ridden solutions. Hence, further testing in these solutions is required.\textsuperscript{150}
Chapter 2

Experimental Methods

Marsilea Booth
Chapter 2. Experimental Methods

The chapter herein describes: 1) reagents and instruments used throughout this work, 2) the synthesis of monomer 3-pyrrolylacrylic acid (PAA), 3) the experimental and electrochemical procedural details, 4) the implantation process into polypyrrole films, 5) characterisation techniques, and 6) the experimental details for DNA sensing.

2.1 Reagents

1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), N-hydroxy succinimide (NHS, 97%), phosphate buffered saline (PBS, pH 7.4), poly(4-styrenesulfonic acid) solution (PSS, 18 wt% in H₂O), lithium perchlorate, propylene carbonate (PC), Triton X, pyrrolepropylic acid and monomer pyrrole were obtained from Aldrich. Dialysis cellulose tubing (average flat width 25 mm) was purchased from Sigma-Aldrich Chemical Co.. Hydroquinone (H₂Q) was obtained from Fluka (≥ 99.0 %), while ferricyanide was purchased from BDH Limited (≥ 99.0 %), and ferrocyanide from M&B Chemicals (≥ 99.0 %). Thiolated mPEG, H₃C-(CH₂CH₂O)₆CH₂CH₂SH, was purchased from Polypure (Norway).

Remaining chemicals were of reagent grade or better and used as supplied unless otherwise stated. All aqueous solutions were prepared using Milli-Q water (18.2 MΩ cm). Polymerisation solutions and most other solutions were deoxygenated prior to use through nitrogen bubbling. The exclusion of oxygen is desired as it can itself be reduced or may interact with radical anions that are important parts of the reaction.

2.1.1 Oligonucleotides

For all experiments alpha DNA synthesized ONs were used, as presented in Table 2.1 and Scheme 2.1. A sequence specific to the blood protein Glycophorin A (GlycoA) was targeted for most experiments. This sequence is a segment of the larger sequence found in Fig. 3 of Siebert and Fukuda, 1986 and has been specifically chosen to span two exon boundaries in order to target the RNA transcript sequence. Due to the length of the ON strands, precaution was taken to minimise any secondary structure (hairpins) and cross hybridisation, which could give rise to hybridisation competition effects. Polyacrylamide gel electrophoresis (PAGE) purification was performed on strands T2 and T3 by Alpha DNA. Although this
sensor targets an RNA transcript sequence, DNA was used for ease. It is believed this sensing platform is transferable to testing for RNA, as was the case in work performed by Lubin et al.\textsuperscript{18} In the case of hairpin probes, a different target DNA sequence was used, see Table 2.1.

Table 2.1 Sequences of oligonucleotides used.

<table>
<thead>
<tr>
<th>Description</th>
<th>Functionality</th>
<th>Label</th>
<th>Sequence, 5' → 3' DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>23'mer probe</td>
<td>5'Amino linker</td>
<td>P1</td>
<td>CGT TTG TGC GTA TCA TTT GTC TG</td>
</tr>
<tr>
<td>23'mer complementary target</td>
<td>None</td>
<td>T1</td>
<td>CAG ACA AAT GAT ACG CAC AAA CG</td>
</tr>
<tr>
<td>50'mer probe</td>
<td>5'Amino linker</td>
<td>P2</td>
<td>CGT TTG TGC GTA TCA TTT GTC TGT GAT GAG ATG TAC TCT TTG TGA CTG AA</td>
</tr>
<tr>
<td>50'mer complementary target</td>
<td>None</td>
<td>T2</td>
<td>TTC AGT CAC AAA GAG TAC ATC TCA TCA CAG ACA AAT GAT ACG CAC AAA CG</td>
</tr>
<tr>
<td>113'mer complementary target</td>
<td>None</td>
<td>T3</td>
<td>AGA AAT TGT GAG CAT ATC AGC ATT AAG TAC CAC TGA GGT GGC AAT GCA CAC TTC AAC TTC TTC AGT CAC AAA GAG TAC ATC TCA TCA CAG ACA AAT GAT ACG CAC AAA CG</td>
</tr>
<tr>
<td>18'mer non-complementary target</td>
<td>None</td>
<td>NT</td>
<td>TCG GCA TCA ATA CTC ATC</td>
</tr>
<tr>
<td>23'mer labelled complementary target</td>
<td>5'fluorescein isothiocyanate (FITC) labelled</td>
<td>T4</td>
<td>CAG ACA AAT GAT ACG CAC AAA CG</td>
</tr>
</tbody>
</table>
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### Table

<table>
<thead>
<tr>
<th>Probe Type</th>
<th>Linker Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>36'mer hairpin probe</td>
<td>5'thiol linker</td>
<td>HP1 ACA CGC TCA TCA AGC TTT AAC TCA TAG TGA GCG TGT</td>
</tr>
<tr>
<td>24'mer complementary target</td>
<td>None</td>
<td>HT1 ACG CTC ACT ATG AGT TAA AGC TTG</td>
</tr>
<tr>
<td>23'mer non-complementary target</td>
<td>None</td>
<td>HNT CAG ACA AAT GAT ACG CAC AAA CG</td>
</tr>
<tr>
<td>12'mer probe</td>
<td>5'Amino linker</td>
<td>P4 CGT TTG TGC GTA</td>
</tr>
<tr>
<td>11'mer detection probe</td>
<td>5'Amino linker</td>
<td>DP1 TCA TTT GTC TG</td>
</tr>
</tbody>
</table>

All DNA samples were stored at -20 °C in buffer solutions when not in use.

#### 2.1.2 Buffers

Phosphate buffered saline (PBS) buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) and 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.1 M, pH 7.1) were prepared using Milli-Q water (18.2 Ω.cm), and either HCl or NaOH added to achieve the desired pH. Standard electrolyte buffer (SEB) for particle suspensions and analysis was prepared containing 0.1 M KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer, 0.01 % v/v Triton X-100 (wetting agent), 3 mM ethylenediaminetetraacetic acid (EDTA) (to chelate any divalent ions such as Ca$^{2+}$ and Mg$^{2+}$ which can cause unwanted aggregation$^{156}$) and HCl to adjust to pH 8.

#### 2.1.3 Particles

The following particles were purchased:

- Particles were obtained from MagQu Co. (MF-COO-0090), with a dextran biocompatible coating, carboxylated surface and a Fe$_3$O$_4$ core.$^{157}$ Manufacturers stated a nominal diameter of 94 ± 22 nm
- NIST-traceable polystyrene particles from Thermo Scientific (“Nanosphere” Size Standards NIST traceable) with a nominal diameter of 102 ± 3 nm (Lot 37294 (PS)).
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- Carboxylated polystyrene particles from Bangs Laboratories with nominal diameters of approximately 115 nm (PSCOOH) and 200-220 nm (B200 COOH).

Quantum dots (QDs) (Q21341MP, 525nm, CARBOXYL modified) were purchased from Invitrogen, Life Technologies. All particles and quantum dots were stored at 4 °C when not in use.

2.1.4 Electrodes

Prior to electropolymerisation working electrodes were cleaned by polishing with 0.05 μm alumina, followed by subsequent washing with Milli-Q water.

The working electrodes used are listed below:

- a glassy carbon electrode (GCE) (eDAQ Pty Ltd, 1.0 mm I.D.), area = 7.9 × 10⁻³ cm²,
- a carbon fibre microelectrode (BASi Ltd. 11 ± 2 μm I.D.)
- a square glassy carbon working electrode (HTW Hochtemperatur-Werkstoffe GmbH, Germany) with polypyrrole film attached (through contact by Kapton tape with remainder masked by Kapton tape), or
- a stainless steel working electrode (Sandvik, Auckland, New Zealand) with an exposed area of 1 × 1 cm (remainder masked by Kapton tape).

The type of electrode used depended on the experiment performed.

2.2 Pyrrole and Functionalised Pyrrole Monomers

Synthesized films were either polypyrrole (PPy) or a copolymer made from the copolymerisation of pyrrole (Py) and functionalised pyrrole. Functionalised pyrrole is used in order to incorporate DNA binding groups into the ICP. Pyrrole synthetic chemistry can be manipulated to synthesize such functionalised pyrroles. Some functionalised PPy possesses limited conductivity, high resistivity⁹¹,⁹⁹ and may prove difficult to grow, hence a copolymer of functionalised pyrrole and pyrrole monomer is often chosen.

In our case the functionalised pyrrole, 3-pyrolylacrylic acid (PAA) was synthesized and used based on its previous success with DNA sensing.⁶⁶,⁷⁹ Pyrrole monomer was distilled under
vacuum and kept under nitrogen atmosphere prior to use, at -20 °C. The copolymer formed between PAA and pyrrole monomer is termed P(Py-co-PAA).

2.2.1 **Synthesis of Functionalised Pyrrole Monomer**

The monomer PAA was synthesized as previously reported, with the overall reaction scheme displayed in Scheme 2.2. The synthesis was repeated three times, labelled MBPyM1, MBPyM2 and MBPyM3.

Formation of p-Toluenesulfonyl chloride (1)

PCl$_5$ (0.44 mol) was added to $p$-toluenesulfonic acid sodium salt (0.4 mmol) giving a yellow slurry, effervescence and heat. After complete mixing the slurry was heated to 80 °C in a silicon oil bath, and stirred for 2 h. The reaction was then left to cool to room temperature, after which the reaction was quenched by pouring onto 600 mL of water and ice. The mixture was immediately filtered and the product was a white/yellow air-sensitive solid.

Formation of 1-(p-Tolylsulfonyl) pyrrole (2)

Pyrrole (0.27 mol, previously distilled under vacuum) whilst dissolved in dichloromethane (previously distilled) was added to a well-agitated suspension of NaOH (0.9 mol) in dichloromethane (previously distilled). The mixture was then cooled to 0 °C using an ice bath, and stirred for 10 min. Following this, a solution of (1) (0.3 mol) in dichloromethane (previously distilled) was added using a separating funnel over a period of 30 min, then the reaction left to stir at 0°C for 30 min. The reaction was then allowed to come to room temperature and remained stirring overnight.

The reaction was quenched by pouring onto ice, and the organic layer was separated. The aqueous layer was extracted with dichloromethane and the combined organic extracts washed with brine to neutrality and dried over MgSO$_4$. Removal of the solvent in vacuo gave (2). $^1$H NMR (CDCl$_3$, δ/ppm), 7.75 (d, 2H), 7.29 (m, 2H), 7.15 (t, 2H), 6.28 (t, 2H).

Formation of 3-Acetyl-1-(p-Tolylsulfonyl)pyrrole (3)

To a suspension of AlCl$_3$ (0.48 mol) in dichloromethane (previously distilled), acetic anhydride (0.16 mol) was added slowly and the mixture was stirred at 25 °C for 15 min.
producing a brown solution which gave off gas and heat. A solution of (2) (0.16 mol) in dichloromethane (previously distilled) was added, and the mixture was stirred at 25 °C for 2 h. The reaction was quenched with ice and the product extracted with dichloromethane. The organic layer was washed with water, dried over MgSO₄ and concentrated to give (3) as a brown solid. Because of the formation of an emulsion NaCl was added to the water fraction, to remove salt contaminants in the organic layer. ¹H NMR (CDCl₃, δ/ppm), 7.81 (d, 2H), 7.73 (t, 1H), 7.35 (d, 2H), 7.14 (m, 1H), 6.68 (m, 1H), 2.42 (s, 3H).

Formation of 1-(p-Tolylsulfonyl)pyrrole-3-carboxylate (4)

Sodium hydroxide aqueous solution (12 % w/w) was cooled to 0 °C. Bromine (375.5 mmol) was carefully added, followed by the addition of dioxane. The resulting hypobromite solution was kept at 0 °C before use.

To a solution of (3) (0.11 mol) in dioxane and water at 0 °C was added a steady stream of a cold hypobromite solution while stirring. During this time the temperature was kept cold by changing the ice. After 1 h acetone was added and the resulting solution acidified with 1 M HCl while still on ice (with stirring as tosyl group is acid sensitive), and the mixture extracted with dichloromethane three times. The combined organic extracts were washed with saturated aqueous sodium bicarbonate, water and brine. The product moved to the aqueous phase during the sodium bicarbonate wash, hence this was re-acidified and re-extracted. The extract was dried over magnesium sulphate, filtered and evaporated to give a brown solid. For MBPyM1 a column was performed at this point, with 1:1 hexane:ethyl acetate mixture. ¹H NMR (CDCl₃, δ/ppm), 7.78-7.83 (m, 3H), 7.13 (m, 1H), 6.68 (m, 1H), 3.80 (s, 3H), 2.12 (s, 3H).

Formation of Methyl 1-(p-Tolylsulfonyl)pyrrole-3-carboxylate (5)

Sulfuric acid was added to a solution of (4) (0.085 mol) in methanol and refluxed overnight at 80 °C. Water was then added to the solution and resulting mixture was extracted with dichloromethane. The extracts were washed with saturated aqueous sodium bicarbonate, water and brine, dried over sodium sulphate, filtered and evaporated under vacuum to give a light yellow solid. ¹H NMR (CDCl₃, δ/ppm), 7.79 (d, 2H), 7.75 (t, 1H), 7.33 (d, 2H), 7.11 (m, 1H), 6.65 (m, 1H), 3.80 (s, 3H), 2.42 (s, 3H).
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Two column purifications were performed for MBPyM3, with a 3:1 hexane:ethyl acetate solvent composition (initial removed brown colour, second separated product).

Formation of 1-(p-Tolylsulfonyl)-3-pyrrolylmethanol (6)

To a solution of (5) (0.063 mol) in anhydrous THF, LiBH₄ was added (0.08 mol) at 60 °C for 24 h. The reaction was quenched by adding water, and the resulting solution was extracted with dichloromethane. The extracts were washed with saturated aqueous sodium bicarbonate, water and brine, dried over sodium sulphate, filtered and evaporated under vacuum to give a light yellow solid. ¹H NMR (CDCl₃, δ/ppm), 7.74 (m, 2H), 7.29 (d, 2H), 7.12-7.09 (m, 2H), 6.29 (m, 1H), 5.20 (s, 1H), 4.46 (s, 2H), 2.39 (s, 3H).

Formation of 1-(p-Tolylsulfonyl)pyrrole-3-carboxaldehyde (7)

Manganese dioxide (previously made from activated carbon and potassium permanganate) was added to a solution of (6) (0.057 mol) in chloroform and the mixture was refluxed at 70 °C for 12 h. The mixture was then filtered and the filtrate concentrated under vacuum to give a brown/yellow oil. ¹H NMR (CDCl₃, δ/ppm), 9.97 (s, 1H), 7.81 (m, 2H), 7.63 (m, 1H), 7.34 (d, 2H), 7.16 (m, 1H), 6.40 (t, 1H), 2.42 (s, 3H).

A column was performed to purify this product using silica gel, and a 2:1 ratio of hexane:ethyl acetate for the solvent.

Formation of 3-(1-(p-Tolylsulfonyl)pyrrolyl)acrylic acid methyl ester (8)

(Triphenylphosphoranylidene) methyl acetate (43 mmol) was added to a solution of (7) (29 mmol) in anhydrous THF. The reaction mixture was stirred at 50 °C for 4 h and concentrated in vacuo to give a light yellow solid. A column was performed following this reaction for MBPyM3 using hexane:ethyl acetate 2:1. ¹H NMR (CDCl₃, δ/ppm), 7.78 (m, 2H), 7.51 (d, 1H), 7.32-7.29 (m, 3H), 7.34 (d, 2H), 7.14 (m, 1H), 6.48 (m, 1H), 6.15 (d, 1H), 3.76 (s, 3H), 2.41 (s, 3H).

Formation of 3-(1H-pyrrol-3-yl) 2-acrylic acid (PAA)

(8) (3 mmol) was placed in 1:1 mixture of methanol and 2 M aqueous NaOH and refluxed for 5 h, after which time the reaction mixture was allowed to cool and methanol was removed in vacuum. The aqueous solution was acidified very slowly on ice, while stirring, with 5 M HCl
to pH 3 and the precipitate was collected and dried under vacuum to give PAA as a pink solid. The \(^1\)H NMR spectrum of PAA is shown in Figure 2.1.

Scheme 2.2 Reaction scheme for the synthesis of 3-pyrrolylacrylic acid (PAA), with assigned numbers to intermediates.

Figure 2.1 \(^1\)H NMR spectrum of 3-(3-pyrrrol)acrylic acid, PAA (in DMSO-\(d_6\)).

1H NMR (DMSO – \(d_6\), δ/ppm) 5.97 (d, 1H), 6.41 (m, 1H), 6.81 (m, 1H), 7.19 (m, 1H), 7.51 (d, 1H), 11.15 (s, 1H), 11.73 (s, 1H).

2.3 Electrochemical Synthesis of Polypyrrole and Copolymers

2.3.1 Polymer Film Synthesis on Glassy Carbon Electrodes

The electrochemical synthesis of copolymer films of PPy and PAA, (P(Py-co-PAA)) for electrochemical DNA sensors, was performed from solutions containing 0.01 M Py, 0.2 mM PAA and dopant, excluding the probe concentration experiments. The thickness of the
polymers grown can be estimated using an equation based on the amount of charge passed during polymerisation.\(^{159}\) For the majority of conducting polymers, solvent and electrolyte used for polymer growth largely determine the polymer morphology, behaviour and electrochemical properties.\(^{160}\) To this end the electrolyte was varied and the effects examined. The two dopants employed were PSS and LiClO\(_4\). Polymerisation solutions contained either PSS (1:10, w/w ratio of monomer to PSS) in Milli-Q water, or 0.2 M LiClO\(_4\) in propylene carbonate to form PSS doped and LiClO\(_4\) doped films, respectively.

Initially two methods were employed for polymer growth: 1) application of a constant fixed potential of 1.0 V until a specified charge had passed, 2) a pulse growth technique, whereby a series of two short pulses at 1.0 V (50 ms) are applied separated by a pulse of 0.0 V (500 ms). The pulse growth technique was employed for further experiments. Optimisation was performed whereby pulses of 1.0 V were applied for 25, 50, 100 or 200 ms, of which it was found that 25 and 50 ms pulses produced reproducible, well-defined results indicative of thin films. These were hence employed for further experiments. A thin film of polymer was desired to aid in sensitivity through increased surface area to volume ratio.\(^{46}\) After polymer growth, cyclic voltammetry (CV) was performed in a potential range from 0 to 0.3 V vs Ag/AgCl for 10 segments with a scan rate of 50 mV s\(^{-1}\).

2.3.2 Altering the Ratio of Pyrrole to Functionalised Pyrrole

Probe density has been shown to be an important parameter for sensor design.\(^{47,161,162}\) To this end, a range of probe densities were investigated for their effect on DNA sensing with H\(_2\)Q as the redox probe. From the mechanistic view of random electropolymerisation, theoretically monitoring the ratio of pyrrole monomer to functionalised pyrrole monomer can mediate the probe DNA surface density\(^{10}\) as well as overcome any steric hindrance.\(^{46}\) Therefore, ratios are achieved by altering the amount of PAA monomer as compared to pyrrole monomer during copolymerisation. The ratios examined were 10:1, 25:1 and 50:1, pyrrole:PAA. Because probe DNA is attached through EDC chemistry to PAA, this allowed formation of a surface with a large probe DNA density, and a more sparsely DNA covered copolymer. The majority of the experiments performed, however, used a ratio of 50:1 pyrrole:PAA. In all of the concentrations examined the total pyrrole monomer (functionalised and non-functionalised) concentration is kept constant, while altering the ratio between the two.
2.3.3 Polymer Film Synthesis for Ion Implantation

Electrochemical growth of thicker polymer films for ion implantation used a stainless steel working electrode and was performed by applying a constant potential of 0.85 V (vs. Ag/AgCl (3 M NaCl)), from a solution containing 0.1 M LiClO$_4$ dopant and 0.1 M pyrrole monomer in propylene carbonate. After growth the films were peeled from the stainless steel substrate, and washed with acetone and secured to a non-conductive surface for implantation experiments.

2.4 Stability Experiments

The electrolyte solution and post-growth treatment were investigated for their effect on the stability of the grown copolymer. Electrochemical stability was measured via electrode impedance changes after incubation in 0.01 M PBS at 28 °C for time periods (30, 60, 90 and 120 min). EIS results for this system can be modelled using a modified Randels’ equivalent circuit, discussed in detail in section 2.7.3. Increases in the charge transfer resistance, $R_{ct}$, may indicate degradation of polypyrrole through non-specific (and undesired) mechanisms such as solution species (e.g. OH$^-$ ions) attack of the polymer backbone.$^{76, 99, 163}$ The change in $R_{ct}$ can be analysed as $\Delta R_{ct}$, calculated as the difference in $R_{ct}$ arising after incubation, divided by the initial $R_{ct}$ value of the freshly grown polymer. Minimising $\Delta R_{ct}$ is targeted for high stability. Stability was investigated for films with different dopants (LiClO$_4$ and PSS), different solution types (aqueous versus organic) and different solution pHs (pH 5.2 and pH 7.4).

2.5 Implantation of Polypyrrole Films

Ion implantation (also known as ion bombardment or ion irradiation) provides a means to modify mechanical, physical and chemical properties of materials.$^{102}$ It works through the controlled physical incorporation of selected atoms into another material, thereby bringing about physical and chemical property changes. Ions are accelerated to energies ranging from hundreds of electron volts to hundreds of kiloelectron volts. A variety of effects are observed from the radiation caused by ion implantation, such as an increase in conductivity, damage to the polymer backbone, amorphisation and damage or loss of polymer counterions.$^{102, 103, 107}$
Current industries utilize implantation for semi-conductor device fabrication, demonstrating the value and ease of implementation of the technique.

The set-up of the ion implantation equipment used in this research can be seen in Figure 2.2. The process was performed by Jérôme Leveneur and Dr. John Kennedy at the National Isotope Centre facility, GNS Science, Wellington, New Zealand. As can be seen, the implantation instrument exists with a number of components, with the ions transported from the source to the target, assured by a set of electrostatic and magnetostatic systems. Within the plasma ions are generated, for example Pt\(^+\) for platinum. A charge can be applied to extract the ions from the plasma chamber. A high vacuum is applied in the chamber (10\(^{-6}\) to 10\(^{-7}\) mbar). The Einzel lens and quadrupoles act to focus the beam, while the magnets act to separate and direct the ion beam toward the sample. The X-Y scanner meanwhile is able to direct the ion implantation to desired surface coverage and ensure uniform implantation. The depth of ion implantation, energy of the ions and fluence (number of ions deposited per unit area) of the implanted ions can be determined through TRIM simulations (Transport of ions in matter), and can be implemented by experimental equipment.

---

Figure 2.2 Schematic representation of the ion implantation system used. Individual parts are labelled, with an inset of a New Zealand map indicating where the ion implantation process took place.
Essentially any ion can be used for ion implantation with the type of ion implanted affecting sample properties. To this end many different ions are used, such as hydrogen, alkali ions and argon. In choosing a specific ion for implantation, for example platinum (Pt) ions, enhanced characteristics of the PPy films may be targeted, for example conductivity, catalytic activity, and biosensing abilities. Lead may be implanted to investigate the effect of incorporation of a lower conductivity metal into PPy films, while platinum may be implanted due to its high conductivity. Implantation allows for incorporation of conducting, metal particles permanently within the PPy framework without the requirement of reducing agent. With control over the implantation fluence, the amount of metal implanted can be selected. It has been shown that a higher concentration of metal ions (gold) ensures close proximity between the particles, as well as larger sizes of metal clusters (in PDMS), which in turn influence electrical contact across the film and hence conductivity.

Synthesized PPy films (possessing an area of approximately 1×1 cm) were secured to a surface for implantation experiments. PPy films were implanted with 15 keV Pt and Pb ions under normal incidence in high vacuum (< 2 × 10⁻⁷ mbar) at room temperature at the GNS Science facility. A range of fluences were investigated for Pt implantation, namely 10¹⁵, 3 × 10¹⁵, 6 × 10¹⁵, 1.2 × 10¹⁶ and 2 × 10¹⁶ Pt at. cm⁻². Meanwhile, implantation fluences for Pb were 2 × 10¹⁴, 5 × 10¹⁴, 1 × 10¹⁵, 2 × 10¹⁵ and 5 × 10¹⁵ Pb at. cm⁻². These fluences were chosen in order to limit the damage to the PPy while maximizing the doping effect. In addition, the implantation current was kept low (about 0.5 µA cm⁻²) in order to limit heating of the film and avoid additional damage. Calculations were performed using Dynamic-TRIM assuming a pristine C₃H₄N PPy film of 1.5 g cm⁻³ density. They revealed a projected range of between 20.5 and 22 nm below the PPy surface depending on the implanted fluence for Pt and between 20.8 and 22.4 nm below the PPy surface for Pb (Figure 2.3). The fluences employed provided a Pt peak concentration varying between 2 and 35 at. %. To limit the influence of changes induced by exposure to high vacuum conditions on the data analysis, all samples were kept for the same length of time inside the vacuum chamber regardless of the implantation duration. Control samples of pristine PPy were included in the study, in which no Pt ions were implanted, however which were exposed to the same vacuum cycle as the implanted samples. All implantation experiments and Dynamic-TRIM simulations were performed by Jérôme Leveneur and Dr. John Kennedy.
2.6 General Characterisation Techniques

Technique descriptions can be found in Appendix A for: nuclear magnetic resonance spectroscopy, scanning- and transmission electron microscopy, UV-vis spectroscopy, Fourier transform infrared spectroscopy (FTIR) and attenuated total reflection FTIR spectroscopy, Raman spectroscopy, Rutherford backscattering spectrometry analysis, fluorescence microscope and fluorometry experiments, and elemental analysis.

2.6.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) was performed for $^1$H capability. Measurements were recorded on a Bruker Avance 300 or Bruker DRX spectrometer, at 300 MHz or 400 MHz. Spectra were recorded in either CDCl$_3$, D$_2$O or deuterated DMSO. Spectra were referenced to either TMS or the CDCl$_3$ peak.

2.6.2 Scanning Electron Microscopy and Transmission Electron Microscopy

Films in preparation for scanning electron microscopy (SEM) were either:

- Grown on a glassy carbon electrode as usual and placed in the sample holder at an angle of about 30°, held in place by carbon tape and a metal block placed close to the electrode end. This allowed viewing of the electrode surface, see Figure 2.4.

Figure 2.3 Dynamic-TRIM simulations of implanted films.
A) Pt implantation at 15 keV with various fluences in PPy and B) Pb implantation at 15 keV with various fluences in PPy.
• Placed as small films on carbon tape in the sample holder, and as a cross-section held in place by carbon tape and metal blocks.

Scanning electron microscope (SEM) measurements were performed with a Philips XL30S FEG (field emission gun) scanning electron microscope equipped with a SiLi (Lithium drifted) EDS detector with a Super Ultra Thin Window, able to measure both secondary electron (SE) and backscattered electron (BSE) images.

Transmission electron microscope (TEM) images were captured with a Phillips CM12 TEM and a Gatan model 792 BioScan camera. PPy films were embedded in resin and subsequently were thinly sliced in order to view cross-sections. Particle analysis meanwhile involved pipetting of 1 µL of solution onto plasma treated copper grids, evaporation and subsequent examination with the microscope.

![Diagram of SEM set-up with glassy carbon electrodes in order to view electrode surface.](image)

2.6.3 **UV-Vis Spectroscopy**

UV-Vis Spectroscopy of liquid solutions was performed on a UV-1700 spectrophotometer (Shimadzu). The UV-vis-absorbance experiments were carried out using a USB2000 miniature fibre optic spectrometer (Ocean Optics). Measurements involved a 20 ms integration time, with 50 spectra averaged, and a boxcar smoothing of 5.
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2.6.4 Fourier Transform Infrared (FTIR) and Attenuated Total Reflection-FTIR Spectroscopy

Reflective Fourier-Transform infrared (FT-IR) spectra were measured using a Thermo Nicolet 8700 FT-IR spectrometer. ATR-FTIR spectroscopy was performed using a Perkin Elmer Spectrum 400 spectrometer equipped with a diamond crystal. Spectra were recorded with resolution of 4 cm\(^{-1}\), for at least 12 runs and ATR and baseline corrected using Omnic spectroscopic software.

2.6.5 Raman Spectroscopy

Raman spectra were collected using a Renishaw Raman spectrometer (system RM-1000) and a Raman imaging microscope (Renishaw, UK) consisting of a single grating spectrograph, a Leica microscope and an air-cooled charge coupled device array detector was used. A 785 nm laser excitation wavelength was used at 26 mW, with 4 cm\(^{-1}\) resolution. Data was processed using Grams32 spectroscopic software (Thermo Fisher Scientific Inc., USA) and Omnic software.

2.6.6 Rutherford Backscattering Spectrometry (RBS) Analysis

Rutherford Backscattering spectrometry (RBS) was performed using a 3 MV Van-de-Graaff accelerator at GNS Science\(^{175}\) with a 2.0 MeV \(^4\)He\(^+\) beam collimated to 1 mm to determine the implanted Pb concentrations in the film. Spectra were measured with a collimated (1.5 mm slit type) Surface Barrier Detector (SBD) positioned at the backscattering angle of 165º and the RBS spectra were analysed with RUMP\(^{176}\) simulations to retrieve the Pb concentration profile. The RBS measurements and RUMP simulations were performed by Jérôme Leveneur and Dr. John Kennedy.

2.6.7 Fluorescence Microscope and Fluorometry Experiments

For fluorescence imaging a DMLS_DC 200F Cambridge CB1 Leica microscope was used with an excitation filter range between 450-490 nm. Fluorescence images were captured generally with a 400× objective, with constant conditions of exposure time and brightness between measurements of the same sample type, initially chosen to view the most accurate
representation of the sample. Fluorescence spectroscopy, meanwhile, was performed using a PerkinElmer LS55 luminescence spectrometer.

2.6.8 Elemental Analysis

Elemental analyses were performed at the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. The elements tested for were C, H, N, S and Cl, and these experiments were performed in order to determine the doping level of PPy films for LiClO$_4$ doped films and PSS doped films. A Carlo Erba Elemental Analyser EA 1108 was used for C, H, N and S elemental analysis.

2.6.1 Conductivity Measurements

The dc conductivity of the PPy films was measured using a Jandel four-point probe conductivity meter. The instrument consists of four equally spaced probes with finite radii. A high impedance current source is then used to supply current to the outer two probes, while a voltmeter measures the voltage across the two inner probes. This allows measurement of the sample resistivity.

A current of 100 µA was passed through the films while the voltage was measured. Calculations of conductivity subsequently used the formula for a thin sheet, where $t$ is the thickness of the film (cm), Equation 1.

$$\rho = \frac{\pi t}{\ln 2} \left(\frac{V}{I}\right)$$

Equation 1

This equation is valid for our platform as the film thickness (5 µm) is smaller than the probe spacing (typically 1 mm).

2.6.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS), also known as Electron Spectroscopy for Chemical Analysis (ESCA), was pioneered in the mid-1960s by Kai Siegbahn and his research group, who subsequently received the Nobel Prize for his work. XPS involves the irradiation of a sample surface with monochromatic X-radiation. The kinetic energy of emitted electrons
is subsequently recorded. In this way, resulting spectra generally consist of a plot of the number of detected electrons per energy interval versus the energy of the emitted electrons (binding energy). The technique is able to provide information about the atomic composition of a sample as well as indications of the structure and oxidation state of the compounds being examined. The ability to examine the first few atomic layers of samples indicates the surface characterising capability of the technique.

When coupled with ablation, elemental concentration depth profiles are able to be determined. In this way, the elemental composition of a surface is captured before a beam of argon ions etches away (sputters) the surface layer (Figure 2.5).

Figure 2.5 Schematic representation of the sample during a depth profile XPS experiment, altered from The ion beam sputter etches the surface of the sample, while the survey spectra are captured at the analysed point after etching periods.

In detail, surface analysis is accomplished by irradiating a sample with monoenergetic soft x-rays and analysing the energy of emitted detected electrons. Typically the photons have limited penetrating power within the sample, on the order of 1-10 micrometers. The atoms in the surface region interact with the photons, causing electrons to be emitted. The kinetic energy of the emitted electron, $E_k$, is measured using an electron spectrometer. The binding energy, $E_b$, can subsequently be calculated using Equation 2.

$$E_b = h\nu - E_k - w$$

Equation 2

53
Where \( w \) is the work function of the spectrometer (a factor correcting for the electrostatic environment in which the electron is formed and measured). Binding energy is a measure of the energy difference between the initial and the final states after the photoelectron has left the atom. The variety of possible final states of the ions is determined by the type of atom, and in this way there is a corresponding kinetic energy of the emitted electrons which is characteristic of the sample atoms. XPS can therefore be used to identify and determine the concentration of elements in the sample surface.\(^ {179} \) Variations in binding energies can occur from chemical potential and the polarizability of compounds, and be measured at higher energy resolution. These chemical shifts in turn can be used to identify the chemical state of the materials being analysed. Deviations from literature values can provide information about the chemical environment of the atom responsible for the peak. Note that as a general rule, binding energies increase as the oxidation state becomes more positive.\(^ {178} \) Although ionization occurs to a depth of a few micrometers, only electrons that originate within tens of angstroms below the sample surface are able to leave the surface without energy loss. It is these electrons which produce peaks in the spectra, and are the most information rich.

2.6.2.1 Experimental Conditions Applied for X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) was recorded on a Kratos Axis Ultra DLD (Kratos Analytical, Manchester U.K.), using monochromatic Al \( K_a \) X-rays (1486.69 eV) with X-ray power of 150 W. Survey spectra were collected with 160 eV pass energy, whilst core-level scans were collected with a pass energy of 20 eV. The base pressure in the system was \( 2 \times 10^{-9} \) Torr. The analysis area for the data collection, using the hybrid electrostatic and magnetic lens system and the slot aperture, was approximately \( 300 \times 700 \) mm. Data analysis was performed using CasaXPS using Kratos’ relative sensitivity factors. Core level scans were calibrated based on a peak fit to the C 1s scan, with the component due to adventitious aliphatic hydrocarbon set to 284.5 eV. Shirley backgrounds were used throughout the analysis. Gaussian-Lorentian product lineshapes were used, with 30% Lorentzian weighting, GL(30). Argon ablation of the film surface was performed using a 5 kV argon beam and an emission current of \( \sim 10.4 \) mA, which was maintained between 10 and 12 mA throughout the experiment.
2.7 Electrochemical Characterisation Techniques

2.7.1 Supporting Electrolyte and the Electrochemical System

In these cells ‘supporting’ electrolyte (inert to the electrochemical reactions taking place at the electrodes and often in quite high concentrations (0.1 M) e.g. Na\(^+\) and Cl\(^-\) in PBS solution) is used to decrease resistance and to carry charge through solution. In this way the ohmic drop (R_s) is affected by the electrolyte type and concentration, as well as by the distance between the reference and the working electrodes. The resistance discussed here is the resistance of the solution, or R_s (Figure 2.6B).

![Diagram showing electrochemical system and equivalent circuit](image)

Figure 2.6 Diagram showing an electrochemical system along with the equivalent circuit. A) A diagram showing the electrical double layer (C_{dl}), electron transfer between the electrode and oxidised and reduced solution species, and the diffusion of oxidised and reduced species into the bulk solution. B) An equivalent circuit for the system shown in A, where the components are solution resistance (R_s), double-layered capacitance (C_{dl}), Warburg impedance (W) and charge transfer resistance (R_{ct}).

2.7.2 Cyclic Voltammetry (CV)

Voltammetry involves the measurement of current as a function of applied potential, under conditions which favour the polarization of the working electrode.\(^{178}\) In particular cyclic voltammetry (CV) involves cycling of the applied potential in a manner shown in the inset of Figure 2.8 using an experimental set-up shown in Figure 2.7.
Figure 2.7 A diagram showing the three-electrode system used for CV measurements. Potential is applied between the reference electrode and the working electrode, while the current is measured between the counter electrode and the working electrode.

In this way a continuous, time-varying potential is applied to the working electrode while the current is measured. The current results from the oxidation and/or reduction of the electroactive species present in solution, the capacitive current observed from formation of a double layer at the electrode surface, as well as any other current contributors (such as adsorbed species). This is explained in Equation 3, where the total current \( I_{tot} \) comprises of a Faradaic contribution \( I_f \) as well as a capacitive component \( I_c \).

\[
I_{tot} = I_f + I_c = I_f + vC_d
\]

Equation 3

The capacitive contribution to the current, \( I_c \), increases with increasing scan rate \( (v) \). The double layer capacitance, \( C_{dl} \), can be explained by the capacitor-like behaviour of an electrode-solution interface. Where no electroactive species is present in solution or at the electrode surface the capacitive current is the only current that is observed. If, however, any electroactive species are present, a Faradaic current contribution will arise. This current is dependent on two parameters, namely the kinetics and transport by diffusion of the electroactive species in solution. The Nernst equation, Equation 4, can be used to describe the relationship between applied potential and the concentration of the electroactive species in the immediate vicinity of the electrode surface.

\[
E = E^e - \frac{RT}{nF} \ln \frac{a_{red}}{a_{ox}} = \bar{E}^e - \frac{2.3026RT}{nF} \log \frac{a_{red}}{a_{ox}}
\]

Equation 4
Where $E^°$ represents the formal electrodes potential, $R$ is the universal gas constant, $T$ is the temperature (K), $F$ is Faraday’s constant, $n$ is the number of electrons that are transferred and $a_{red}$ and $a_{ox}$ are the chemical activities of the reduced and oxidised species, respectively. Chemical activity is defined as the activity coefficient multiplied by the concentration of the species; however as activity coefficients tend towards 1 at low concentrations, activities are often replaced by concentrations.

With the assumption that the concentrations at the surface of the electrode are governed by the Nernst equation, as the potential becomes more negative the concentration of the oxidised species at the electrode surface will decrease. If we also assume that the rate of electron transfer is very rapid (the reaction is mass transfer limited), the current ($I$) that is measured as the potential decreases will be directly related to the diffusion rate of the oxidised species to the electrode surface. This diffusion is governed by Fick’s law. As the potential is scanned to increasingly negative values, the concentration of oxidised species at the surface depletes. According to Fick’s law of diffusion, there is subsequently a higher flux of oxidised species to the surface, and hence a higher cathodic current. As the potential applied becomes more negative, the concentration of oxidised species at the surface will eventually become zero. At this point the volume of solution depleted of oxidised species will also be large; therefore the current will begin to decrease. This gives rise to the cyclic voltammogram (CV) curve in the reduction scan observed in Figure 2.8. Subsequently the same happens but for reduced species as the potential is scanned to become increasingly positive.

A parameter of interest is current peak height ($I_p$) as it is directly proportional to analyte concentration, as is described by the Randles-Sevčík equation (Equation 5).

$$I_p = -0.4463nF \left(\frac{nF}{RT}\right)^{1/2} c_0^{\infty} D^{1/2} v^{1/2}$$

Equation 5

Where most of the parameters have been earlier described, but additionally $c$ is the analyte concentration and $D$ is the diffusion coefficient. From this it can be concluded that for a reversible Nernstian system, $I_p$ depends on the square root of the scan rate and that the peak position ($E_p$) is independent of $v$. Moreover, the difference between $E_p$ and the point where the current is half that at $E_p$ ($E_{p/2}$) is 59/n (mV) at 25 °C, and $I_{pa}/I_{pc} = 1$. For quasi-
reversible redox reactions the voltammograms resemble those for reversible reactions, however with slight broadening of the peaks and larger peak separation.

In this research CVs are also performed on electrodes coated with PPy, which may differ from the bare electrode CV. After polymerisation if the redox couple has limited approach to the electrode surface, peak separation will increase as it is a function of the rate constant for electron transfer. If however, the reaction remains diffusion limited the oxidation and reduction peak separation will remain small. Analysis of the PPy-coated electrode in solution with the absence of redox active species will still yield CVs with $I_p$ proportional to $v$. In this case it will act as adsorbed species, where the current will decrease to zero after all of the conducting polymer has been oxidised and the same in the reversed scan with reduction. The redox reaction of PPy deviates somewhat from a strictly Nernstian behaviour, arising from slow ion mobility within the polymer film and an inherent property of PPy structure.\(^{181}\)

2.7.2.1 Experimental Conditions Applied for Electrochemical Experiments

Potential pulse polymerisation was performed on a CHI 650 or 440 electrochemical workstation (CH Instruments). All electrochemical experiments involved a three electrode cell involving an Ag/AgCl reference electrode (3 M NaCl, +0.197 V vs. SHE), a platinum
(either a spiral or straight wire or a platinum sheet, dependant on the surface area of the working electrode) counter electrode, and a working electrode. Cyclic voltammetry (CV) was performed either on a CHI 650 or 440 Electrochemical Workstation (CH Instruments), a BAS 100B Electrochemical Analyzer (Bioanalytical Systems, Inc.) or a Bio-Logic potentiostat (VSP-300).

Scan rates of 50 or 100 mV s\(^{-1}\) were adopted as the standards for all CV films in this thesis unless otherwise specified. Often CVs were employed to confirm the cleanliness of the bare electrodes. In these instances a 0.1 M NaCl PBS buffer solution was used, and subsequently a PBS buffer solution with 5.0 mM potassium [Fe(CN)\(_6\)]\(^{3/-4}\) solution (1:1 molar ratio).

### 2.7.3 Electrical Impedance Spectroscopy (EIS)

The electrical term resistance (\(R\)) shown in Ohm’s law, Equation 6, is the ability of a circuit element to resist the flow of electrical current.

\[
R = \frac{E}{I}
\]

Equation 6

While resistance can be used for electrical circuits, impedance is required where electrochemical cells are involved as both resistance and capacitance values must be considered. If a circuit contains only resistors, impedance would be equivalent to resistance. Impedance can be defined as a measure of the total opposition a device or circuit offers to the flow of an alternating electrical current (AC) at a given frequency.\(^{182}\)

Electrical impedance is generally measured using electrical impedance spectroscopy (EIS), which involves the application of a constant potential over which a small, sinusoidal potential amplitude (typically 5-10 mV) is overlaid, Figure 2.10A. The frequency of the applied amplitude potential is then modulated from high to low frequencies. The output is examined as a function of signal input. The cell impedance (\(Z_{cell}\)) has both a phase and an amplitude and can be divided into real (\(Z_{re}\)) and imaginary (\(Z_{im}\)), Equation 7.

\[
Z_{cell} = \frac{E_{cell}}{I_{cell}} = Z_{re} + jZ_{im}
\]

Equation 7
The real part of an impedance vector is the resistance, while the imaginary part is reactance. A phase angle for impedance expresses the balance between the capacitive and resistive components in the series circuit. As can be seen in Figure 2.9 there is a time-delay for current response when elements such as capacitors or inductors are involved. Therefore, where a shift in amplitude and phase is observed the type of components present may be deduced. Theoretically investigating the response at a range of frequencies may aid in the separation of the individual system components, Figure 2.10. However, sometimes separation is not possible. Progress in the assignment of equivalent circuit models has aided in the separation of components but assignment of an equivalent circuit to a system must be performed with caution. The best model is one which proceeds from identification of the expected components present and output comparison to experimental data. An equivalent circuit with a large number of components may improve the experimental fit, but may also run the risk of having superfluous components and not be applicable in terms of physical cell properties. Even if the model is not a true explanation of the electrochemical phenomena taking place, it can help to provide insight into interfacial processes occurring at the electrode surface. A physical model for a system may provide more information, however due to the complexity of polymer coated electrodes, none of the current theories can be regarded as satisfactory in all respects.\textsuperscript{160}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phasor_diagram.png}
\caption{Phasor diagram indicating the relationship between alternating current ($I$) and voltage ($E$) over time in A) a capacitor and B) a resistor. Diagram adapted from Ref.\textsuperscript{82}}
\end{figure}
Chapter 2

Figure 2.10 Principles of electrical impedance spectroscopy, the equivalent circuit used and the resulting signal output. A) A diagram of the principle of EIS. The arrows indicate the AC potential applied and the resulting AC current obtained. B) The equivalent circuit used, with indications of the path of least resistance at high frequencies and low frequencies. Also shown are the circuit positions of the counter electrode (CE), reference electrode (RE) and working electrode (WE). C) An example of EIS signal output displayed as a Nyquist plot, with the key features highlighted.\(^{180}\)

The Randels’ equivalent circuit (Figure 2.6B and Figure 2.10B) describes the system used, however with a modification to the double layer capacitor. The equivalent circuit comprises of solution resistance \( (R_s) \), Warburg impedance arising from the diffusion of ions \( (Z_W) \), a constant phase element \( (CPE) \), and charge transfer resistance \( (R_{ct}) \), the latter two of which are the contribution of Faradaic impedance.\(^{44, 46, 160, 183}\) A constant phase element (Equation 8) is used in place of the typically encountered double layer capacitor as it models the data in a superior manner.\(^{160, 184}\)

\[
Z_{CPE} = A(j\omega)^{-n}
\]

Equation 8

Where \( \omega \) is the parameter frequency, \( A \) is the CPE coefficient and \( n \) determines the extent of deviation from the Randels and Ershler model.\(^{46}\) When \( n = 1 \) the CPE is equivalent to a capacitor. It is believed the CPE physically amounts to the existence of inhomogeneities present in the PPy film surface reactions.\(^{46}\) As PPy is a semiconductor there is less homogeneity, less even charge distribution and fewer free states as compared to conductors meaning the semiconductor varies along films. This component also describes the fact that some ions in solution balance the charge of the electrode at the electrode:solution interface.

EIS allows probing of the surface properties of the electrode in a non-destructive, highly sensitive manner, thus making it suitable for surface DNA hybridisation analysis. A typical way to display EIS data uses a Nyquist plot with \( Z_{re} \) on the x-axis, versus \(-Z_{im}\) on the y-axis. The semi-circular arc observed in Figure 2.10C in the Nyquist plot is influenced by \( R_{ct} \) of 61
electrons across an interface. For PPy covered electrodes this is kinetically governed by the charge transfer being limited across the solution:PPy or PPy:GCE interfaces, rather than being limited through the electrolyte solution. Typically a redox active species (i.e. a redox couple) is present which is able to support electron transfer through the solution, such as the ferri/ferrocyanide redox couple. A straight line at a 45 degree angle may follow the semi-circular arc, indicative that response at low frequencies is dominated by diffusion-controlled processes. Although Nyquist plots are commonly used for the presentation of impedance data other methods of display are available such as Bode plots (impedance or phase angle versus frequency, preferred when there are several rate-determining processes in the system), and admittance plots (Y, the reciprocal of impedance, preferred when parallel connections are involved).

2.7.3.1 Experimental Conditions Applied for Electrical Impedance Spectroscopy

Electrochemical impedance spectroscopy was performed on either an EG&G potentiostat/galvanostat (Princeton Applied Research, model 280) coupled to an EG&G 1025 Frequency Response Analyzer, or a Bio-Logic potentiostat (VSP-300) system. A three-electrode system was used as described for EIS experiments, see Figure 2.11. Impedance measurements were performed with 10 mV sinusoidal modulation amplitude at an applied bias potential (specified within the text as either 0.23 V or 0.12 V) and collected for harmonic frequencies between either:

- 0.1 MHz and 0.1 Hz at 12 steps per decade for 1.0 mm I.D. glassy carbon working electrodes or
- 100 kHz and 10 Hz at 12 steps per decade for polypyrrole films adhered to glassy carbon square electrodes.

Applied bias potentials were 1) in a solution of PBS containing 5.0 mM \([\text{Fe(CN)}_6]^{3-/4-}\) (1:1, mol:mol) at an applied bias of 0.230 V, or 2) PBS solution containing 1.0 mM H$_2$Q at an applied bias of 0.120 V. Due to the photo-degradation of H$_2$Q, this solution was prepared daily.

Data were analysed using the ZView software (version 2.80, Scribner Associates Inc., North Carolina), or EC-Lab Express Z fit function (V5.40, Bio-Logic). The circuit model used for
fitting was constructed based on the system used and experimental results as well as previous experiments within the research group and other research groups.

Experiments were performed in at least duplicate to ensure reproducibility. Although absolute sensor responses were not identical, the observed trends were reproducible; hence changes in signal are described versus absolute signal values. Variations in the electrode surface and exact film content may account for any diversity observed.

Figure 2.11 Three-electrode electrochemical cell for EIS measurements. Cell contains a glassy carbon working electrode, curly platinum counter electrode and Ag/AgCl (3 M NaCl) reference electrode immersed in 0.1 M NaCl in PBS solution.

2.8 Scanning Ion Occlusion Sensing and Light Scattering Analysis (SIOS)

2.8.1 Scanning Ion Occlusion Sensing (SIOS)

Scanning Ion Occlusion Sensing (SIOS) technology is an adaptation of the principles of resistive pulse-sensing, which had a resurgence of interest for Coulter based techniques after the work of Kasianowicz et al. in 1996. The simplicity, measurement sensitivity, and potential for individual single-object output capabilities show the allure such a technique possesses. A qNano instrument is used for the resistive pulse sensing performed here.
2.8.1.1 The qNano Instrument from IZON Science

The commercially available tuneable pore instrument, the qNano (IZON Science) was used for resistive pulse sensing and characterisation of the particles,\textsuperscript{52, 54, 145} Figure 2.13. The qNano consists of two main components, the instrument platform (Figure 2.13A) and an elastomeric nanopore (Figure 2.13B). The conical nanopores\textsuperscript{145} are fabricated in TPU (Elastollan 1160D, BSAF). The nanopore membrane was wetted prior to sampling. Sampling began after a stable background current signal was achieved; such as Figure 2.12.

![Image](Figure 2.12 A sample of a stable background current signal for SEB solution.
Conditions used include 47 mm stretch, 0.32 V applied and an NP100 pore. In this case the baseline current is 71.5 nA, while the noise is 7 pA. Using the instrument platform the nanopore can be mechanically actuated to adjust the pore size (Figure 2.13C). If clogging of pores by large aggregates occurred the pore was cleared using pressure, fluid movement in the upper fluid cell or pore cleaning. Adjusting pore size also affects the current signal. A minimum of 200 (early experiments) - 500 (later experiments) particles were analysed for each measurement. Control particles of carboxylated polystyrene (PSCOOH) were run with each pore prior to sample particle measurement, and used to calibrate signal output.

A variable pressure module (VPM) existing as a water-based manometer (instrument for measuring pressure), was used in combination with the qNano, Figure 2.13D. This allowed application of pressure to the fluid cell of between 0 and 15 cm of water (equating to 0-1.5 kPa).\textsuperscript{156} Pressure is applied by adjusting the manometer lever, which transfers pressure through the connecting tube attached to the top of the fuel cell. A vacuum can also be applied creating a negatively applied pressure (pressure from lower to upper fluid cell) to the fluid cell.
Figure 2.13 The commercially available qNano instrument, by IZON Science. A) a view of the instrument, B) an IZON Science elastomeric nanopore, with inset in the bottom right displaying the upper fluid cell, C) the nanopore in the qNano set-up with the upper fluid cell in place, and D) the variable pressure module (VPM) with gauge and plastic tubing here in the storage position.

2.8.1.2 IZON software – IZON control suite

The interface software V2.2 IZON software is used for data collection. This software allows for concentration calibration, size characterisation (against a calibration particle group of known size), and measurements of duration properties (Full width half maximum (FWHM) measurements and baseline duration measurements), see Scheme 2.3.

Scheme 2.3 Overall scheme of the qNano and signal output. A) Scheme of the nanopore set-up in the qNano, and B) transient current drops appearing as particles pass through the nanopore, with an enlarged trace indicating the calculations for baseline duration, FWHM and magnitude (dI).

An example of the user interface is shown in Figure 2.14. As the particle passes through the nanopore, ions in solution are prevented from flowing through the pore, thereby causing an increase in resistance (and a decrease in current). These are known as translocation events or blockade events. The frequency of the translocation events provides concentration information, Figure 2.14B. Particle characteristic information, meanwhile, can be extracted
from the current-pulse signature e.g. magnitude and duration, Figure 2.14A and C and Scheme 2.3B. Individual traces of blockade events can also be captured by the software.

Duration can be measured as both the time taken for the baseline duration to return to a baseline current value, as well as a full-width half maximum (FWHM) measurement, Scheme 2.3B. The use of FWHM can reduce the measurement error introduced in deciding when the current value has returned to baseline. Note that duration does not necessarily measure the time taken for the particle to pass through the total length of the nanopore, but is in fact the time taken for the particle to traverse the sensing zone. In particular for conical pores with large cone angles the sensing zone is very localised around the pore restriction, in this way individual particles can be analysed. The principle behind this technique is found valid for measuring non-conducting particles.

Drops in current are measured to be blockade events (signal that is not due to noise) if the current deviation is more than $8 \times$ the level of the root mean square (RMS or background) noise. The potential applied and stretch must be chosen so that the current is high enough that the particle blockade events are large enough to be measured, and the background noise must be low enough not to generate blockade signals. This can prove difficult where multiple sized particles are present or where aggregates are problematic, but can be optimised for the particle system being used.

Figure 2.14 A typical user-interface screen during sample analysis. Screen indicates A) a histogram of blockade height and particle count, B) a rate plot for particle count, and C) a typical current signal where the translocation events of particles can be seen.
2.8.1.3 Characterising Particle Size

The current methods used to acquire particle size characterisation are microscopy analysis (e.g. TEM and SEM) and light scattering technology (e.g. DLS). Microscopy analysis suffers from time consuming analysis and complicated equipment. DLS, meanwhile, can prove inherently biased by small subpopulations, can encounter problems with large particle analysis and requires very low particulate concentrations and limited particle movement within the sample. DLS offers measurement of particles between about 0.005-1 µm and although the repeatability of the technique is good, the resolution of the technique is not as good. Other techniques for particle sizing exist, such as ultracentrifugation, chromatography and gel electrophoresis.

Using SIOS technology and the Control Suite V2.0, particle size, duration and traces of individual translocation events can be extracted from experimental data. The pulse magnitude is used for size determination after calibration against a particle set of known size, Figure 2.15. Individual particle analysis allows for low concentration samples, and typically low volumes are required for sample analysis (40 µL). Current capability of this instrument is reported at analysis of 50 nm – 10 µm particles. A disadvantage of the qNano lies in the fact that an electrolytic fluid is required. Ions in the fluid are required to provide a background current flow through the nanopore, which is then altered as particles pass through the pore. Furthermore, the particle solution must be quite dilute to prevent blocking of the pore.

It should be noted that for qNano experiments (and in DLS), hydrodynamic size is obtained which includes a layer of adsorbed ions and molecules from the dispersion medium.

![Illustrative description of different particles through a nanopore and resulting blockade events. With A) a particle with large diameter, and B) a particle with a small diameter. Figure adapted from 156.](image-url)
A fine balance exists with resistive pulse work between the pore size used and the measureable signal. A larger pore size reduces clogging, however does not allow measurement of small particles or fine discrimination between particles with similar surface properties. A small pore size also allows selective gating of larger objects from analysis. The use of variable pore size technology greatly assists in tuning a happy medium between pore clogging and sensitive measurements.

After achieving a stable baseline current, calibration particles were introduced into the upper fluid cell. An appropriate nanopore stretch and applied potential were sought in order to achieve a high current baseline (ensuring translocation events are sufficiently high for analysis) while retaining low noise. Once these conditions had been found a calibration data set of at least 500 particles was collected. Subsequently the upper fluid cell is washed, and the sample introduced while all conditions (stretch, applied potential etc.) remained constant. A sample data set was then collected.

Note that prior to analysis samples (excluding NIST traceable and calibration particles) were suspended in filtered SEB solution, sonicated for 1 min, and filtered with a 0.45 µm filter unit (Millipore). Dilution of particles in electrolyte was executed so that concentrations were approximately $10^9$ (unfiltered calibration particles) to $10^{12}$ particles mL$^{-1}$ (samples to be filtered).

### 2.8.2 Characterising the Zeta Potentials of Particles

Zeta potential ($\zeta$) is used as a measure of the charge stability of a particle suspension, and is essentially the electrokinetic potential of a colloidal system. Although the absolute charge of the particles is not provided, the zeta potentials may be related to the particle surface charge. In an aqueous solution particles will acquire a surface charge, generally through either ionization of surface functionalities or through adsorption of charged species. These charges will also alter the layers surrounding the particles. The zeta potential can be defined as the potential difference between the bulk solution and the stationary layer attached to the dispersed particle (slipping plane, Scheme 2.4). Solution composition is highly influential on zeta potential, in particular the ion type, concentration and solution pH.

In terms of physical effect, zeta potential is one of the main forces mediating interparticle interactions. High zeta potentials (typically $> \pm 30$ mV) of the same charge (either positive...
or negative) will repel one another and maintain particle stability, while particles with low zeta potentials are prone to aggregation.

Scheme 2.4 Schematic representation of a particle and associated zeta potential. The particle here is negatively charged, hence the layer of tightly associated positive ions present in the Stern layer. Inset is a graph describing the layers surrounding a negatively charged particle, including the ζ potential.

Zeta potentials are not directly measureable, but are calculated using theoretical models as well as the experimentally-determined electrophoretic mobility of particles. Phase Analysis Light Scattering (PALS) can be used to determine particle mobility, and modern Zetasizer instruments are able to calculate zeta potentials. Electrophoretic mobility can also be determined using a variable pressure module (VPM) technique combined with the qNano, developed and detailed by Vogel et al.\textsuperscript{52, 187}

2.8.2.1 Zeta Potential Characterisation with Phase Analysis Light Scattering (PALS)

For particle surface charge characterisation through zeta potential measurement, a commonly used technique is PALS. PALS calculates zeta potentials through application of an electric field across the suspension of particles. Any particles possessing a zeta potential will migrate toward the electrode of opposite charge, and do so with a velocity proportional to the magnitude of the zeta potential. Velocity is measured using the phase shift of an incident laser beam caused by the moving particles and in turn gives a measure of particle mobility. Particle mobility can then be converted to zeta potential by inputting the dispersant viscosity
and application of the Smoluchowski or Hückel theories.\textsuperscript{189, 190} PALS technology offers high performance over laser Doppler electrophoresis,\textsuperscript{190} the latter of which uses the laser frequency shift in place of the phase shift used in PALS. A disadvantage of the technique is the practical problem of contamination, where a small contamination can seriously affect results due to the sensitivity of the technique.

Zeta potentials were measured on a Malvern Zetasizer Nano ZS, with PALS. Average zeta potentials were calculated for the different particles dispersed in filtered SEB solution (pH 8.0), and data were obtained from at least 12 measurement cycles and repeated three times. Zeta potential measurements were performed after washing (in order to remove residual EDC, NHS and DNA) and without filtering of the particles.

2.8.2.2 Zeta Potential Characterisation with Scanning Ion Occlusion Sensing (SIOS)

Theoretical Basis for Particle Zeta Potential Characterisation

There are a number of mechanisms that act to transfer particles from one fluid cell to the other, namely diffusion, electrophoresis, electro-osmosis,\textsuperscript{148} and hydrodynamic pressure (dependent on experimental conditions such as applied external pressure). These mechanisms can be combined and described by the Nernst-Plank approach,\textsuperscript{52} Equation 9.

\[
J = \frac{\varepsilon (\zeta_{\text{particle}} - \zeta_{\text{pore}})}{\eta} E + \frac{Q_P}{A}
\]

Equation 9

where \( J \) is the total particle flux, \( C \) is the concentration of the particles, \( \varepsilon \) and \( \eta \) are the permittivity and kinematic viscosity of the electrolyte, respectively, \( \zeta \) indicates zeta potential, \( E \) is the electric field, \( Q_P \) is the volumetric flow rate which is driven by the applied pressure, and \( A \) is the cross-sectional area of the pore constriction. The equation can be broken down into the contributions due to electrophoresis (\( \zeta_{\text{particle}} \), expected to be the most significant contributor), electro-osmosis (\( \zeta_{\text{pore}} \)), and hydrodynamic transport (pressure).\textsuperscript{52} Diffusion has been omitted in this analysis as the contribution on particle movement is negligible when compared to the other contributors, as the buffer solution in the fluid cell is kept constant. Electro-osmotic and electrophoretic contributions can be estimated using the Smoluchowski equation.\textsuperscript{52} Hydrodynamic transport meanwhile can be thought of as a combination of the
externally applied pressure and the inherent pressure in the fluid cell arising from
gravitational head and meniscus effects from the upper fluid cell’s 40 µL volume. The
conical pore geometry (small and large pore opening diameters and pore length) can be
estimated from evaluating baseline current and blockade (signal) magnitude of a calibration
particle of known size. In this way, the equation consists of many known components.
Note that the calculations relating to zeta potential and Equation 9 were performed by Dr.
Robert Vogel.

![Scheme 2.5 Scheme indicating the mechanisms acting on particles as they pass through the nanopore.
In this case the particle is negatively charged, while the applied potential is positive (0.48 V). This causes a
force from the upper to lower fluid cell from electrophoresis, while electro-osmosis opposes this force. External
applied pressure also creates a force from the upper to lower fluid cells (though this can be reversed if a vacuum
is applied).

Experiments can be performed to calculate zeta potential, whereby both fluid cells contain an
equal concentration of particles. A fixed applied potential and nanopore stretch is applied,
and this remains constant throughout the experiment. The VPM addition can then be used to
apply a range of pressures to the upper fluid cell ensuring particles flow from the upper fluid
cell through to the lower fluid cell (or vice versa if begin with a vacuum). Data is then
recorded, and every 30 s collection is paused while the pressure is adjusted by 50 Pa (-0.5
cm). Data collection is then resumed and the 30 s countdown started once a stable baseline is
again achieved. In this way the pressure is changed incrementally from a positive to negative
pressure. At some point during this process the particles will start to linger very close to the
nanopore and will eventually swap direction and flow from the lower fluid cell to upper fluid cell. The switching point, also known as the inflection pressure, is of interest here as it is when the particle flux, \( J \), in Equation 9 is zero. In this way the applied pressure exactly opposes the effects of electro-osmosis, electrophoresis, and inherent pressure. At this point, because values for \( \zeta_{pore} \), inherent pressure and applied pressure as well as pore geometry are known, the values of inflection pressure can be included to calculate zeta potentials.

**Experimental Details for Particle Zeta Potential Characterisation**

For the VPM and qNano technique, equipment was used as detailed in section 2.5. Briefly, a potential of 0.48 V was applied with equal concentration of particles in both fluid cells. As discussed in particle size characterisation experiments (section 2.8.1.3), filtration of sample particles following sonication was performed. Again, dilutions of particles were on the order of \( 10^9 \) (unfiltered calibration particles) to \( 10^{12} \) particles mL\(^{-1} \) (samples to be filtered).

Following a steady baseline current, a pressure is applied to the top fluid cell (determined by the water-based VPM (IZON Science)), and at 30 s increments varied by 50 Pa. The range of pressures applied was from 300 Pa to -600 Pa (vacuum). The pressure system used by Vogel et al.\(^52\) utilised a continuously adjusting pressure system. This set-up meanwhile, used 50 Pa increments and subsequently a difference in data analysis and display can be observed. The stretch of the membrane (and hence pore size) and applied potential were kept constant throughout measurements. When particle clogging occurred the run was stopped, pore cleaned and solution changed. Zeta potential calculations were subsequently performed by Dr. Robert Vogel, with associated errors extracted from the pressure uncertainty for inflection pressures.

**2.9 DNA Modification and Sensing Experiments**

Experiments involving DNA attachment and incubation were performed for three sensing platforms. The first two are electrochemical DNA sensors, GCEs with P(Py\(-co\)-PAA) polymer, and platinum implanted PPY films, while the third platform uses particles with resistive pulse sensing.
2.9.1 **Surface Derivatisation Efficiency**

The number of carboxylic groups on the surface of the polymer and particles was determined according to the method reported by Sano et al. and adopted by others. This method is based on the assumption that one molecule of toluidine blue (TB) binds with one carboxylate group.

In this method the surface of interest (either a GCE coated with P(Py-co-PAA) or a solution of carboxylated particles) was incubated in TB solution \(2 \times 10^{-4} \text{ M}\) in 0.1 mM NaOH for 1 h at 40 °C. Following incubation the surface was washed 3 times with fresh 0.1 mM NaOH solution. The samples were subsequently placed in a clean Eppendorf tube with 1 ml of 50 % acetic acid for 30 min at 40 °C to release bound TB into solution. The absorbance was measured with a UV-1700 Shimadzu spectrophotometer, and the absorbance at a wavelength of 360 nm noted. Using a calibration curve of toluidine blue concentration versus absorbance, the number of reactive carboxylic acid groups in the films and on the particles can be calculated. Unfortunately this experiment has reported non-specific adsorption, therefore preventing direct quantification of the number of carboxylate species, but more a qualitative comparison between similar films or particles. Additionally, because this experiment relies on electrostatic interactions anything which interferes with the attraction between the TB and the acid group will affect results.

2.9.2 **Probe Attachment and Target Hybridsation at Polypyrrole Films**

Post polymerisation and prior to probe incubation, the films were cycled by CV as stated in section 2.3.1. Probe attachment to copolymer films was performed based on work previously described, using either well established EDC carbodiimide chemistry with NHS added, or thiol attachment chemistry. With carbodiimide chemistry EDC reacts with the carboxylate groups from the functionalised pyrrole, forming an activated ester intermediate, Scheme 2.6. This intermediate is then able to react with primary amines to form a peptide bond. The modifications present on the probe DNA (either a terminal amino or thiol group) allowed ease of probe attachment.

Probe incubation was performed in two ways, either by:
- Incubation of GCE coated with P(Py-co-PAA) at 28 °C for 1 h in 50 μL PBS containing 80 μM ON probe, 50 mM EDC and 80 mM NHS, followed by thorough washing with PBS solution. A plastic piece of tubing was used as an incubation chamber in order to form contact between the polymer surface and the probe solution, as illustrated in Figure 2.16 shown fitted over the GCE. Or
- Through overnight incubation of a solution containing thiolated hairpin probe DNA and mPEG (at a ratio of 1:25\textsuperscript{195}), followed by thorough washing with PBS for the Pt implanted PPy films. The thiol functionalities of the ON and mPEG encouraged favourable interactions on the Pt surfaces.\textsuperscript{43, 195} Probe solution was pipetted onto the film surface and kept in an enclosed area (to prevent evaporation).

Following thorough washing, electrodes were then immersed in solution in preparation for EIS or other experiments. Probe DNA solution was prepared using PBS buffer at pH 7.4 except when stated, whereby a pH 5.2 was used. Fresh EDC/NHS solution was prepared for each experiment.

Scheme 2.6 Chemical scheme showing the conversion of carboxylate species to amide bonds between an R group and probe DNA. R is either an acid group from PAA in a P(Py-co-PAA) copolymer film or a carboxylate group on a particle surface.
Subsequently, target hybridisation was performed by either:

- Incubation for 1 h at 42 °C for a range of concentrations between 2.5 nM and 2.0 mM for GCEs (eDAQ Pty Ltd) in PBS solution, using an incubation chamber as for probe incubation, Figure 2.16. Hybridisation period and temperature were based on previous work.\textsuperscript{46, 48, 79} Electrodes were thoroughly washed with PBS solution post-hybridisation.

- Through incubation of Pt implanted PPy films with target DNA, performed at 37 °C\textsuperscript{195} for 2 h (concentration of 5 and 50 µM), followed by thorough washing with PBS. Target solution was pipetted onto the film surface and kept in an enclosed area (to prevent evaporation). Thorough washing was performed following hybridisation.

Non-complementary DNA incubation to test for non-specific DNA binding was performed by replacing target DNA with non-complementary DNA and identical incubation conditions. Concentrations of non-complementary target were varied between 50 µM and 5 mM.

Electrodes were then immersed in solution in preparation for EIS or other experiments. Complementary and non-complementary target was prepared using PBS buffer (pH 7.4)

![Figure 2.16](image)

**Figure 2.16** A GCE (eDAQ Pty Ltd) fitted with the incubation chamber. Probe solution was pipetted into the chamber ready for incubation of probe or target. After introducing the ON solution the chamber was wrapped with parafilm and the set-up placed within a closed water bath at the desired temperature.

### 2.9.3 Probe Attachment and Target Hybridisation at Particle Surfaces

The same EDC/NHS chemistry is used for probe attachment to particles, explained in Scheme 2.6. Grafting of probe DNA onto magnetic particles consisted of addition of 100 mM EDC and 100 mM NHS to a solution of a volume of 300 µl MES buffer (pH 7.1) containing 9.5 µM probe DNA and ~ 9 × 10\textsuperscript{12} carboxylated dextran particles.mL\textsuperscript{-1}. Subsequent shaking
at room temperature followed, with varying times for optimisation experiments.\textsuperscript{196} After reaction the particles were diluted with filtered SEB buffer.

Subsequent DNA incubation (target and non-complementary DNA) was performed in SEB buffer with $10 \times 10^{-6}$ M target DNA, or $50 \times 10^{-6}$ M non-complementary DNA. The particles and solution were mixed and shaken for varying times for optimisation experiments. Following hybridisation the particles were diluted with SEB buffer.

It was found that washing of magnetic particles caused unwanted aggregation, hence this was not used for further experiments. As the particle solutions were diluted for qNano analysis ($27 \times$ and $3 \times$ for probe and target solutions, respectively), the concentrations of EDC and NHS (in probe-grafted particle solutions) and non-hybridised target DNA (in solutions of probe-grafted particles incubated with target DNA) should not affect the qNano results.

2.9.3.1 Quantum Dot Modification and Use

The same EDC/NHS chemistry explained in Scheme 2.6 is used for QD modification. The QDs are carboxylate functionalised, hence incubation of 1 µM QDs with 6 mM and 8 mM of EDC and NHS, respectively, and 40 µM of amine functionalised detection probe DNA was performed, based on protocol advice from Invitrogen and the literature.\textsuperscript{83,197} Shaking for 2 h at room temperature followed. Only a small amount of EDC and NHS were used as incomplete modification (to limit aggregation) was desired. Following modification of QDs, incubation with “Target” particles was performed overnight.

2.10 Sensing Techniques and Data Analysis

2.10.1 Detecting Target DNA Hybridisation Using EIS

The first two sensing platforms explored use EIS as a sensing technique to detect target DNA, with the instruments stated in section 2.7.3.1, and with the conditions described in section 2.7.3. Any increases in $R_{ct}$ caused by target incubation can be normalised against the $R_{ct}$ value for the probe-grafted film (prior to incubation, $R_{ct}^0$), giving $\Delta R_{ct}/R_{ct}^0$. This allows a measure of 1) whether or not target hybridisation has occurred, and 2) information on the amount of target hybridisation which has occurred based on the size of $\Delta R_{ct}/R_{ct}^0$. Therefore this value, $\Delta R_{ct}/R_{ct}^0$, can be plotted against DNA concentration to give sensor response curves.
When analysing results from electrochemical DNA sensing, averages were taken of the measurements and errors reported as the standard deviations from the multiple repeats.

2.10.2 Detecting Target DNA Hybridisation with SIOS and VPM Analysis

Using equipment as detailed in section 2.8.2.2, the VPM technique and qNano were used to calculate zeta potentials in order to detect target DNA hybridisation. Additionally, the FWHM values (section 2.8.1.2 and section 2.8.1.3) and particle size were investigated for different particle sets in the following statistical analysis.

Previous research have used a number of techniques to compare data arising from particle groups, including Gaussian fitting to histograms, size and duration histograms, and fitting of the duration data with first-order kinetics between the blocked and open state with a sum of two exponentials equation. The latter method is able to provide information regarding the differences between groups, but can be complex to interpret. Gaussian fit on the other hand can be hard to identify changes sensitively, particularly where large distributions are present. The method chosen, however, puts a statistical significance to the differences between groups, as well as providing a numerical measure of the difference. Two parameters were chosen for analysis, particle size (calibrated to control particles and calculated by current pulse magnitude) and FWHM duration. FWHM duration can also be a measure of particle mobility, \( \mu \), Equation 10.

\[
\mu = \frac{qD}{K_BT}
\]

\text{Equation 10}

where \( q \) depicts the charge on the particle, \( D \) represents the diffusion coefficient, \( K_B \) represents Boltzmann’s constant and \( T \) represents temperature. The diffusion coefficient in turn can be explained by Equation 11,

\[
D = \frac{K_BT}{6\pi\eta R}
\]

\text{Equation 11}
where $\eta$ represents viscosity and $R$ represents the radius of the particle. In this way it can be seen that FWHM duration is a response variable, while size is an explanatory variable. Following this, and with the assumptions of 1) independence of the data and 2) that the variation in FWHM duration over size is almost constant, the data can be plotted and transformed to display the data on a log-log scale. The data, for example, may consist of data collected from probe-grafted particles, labelled “Probe” and particles after target incubation labelled “Target”.

It is proposed that the data can be analysed with what is traditionally known as an analysis of covariance (ANCOVA) model to quantify the relationship. However, one can simply regard this as a linear model between (the logarithm) of FWHM duration and (the logarithm) of particle size. The change in signal is modelled for the “Probe” and “Target” groups by changing the intercept, conditional on the group. This model is a sub model of the full interaction model which would also allow the slope of each line to change dependent on the group. The following model was used:

$$\log(\text{FWHM duration}_i) = \beta_0 + \beta_1 \log(\text{Particle size}_i) + \beta_2 \times (\text{Probe or Target}_i) + \epsilon_i$$

Where Error: $\epsilon_i \text{ iid } N(0, \sigma^2)$

Equation 12

The $\text{Probe or Target}_i$ contribution is a dummy variable which takes the value zero when the observation comes from the “Probe” group and one when the observation comes from the “Target” group. This has the net effect that the intercept changes (as desired) dependent on group membership. The method of least squares was used to estimate the values of the model parameters, $\beta_0, \beta_1, \beta_2$, and $\sigma$, where $\beta$ represents the contribution to the signal from the “Target” group. P-values calculated from the summary results of the difference in intercepts from the linear model fit were used to assign statistical significance to a difference in signal, as well as confidence intervals (level of 0.95) calculated. Residual plots were checked to confirm the validity of assumptions. Sufficient data was collected in this study to invoke the central limit theorem. This simply means that although there may be some concerns about the normality of the residuals (the estimated errors), this will not affect our inference about the mean, the mean here being the regression lines.
One might ask why “Probe” and “Target” data were not evaluated individually. The model formulated means not only can the regression coefficients be estimated simultaneously, but also that all of the data is used in estimating the standard deviation of the errors, $\sigma$. This means more data is used in estimating the standard error (the standard deviation around the lines) and will amount to narrower prediction intervals. The statistical program R was used for data analysis.

2.11 Ethics Approval for Investigation of Forensically Important Samples

Ethics approval (reference 2009/523) was sought in order to perform experiments with real samples similar to those encountered in forensic analyses. Ethics approval is important in cases such as these, whereby consideration of sample collection (in case of discomfort, pain, embarrassment, psychological or spiritual harm), sample use, sample storage and information gathering and storage is imperative. Ethics approval was granted conditionally on 02-December-2009, and considerations to resolve ethical issues involved were discussed. As the project progressed it transpired that the only samples able to be examined (due to time and capability constraints) were blood samples, examined through autonomous finger pricks as discussed in the ethics approval.

2.12 Testing of Forensically Important Samples

Electrochemical DNA sensors developed with GCE electrodes coated with P(Py-co-PAA) polymer, and with EIS as a detection technique, were investigated for their use with real forensic samples namely the body fluid blood.

2.12.1 Incubation with the Body Fluid Blood

Initially P(Py-co-PAA) polymer was grown on GCE electrodes, followed by CV cycling, probe immobilisation, and an initial EIS measurement (Sections 2.3.1, 2.7.3 and 2.9.2). Subsequently, the GCE electrode surfaces were incubated with the body fluid blood. This was performed using a 1:10 dilution in PBS solution of real blood to a total volume of 22 $\mu$L. Incubation was performed for 30 min at room temperature, using an incubation chamber as seen in Figure 2.16. Following incubation the electrode was washed thoroughly with PBS solution and another EIS measurement taken. This experiment allows analysis of non-specific binding and should highlight false positive signals produced through blood incubation.
2.12.2 Attempts to Reduce Non-Specific Binding of Blood Components

In order to reduce non-specific binding of blood components to the polymer surface a number of methods were trialled.

2.12.2.1 Trying to remove non-specifically adsorbed species

Assuming that a false positive signal is generated through blood species adhering to the polymer surface (as opposed to chemically altering the polymer itself), attempts to remove some of the non-bound species were performed.

Application of a potential to the electrode after blood incubation

Performed in PBS solution, a potential was applied, initially +0.6 V, for a period of 3 min then 0 V for 3 min. The electrode was then washed and in new PBS solution a potential of -0.8 V was applied for 3 min followed by 0 V for 3 min. Another EIS measurement was subsequently taken.

Washing the electrode with PBS containing surfactant

A non-ionic surfactant, Triton X-100, was used in order to remove lightly bound species from the polymer surface. A thorough wash of the electrode surface with PBS with Triton X added (< 0.1 w/w) was performed. Subsequently an EIS measurement was taken. Soaking of the electrode following this in the PBS/Triton X solution was performed for 30 min followed by another EIS measurement.

2.12.2.2 Preventing unwanted blood species adherence

Use of a dialysis membrane

A membrane of dialysis tubing between the GCE polymer surface and the incubated blood was employed. Following immobilisation of probe DNA, PBS-wet dialysis tubing was used to cover the electrode surface and held in place by an o-ring, Figure 2.17. The dialysis cellulose tubing is reported to retain proteins with molecular weights of 12,000 or greater. An incubation chamber (Figure 2.16) was used to then incubate with blood (1:10 dilution with PBS) for ½ h at room temperature.
Chapter 2

Figure 2.17 Image of electrode with dialysis tubing covering the surface, held in place with an o-ring.

Use of a nanofibrous nylon mesh membrane

A nanofibrous nylon mesh membrane prepared by Scampicchio et al.\textsuperscript{203-205} was cut-to measure and used in place of the dialysis tubing. The porosity for similar nanofibrous membrane (NFM) films was measured as 90-95 %.\textsuperscript{204} Again, following probe immobilisation a thin layer of wetted nylon mesh was placed over the electrode surface and held in place using an o-ring.

2.12.3 Investigating Target DNA and Blood Incubation

Once the best method for reducing non-specific binding had been found (use of a dialysis tube membrane) a more in-depth study was performed. Initially a positive control was performed, followed by investigations into the effect of blood with target DNA incubation.

2.12.3.1 Synthetic Target DNA Incubation with and without a dialysis membrane (Positive Control)

It was of interest whether a dialysis membrane would limit diffusion of target DNA to the probe-grafted DNA surface. Therefore after placing a dialysis tubing membrane over the GCE polymer surface, incubation with target DNA followed (1 × 10\textsuperscript{-4} M). An EIS measurement followed.

2.12.3.2 Incubation of Synthetic Target DNA with and without Blood (False Negatives)

Following probe immobilisation the GCE polymer surface was covered with wetted dialysis tubing. Incubation with whole blood (1:10 dilution with PBS) and target DNA (1 × 10\textsuperscript{-4} M) simultaneously was then performed. Thorough washing with PBS was performed and another EIS measurement. This experiment was repeated with the incubation broken into two steps. Firstly performed was incubation of the probe-immobilised P(Py-co-PAA) dialysis
membrane covered electrode with blood (1:10 dilution with PBS) for ½ h at room temperature. Thorough washing was then performed. Subsequent incubation with synthetic target DNA for 1 h at 42 ºC was followed by washing and an EIS measurement.
Chapter 3

Developing Electrochemical Polypyrrole-Based DNA Sensors

Marsilea Booth
Chapter 3. Developing Electrochemical Polypyrrole-based DNA Sensors

Previous research within our group\(^\text{22,46,79}\) indicated promising results with PPy and functionalised PPy copolymers for DNA sensing. Electrochemical growth together with electrochemical impedance spectroscopy (EIS) as a detection technique, were able to provide the sensing surface and tools to create successful DNA sensors. When developing sensitive and selective sensors, in-depth characterisation can aid in understanding the sensing platform. Although this sensor platform has been previously developed some parameters were improved through characterisation and subsequent optimisation. One of these parameters was stability, an important property for practical and successful sensors. Investigation into sensor stability, as well as sensor response with different target DNA strands was performed\(^\text{206}\). Much research-to-date on DNA sensor development has focused on short target DNA strands as model genes. In the study here, the effect of the length of oligonucleotide probe and target strands on sensor response is investigated, as a significant step towards real world applications for DNA detection\(^\text{207}\). Furthermore, once a stable, reproducible sensor surface was achieved, the effect of the redox couple used in EIS was investigated\(^\text{208}\).

In this way the objective being sought in this section pertained to:

- Develop, characterise and understand a stable, sensitive, selective, and robust DNA sensor platform.

3.1 Synthesis of 3-Pyrrolylacrylic acid and Monomer Choice

As mentioned in section 2.2.1, synthesis of a functionalised pyrrole monomer, 3-pyrrolylacrylic acid (PAA) was performed. This monomer is chosen due to its ability to act as an anchor between probe DNA and the polymer surface. Investigation into a second monomer for copolymerisation used the commercially available pyrrolepropylic acid (PPa), as used in research by Chen et al.\(^\text{99}\). This monomer also has the ability to form an anchor for DNA attachment, however the functionalisation originates from the nitrogen. Comparisons of copolymers of the two functionalised copolymers were performed. P(Py-\textit{co}-PPa) exhibits
lower electrochemical activity than P(Py-co-PAA) (observed through less pronounced oxidation and reduction peaks), and lower charge storage (observed through lower currents) likely to be from the functionalised position and subsequent disruption in planarity and π-electron conjugation. Additionally, the unsaturated carbon side chains present with PAA may contribute to the superior properties and prove more suitable for DNA sensing. For these reasons P(Py-co-PAA) was chosen for further analysis.

Figure 3.1 Chemical structures and CVs for A) P(Py-co-PAA) and B) P(Py-co-PPa) copolymers. As can be seen from the above CVs, P(Py-co-PAA) shows better electrochemical properties.

3.2 Developing Reproducible and Stable Copolymer Films

3.2.1 Growth Method

Two methods were employed for polymer growth: 1) application of a constant fixed potential of 1.0 V until a specified charge had passed through amperometric I-t curve application, 2) a pulse growth technique, whereby a series of two short pulses at 1.0 V are applied separated by a pulse of 0.0 V. Disadvantages of the first of these include that initial charging of the surface can produce inconsistent results. Additionally as the monomer surrounding the electrode gets oxidised and subsequently polymerises there is little time for monomer from
the bulk solution to diffuse to the electrode surface. In this way depleted species as time progresses means inconsistent polymerisation. With pulse growth, the period of 0.0 V application allows time for solution species to diffuse to the electrode surface. This imparts control over polymer growth, echoed in the more reproducible films produced, Figure 3.2. The physical meaning of the lower and less pronounced semi-circle observed for the amperometric I-t curve grown film may be to do with properties such as the thickness of the film. In this case it could be that a thick polymer film is grown so that both ionic (counterion) and electron transfer can occur, giving rise to a double semi-circle EIS spectrum. Consequently, a different equivalent circuit must be used, for example one where a diffusion resistance component is introduced, which has a dependence on angular frequency. For this reason pulse growth was chosen as an appropriate method for polymer growth.

![Figure 3.2 Comparison of the results from growth using A) an amperometric I-t curve, and B) pulse growth. The bare electrode is represented by black squares, while blue circles represent the EIS curve from the copolymer. The EIS curves show that the pulse growth technique gives rise to a system modelled by the Randels’ equivalent circuit.](image)

After the growth technique had been determined, optimisation of the length of the pulses was performed. Pulses of 1.0 V were applied for 25, 50, 100 or 200 ms, of which it was found that 25 and 50 ms pulses produced reproducible, well-defined results indicative of thin films. These were hence employed for further experiments.
Chapter 3

3.2.2 Electrode Size

Research within the group indicated enhanced sensitivity can be achieved with the use of microelectrodes (11 ± 2 µm in diameter) in place of the macroelectrodes (1.0 mm in diameter) previously used. Initial experiments showed differences between the macro- and micro-electrode results (Figure 3.4); however microelectrodes did not seem significantly improve sensitivity. This may be a feature of the microelectrode used, as a clear difference is observed between the electrode used here and the electrode used in the reference in terms of surface area (Figure 1A and 1C of reference). Underlying electrode surface morphology was discussed by Kannan et al. as affecting polymer film morphology and hence affecting sensor response. Due to the improved ease of using macroelectrodes as well as the reproducible results, macroelectrodes were used in place of microelectrodes for further experiments.
3.2.3 Polymer Stability

As discussed in section 1.5, of the ICPs PPy is extensively studied in particular for biosensing applications. High electrical conductivity, retained electroactivity in neutral solutions and biocompatibility all contribute to its popularity. A significant drawback of PPy however, exists in its susceptibility to degradation, limiting the ICPs practical use. Commonly this degradation exists as polymer overoxidation, and can transpire from the application of high potentials, the presence of nucleophiles (such as OH⁻), and dedoping of the polymer. Because stability is such a key property for practical sensors investigations into copolymer stability were performed. The effects of electrolyte solution, post-growth treatment and solution pH on polymer stability were examined.

3.2.3.1 Growth Electrolyte

The counterion incorporated in the polymer has been known to affect polymer stability. Large counterions may be used in order to prevent solution species attacks, as well as decreasing the likelihood of dedoping. For this reason the effect of electrolyte solution on polymer
stability was investigated. Two dopants were used, a small dopant (LiClO₄) and a much larger polymeric dopant, poly(4-styrenesulfonic acid) (PSS). The results support the literature,²¹¹ whereby the use of PSS as a dopant produces a more stable polymer film than LiClO₄ doped polymer. It should be noted that although $\Delta R_{ct}$ appears significant for the polymer films, once the probe single-stranded DNA (ssDNA) has been attached the stability is much improved (Figure 3.5).

![Figure 3.5 Comparison in the stability of four copolymer films and the chemical structure of PSS. The four films are probe immobilised P(Py-co-PAA)/PSS, P(Py-co-PAA)/PSS copolymer film, P(Py-co-PAA)/LiClO₄ film grown from propylene carbonate (PC) solution and P(Py-co-PAA)/LiClO₄ film grown from aqueous solution. Additionally shown is the chemical structure of PSS.]

### 3.2.3.2 Post-growth Treatment and Solution pH

Post-growth treatment of the polymer film may also play a role on polymer stability. As seen in Figure 3.6A, it appears that CV cycling over a limited range $^{100, 125}$ improves the stability of the polymer film. Cycling is thought to aid the ICP film stability by morphological restructuring to form a more compact structure, as well as helping the film reach an equilibrated redox response. $^{163, 212, 213}$ Aqueous solution for growth may be favoured in order to maintain consistency of the solvent type during experiments. $^{212, 214}$ This may explain the instability of films grown in organic solutions (such as PC) which are then transferred for analysis into aqueous solutions. The pH of the incubation solution also affects the stability of the polymer film, with a higher pH solution (PBS pH 7.4) appearing to ensure slightly higher stability (Figure 3.6B). This may be dependent on the polymer, solution and potentials being applied. $^{100}$ For this system stability at pH 7.4 is advantageous as pH 7.4 is physiological pH.
Based on these findings, films with PSS or LiClO$_4$ dopants were used for DNA sensing, with post-growth cycling treatment and with analysis at a pH of 7.4.

### 3.3 Characterising Copolymer Films

#### 3.3.1 SEM Analysis

Characterisation of the copolymer surfaces by SEM images illustrates the different morphologies arising from the dopant and solvent systems used (Figure 3.7 B-D) with no such morphologies observed for bare electrodes (Figure 3.7A). The polymer grown with LiClO$_4$ as a dopant in organic solvent shows a smooth under-layer topped with occasional growths and outcrops of polymer, as supported by literature.$^{215}$ Meanwhile, polymer doped with PSS and grown in aqueous solution shows a rougher but more homogeneous morphology.$^{216}$
Figure 3.7 SEM images of electrodes and copolymer films. A) A bare glassy carbon electrode, B) an electrode with a coating of LiClO$_4$ doped P(Py-co-PAA) copolymer, as viewed side-on at the edge of the electrode, C) an electrode with a coating of PSS doped P(Py-co-PAA) copolymer, and D) a higher magnification image of P(Py-co-PAA)/PSS copolymer.

3.3.2 **FTIR-ATR and Cyclic Voltammetry**

Characterisation of the copolymer P(Py-co-PAA) by ATR-FTIR for both dopants was performed. Spectra of both P(Py-co-PAA)/PSS and P(Py-co-PAA)/LiClO$_4$ display bands suggestive of PPy films. The PSS doped polymer indicates slightly shifted bands, Figure 3.8, supported by literature. The presence of PSS within this film is confirmed through observed characteristic bands.
Figure 3.8 FT-IR spectra of two copolymer films, P(Py-co-PAA)/PSS (grey) and P(Py-co-PAA)/LiClO₄ (black). Neither spectrum displays a significant band at c.a. 1710 cm⁻¹ which one may expect from the C=O stretching mode in PAA. It is believed that monomer PAA is incorporated into the copolymer in small quantities not giving rise to a strong enough carboxylic stretching band in the spectra. Two additional bands are observed for the PSS doped polymer confirming the presence of PSS in the conducting polymer film.

Cyclic voltammograms of the films show two doping and de-doping redox peaks for each film (Figure 3.9). A linear relationship is observed between the peak current and the scan rate for both copolymers (see insets, Figure 3.9) indicating that the redox processes are charge-transfer limited.²¹⁷ The voltammogram of P(Py-co-PAA)/PSS (Figure 3.9) presents more prominent redox waves as compared to the voltammogram of P(Py-co-PAA)/LiClO₄, indicating more readily oxidised and reduced polymer.

Figure 3.9 Cyclic voltammograms of an electrode coated with (A) the copolymer P(Py-co-PAA)/PSS, and (B) P(Py-co-PAA)/LiClO₄ film. Cyclic voltammograms are measured in 0.1 M NaCl PBS solution at a scan rate of 50 mV s⁻¹. Insets show the dependence of peak current on scan rate, indicating relationships typical of thin surface layers.
Chapter 3

3.3.3 **Surface Derivatisation Efficiency**

The presence of chemically-accessible carboxylate species was investigated by reaction with toluidine blue. Assuming binding to surface –COOH groups in a 1:1 molar ratio, the density of the functional carboxylic acid groups was quantified as about \( (10 \pm 3) \times 10^{16} \) cm\(^{-2}\). This is comparable to calculations by Kannan et al.,\(^{48}\) despite the different macroelectrodes used. Care must be taken as non-specific binding of toluidine blue is known to occur, however the experiment suggests a high number of carboxylic acid groups are chemically-accessible for probe attachment.

3.3.4 **Electrical Impedance Spectroscopy**

EIS was used as a detection technique for DNA hybridisation as well as a characterisation technique. An example of typical EIS spectra for a bare electrode and a P(Py-co-PAA) coated electrode can be seen in Figure 3.10. The exact charge transfer resistance values are dependent on the polymer grown, which in turn depends on the electrode surface of the bare GCE, the polymer solution, growth method, charge passed etc. Because of this absolute charge transfer values were not compared, but the relative changes in charge transfer resistance after a process were instead analysed. The decrease in charge transfer resistance observed in Figure 3.10 after polymer growth is thought to be a feature of the increased “effective” area gained by the copolymer growth, Figure 3.7.

As described in section 2.7.3, the semi-circular arc observed in the Nyquist plots are indicative of the charge transfer resistance where the charge transfer is limited across the polymer:solution or polymer:electrode interfaces rather than being limited through the bulk solution. The bare electrode and copolymer surfaces grown here display depressed semi-circles, presumably due to the surface roughness of the electrodes and inhomogeneity of the polymer surface. These inhomogeneities may amount to either microscopic roughness events, or microscopic chemical inhomogeneities.\(^{218}\) The irregular polymer growths (section 3.3.1) and surface features of the GCEs may act to slow the Faradaic processes of ionic exchange at the polymer:electrolyte interface, thereby giving rise to the depressed angle.\(^{184,219}\) In the equivalent circuit model a CPE is used to address this non-uniformity. For ease of viewing, some Nyquist plots presented in this thesis are not shown on equal scales for \( Z_{\text{real}} \) and \( -Z_{\text{im}} \).
Despite this improving the viewing of the EIS curves, all are in fact depressed semi-circular arcs.

![EIS spectra](image)

Figure 3.10 EIS spectra of a) a bare GCE electrode and b) a P(Py-co-PAA) polymer coated electrode. EIS spectra are measured in PBS solution with 5.0 mM Fe(CN)$_6^{3-4^{-}}$.

3.4 Copolymer Investigations

Following characterisation of the synthesized polymers, investigations were made into the polymer properties such as non-specific DNA interactions and sensor response.

3.4.1 Non-specific DNA Interactions

In order to confirm covalent immobilisation of probe DNA onto the polymer surface a negative control experiment was performed in which pristine PPy was treated under identical probe immobilisation conditions, and impedance was measured. PPy was used in order to only investigate polymer interactions and remove the complication of interactions between the functionalised monomer and the DNA. The experiment was also performed with copolymer films and non-functionalised DNA with identical outcomes. Results displayed a considerable increase in $R_{ct}$ for the polymer doped with LiClO$_4$ (Figure 3.11A), suggesting that non-specific adsorption of DNA was occurring on the PPy/LiClO$_4$ surface. This is confirmed by fluorescence imaging of a PPy/LiClO$_4$ film following exposure to fluorescently labelled DNA (Figure 3.11). The same experiment repeated with PSS doped PPy, interestingly, did not show a significant increase in $R_{ct}$, and no fluorescence was observed (Figure 3.12). It is believed that non-specific ON binding is prevented in this case due to the size and nature of PSS dopant, as discussed in the following.
It is suggested that the incorporation of PSS into polymer films, as it is a large polymeric anion, provides an excess of negative charges, while in the case of \( \text{ClO}_4^- \) as a dopant there is no such excess. This is supported by elemental analysis, where sulphur, chlorine and nitrogen were used to calculate mole fractions of PSS, \( \text{LiClO}_4 \) and pyrrole species (both pyrrole and functionalised pyrrole) respectively. Results revealed that PSS doped copolymer contains c.a. one negative charge from a styrene sulphonate monomeric unit per 2.45 pyrrole units, while copolymer doped with \( \text{LiClO}_4 \) contains c.a. one negatively charged \( \text{ClO}_4^- \) anion per 2.72 pyrrole units (Figure 3.13). The results for PSS doped films measured by XPS (Figure 3.12) gave an elemental ratio of 1.9:1 for N:S suggesting on average one styrene sulphonate anion to every 2 pyrrole units (quantification performed using peak areas previously divided by empirically derived atomic sensitivity factors\(^{220}\)). The difference in ratio as measured by elemental analysis and surface sensitive XPS could arise from a higher surface concentration of PSS than in the bulk material.\(^{221}\) XPS data suggests that not all PSS is directly interacting with \( \text{P(Py-co-PAA)} \) charged species (Figure 3.12B). Calculated from XPS spectra, the ratio between N1s neutral species and N1s charged species is 5 suggesting that while there may be considerable styrene sulphonate anions present, not all are participating in electrostatic interactions (doping) with the copolymer. Some of the PSS may simply be entangled with the \( \text{P(Py-co-PAA)} \). That presents an excess of negative charge for PSS doped copolymer. A higher negative charge on a surface is expected to repel negatively charged ONs, as has been shown with other biological entities such as antibodies.\(^{222, 223}\) It is important to prevent non-specific binding in order to maintain practicality for selectivity and sensitivity in DNA sensors.

### Table 3.1 Results from elemental analysis of \( \text{P(Py-co-PAA)} \) copolymer films

<table>
<thead>
<tr>
<th>Dopant</th>
<th>Element, %</th>
<th>N:dopant ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>H</td>
</tr>
<tr>
<td>PSS</td>
<td>18.0</td>
<td>18.7</td>
</tr>
<tr>
<td>( \text{LiClO}_4 )</td>
<td>14.4</td>
<td>13.8</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\)Oxygen content has been calculated by the remaining mass balance with the assumption that the content of the sample is primarily composed of C, H, O, N and S or Cl. The N:dopant ratio has been calculated assuming that one nitrogen is indicative of one pyrrole monomer and that one sulphur or chlorine is indicative of one styrene sulphonate or \( \text{ClO}_4^- \) anion respectively. The ratio is then calculated on an atomic percent basis.
Figure 3.11 EIS spectra and fluorescence images of the non-specific DNA interactions for LiClO₄ doped films. A) Electrical impedance spectra i) directly after electropolymerisation of a LiClO₄ doped polypyrrole film (black circles), ii) after incubation with 80 µM probe ON immobilisation solution (grey triangles). An increase in $R_\text{ct}$ suggests non-specific DNA binding. The symbols represent the experimental data while the solid lines indicate the fitted data. B) Fluorescence microscope image (400x objective) of the pristine polypyrrole film and C) Fluorescence microscope image (400x objective) after incubation with fluorescein-labelled DNA (260 µM).

Figure 3.12 EIS spectra and fluorescence images of the non-specific DNA interactions for PSS doped films. A) Electrical impedance spectra i) directly after electropolymerisation of a PSS doped polypyrrole film (black circles), ii) after incubation with 80 µM probe ON immobilisation solution (grey triangles). The symbols represent the experimental data while the solid lines indicate the fitted data. B) Fluorescence microscope image (400x objective) of the pristine polypyrrole film and C) Fluorescence microscope image (400x objective) after incubation with fluorescein-labelled DNA (260 µM). Non-specific binding of fluorescein labelled DNA to the polymer surface was not observed.
Figure 3.13 A) Survey XPS spectrum of a P(Py-co-PAA)/PSS copolymer film. Copolymer film was grown on indium tin oxide (ITO) coated glass. B) High-resolution deconvolution analysis of the N 1s peak. Quantification for the calculation of a N:S ratio was performed using peak areas previously divided by empirically derived atomic sensitivity factors. The high-resolution deconvolution analysis of the N 1s peak of a P(Py-co-PAA)/PSS copolymer film displays a peak at 401 eV arising from C-N^+ type species, while the peak at 402 eV arises from C=N^+ species. These two peaks indicate positively charged species in the polymer and hence the polymer and dopant interaction level.

3.5 DNA Sensing using Electrochemical Polypyrrole-based Sensors

As discussed in section 3.2, films with PSS or LiClO_4 as dopants were chosen for further analysis for DNA sensing. Shown in Figure 3.14 is a typical set of EIS curves with the response of a fabricated sensor to different target DNA concentrations. EIS spectra were fitted using a modified Randels’ equivalent circuit (inset of Figure 3.14, and discussed in section 2.7.3). An increase in $R_{ct}$ after target incubation results from an inhibition of interfacial electron transfer due to the increased negative charge arising from the double-stranded DNA complex (Scheme 3.1). This can be normalised against the $R_{ct}$ value for the film prior to target incubation ($R_{ct}^0$), giving $\Delta R_{ct}/R_{ct}^0$. Selectivity against non-complementary DNA was tested by incubation of the sensor film with non-complementary DNA (Figure 3.15). No significant $\Delta R_{ct}/R_{ct}$ was observed ($\Delta R_{ct}/R_{ct}^0 = 0.07$ as compared to $\Delta R_{ct}/R_{ct}^0 = 0.32$ with complementary DNA) indicating this sensor platform is highly selective. Selectivity can be defined as the extent to which particular analyte(s) can be determined in a complex mixture without interference. Not to be confused with specificity, which as an absolute value can be thought of as the ultimate selectivity and hence is rarely achieved. Selectivity was further investigated through incubation with multiple DNA sequences simultaneously (non-complementary target and complementary target of equal concentrations). The signal
observed was equal to the signal achieved when only complementary target DNA was present. Shown in Figure 3.14B is a fluorescence image of a copolymer P(Py-co-PAA)/PSS film after probe immobilisation and subsequent incubation with 10 µM fluorescently-labelled complementary target DNA. The observation of the fluorescence illustrates the validity of the sensor platform.

Figure 3.14 EIS and fluorescence results for probe immobilised P(Py-co-PAA) after target DNA incubation. A) Electrical impedance spectra, presented as Nyquist plots (-Z_{im} vs. Z_{re}) for an electrode coated with P(Py-co-PAA)/PSS as measured in PBS solution with 5.0 mM Fe(CN)_{6}^{3-/4-}: (a) prior to immobilisation of probe ONs, (b) after exposure to probe ONs, (c) after incubation with 60 µM, (d) after incubation with 100 µM, and (e) after incubation with 1 mM complementary target ON. Inset: equivalent circuit model used for modelling the data. Experimental data are shown as symbols while the fitting data are shown as lines. B) Fluorescence image (400× objective) of a P(Py-co-PAA)/PSS copolymer film with immobilised probe DNA and subsequent incubation with fluorescently-labelled target DNA.

Scheme 3.1 Scheme showing the redox couple with A) a probe immobilised surface, and B) following target hybridisation.
A) When probe DNA are immobilised on the copolymer surface, a number of redox ions are able to approach the surface and become reduced/oxidised causing electrons to flow. After target hybridisation however, the increased negative charges from the extra DNA reduce the number of redox ions that can approach the surface, thereby causing fewer electrons to flow.
Figure 3.15 Electrical impedance spectra after a) immobilisation of ON probe from 80 µM solution (black triangles) and b) after incubation with non-complementary target solution (100 µM). The symbols represent the experimental data while the solid lines indicate the fitted data.

### 3.6 Effects of Dopant on Sensor Performance

In order to investigate the sensing ability of films with different dopants a range of complementary target ON concentrations ($2.5 \times 10^{-8}$ to $2.0 \times 10^{-3}$ M) were investigated for each film (Figure 3.16). Copolymer with PSS as a dopant delivers a higher response than LiClO$_4^-$ doped copolymer. The sensitivity of the sensors was calculated using the slope arising from the linear portion of the semi-logarithmic plot of concentration vs. $\Delta R_{ct}/R_{ct}^{0}$. The sensitivity of the PSS doped polymer was found to be $1.38 \pm 0.04$ units/log[mol L$^{-1}$], while the LiClO$_4$ doped polymer was found to have a sensitivity of $0.39 \pm 0.03$ units/log[mol L$^{-1}$] (over the range $\sim 5 \times 10^{-5}$ to $1 \times 10^{-3}$ mol L$^{-1}$). Both can be seen to readily detect target in the µM range. Sensitivity can be defined as the signal output per unit concentration of a substance and the slope of the calibration curve. This should not be confused with the limit of detection (LOD) or quantification (LOQ) which both take into account the blank and signal fluctuation (noise). The LOD is the smallest amount of sample producing a signal that can be distinguished from the background noise. Generally a signal-to-noise ratio of 2:1 can be used to define the detection limit. For the developed sensors the LOD is not reported as sensitivity values are not exceptionally high. Despite possessing only satisfactory sensitivity, the sensors display interesting results.

It is suggested that the PSS doped copolymer possesses a rougher surface morphology and contains more surface negative charges than the LiClO$_4$ doped copolymer. Morphology plays...
a major role in increased sensitivity, as rougher surfaces may possess greater surface area and pore volume (Figure 3.7). Also, as discussed by Gębala and Schuhmann \(^{44}\) charged polyelectrolytes accumulate a fraction of counterions in order to compensate for associated negative charges. A higher number of counterions affect the potential decay from the electrode surface (helping to disperse the potential across the polymer layer), and hence influence ONs tethered to the electrode surface through interactions with the ionic double layer and electric field. These interactions may affect probe ON orientation and behaviour. \(^{44}\) This, coupled with coloumbic repulsion, affects probe accessibility and hybridisation efficiency and hence the sensing ability of the polymer films. Subsequent DNA experiments were performed on the P(Py-co-PAA)/PSS copolymer for its higher response.

![Graph](image)

Figure 3.16 Calibration curves corresponding to the changes in charge transfer resistance for P(Py-co-PAA)/PSS based sensors (black squares) and P(Py-co-PAA)/LiClO\(_4\) sensors (grey circles) after incubation with complementary ON target of different concentrations (2.5 \(\times\) 10\(^{-8}\) to 2.0 \(\times\) 10\(^{-3}\) molL\(^{-1}\)). Error bars are standard deviations. Solid lines display the fit to theory with apparent association constant \(K = 2.0 \times 10^4\) M\(^{-1}\). \(^{48}\)

### 3.7 Effects of Probe and Target Length on Sensor Performance

#### 3.7.1 Effect of Target Length

A series of three complementary target ON lengths (23’mer (T1), 50’mer (T2) and 113’mer (T3)) were investigated with P(Py-co-PAA) copolymer sensors (Figure 3.17). In all cases the same 23’mer probe sequence was used. Sensitivity was calculated for each target length.
The results showed (over the range $\sim 5 \times 10^{-5}$ to $2 \times 10^{-4}$ mol L\(^{-1}\)) that the highest sensitivity was achieved towards 113'mer targets (5.86 units/log[mol L\(^{-1}\)]), while the sensitivity towards 50'mer targets was $2.19 \pm 0.02$ units/log[mol L\(^{-1}\)] and was $1.38 \pm 0.04$ units/log[mol L\(^{-1}\)] towards 23'mer targets. To understand the higher sensitivity achieved with longer targets, one must look at the net charge possessed by the different lengths of ONs. Longer ON strands possess more negative charges and attracted cations from solution.\(^{44,65}\) It has been proposed that the Donnan exclusion principle may be used to explain sensitivity in an ICP DNA sensor platform such as ours.\(^{48}\) This principle may also be extended to explain the increased signal observed for longer DNA targets. The maximum attainable signal for such a system is affected by the pore size of the polymer, surface immobilisation density as well as the effective charge concentration within the pores, relative to the background electrolyte concentration outside the pores. The observed electrochemical rate constant, when fast electron transfers are concerned, is dependent on the concentration of the redox species present at the measured interface.\(^{227}\) The increase in charge per surface area in our system causes a decrease in concentration of the redox species at the polymer:solution interface in turn affecting the charge transfer resistance. This can be explained using following equation.\(^{48}\)

\[
\frac{\Delta R_{ct}}{R_{ct}} \approx \left( \frac{[Z^-]_2}{[Na^+]_o} + \sqrt{\left( \frac{[Z^-]_2}{[Na^+]_o} \right)^2 + 4 \left( \frac{\gamma_{\pm,o}}{\gamma_{\pm,i}} \right)^2} \right)^{3.5} - 1
\]

Equation 3.1

where $[Z^-]$\(_1\) denotes the fixed charge concentration within the pores when surface probe ONs are attached, and $[Z^-]$\(_2\) denotes the fixed charge concentration after hybridisation. The concentration of Na\(^+\) in the bulk solution is represented by $[Na^+]_o$ and $\gamma_{\pm,o,i}$ denotes the mean ionic activity coefficient for pore (i) and bulk (o) solution. The relative change in $R_{ct}$ is greatly affected by the difference in charge between non-hybridised and hybridised states, i.e. the difference between $[Z^-]_1$ and $[Z^-]_2$. The experiments described here are based on the same polymer and non-hybridised ON system, but differ in the amount of effective charge held within each pore after hybridisation. This explains the increased signal observed when larger
DNA target strands are hybridised, arising from a much larger fixed charge concentration within each pore.

Figure 3.17 Sensor response curves corresponding to the changes in charge transfer resistance for P(Py-co-PAA)/PSS film based sensors after incubation with complementary ON target of different lengths. Shown are 23’mer (black squares), 50’mer (dark grey circles), 113’mer (grey triangles) targets for a range of concentrations between $2.5 \times 10^{-9}$ to $2.0 \times 10^{-3}$ M. Error bars are standard deviations. Solid lines display the fit to theory with apparent association constants $K = 2.0 \times 10^4$ M$^{-1}$ (T1), $3.5 \times 10^5$ M$^{-1}$ (T2) and $4.0 \times 10^5$ M$^{-1}$ (T3).

3.7.2 Effect of Probe Length

In order to increase sensitivity and perhaps selectivity, a longer probe ON was explored. It should be mentioned that Lubin et al. (2009), in working with longer probes, found that an increase in signal was coupled with a decrease in selectivity. This aspect is not investigated here. Herein it is investigated whether having a longer probe DNA strand affects the magnitude of response of the sensor. It is expected that the number of interacting base pairs to influence the resulting signal, in that more interacting base pairs should result in a higher signal. As predicted by the model suggested in section 3.7.1, a larger signal may also result from an increase in effective fixed charge within the pores arising from using a larger probe (e.g. when probe length is longer than target length). Both phenomena were indeed observed in experiments where a 50’mer probe (P2) was investigated and the results compared to the response with a 23’mer probe (P1). By testing both P1 and P2 with the same targets a comparison may be performed as the targets should exhibit similar behaviour (e.g. ability for secondary folding of the ON or differing diffusion rates). The highest sensitivity was achieved with P2 across all three concentrations tested ($2.5 \times 10^{-9}$, $2.5 \times 10^{-8}$ and $5.0 \times 10^{-6}$ M). The results for T1 with both probe lengths are shown in Figure 3.18. In this case, the
increase in sensor response when a longer probe DNA length was used is likely to arise from
the increase in negative charges subsequently present at the polymer-probe/solution interface,
as discussed previously.

Figure 3.18 Changes in charge transfer resistance ($\Delta R_{ct}/R_{ct}^0$) for P(Py-co-PAA)/PSS based sensors with P1 (23'mer) probe attached and subsequent target (T1) incubation (black squares), and P(Py-co-PAA)/PSS based sensors with P2 (50'mer) probe attached and subsequent target (T1) incubation (grey circles). Target concentration was set at $2.5 \times 10^{-9}$ M, $2.5 \times 10^{-8}$ or $5.0 \times 10^{-6}$ M. Error bars are standard deviations.

3.7.3 Further Information about Fitting the Experimental Data

Experimental data collected from EIS experiments examining the effect of dopant and target
length on sensor response (Figure 3.16 and Figure 3.17) have been fitted using the developed
patch model described in the reference by Kannan et al.. The experimental data were
fitted to theory with different association equilibrium constants ($K$), for example
$K = 3.5 \times 10^5$ M$^{-1}$ for a probe coated P(Py-co-PAA)/PSS copolymer film with target, T2. This
is comparable to association constants observed by Wong, Gooding et al., for example a
P1/MCH sensor (mercaptopropyl/ mercaptohexanol and 20'mer DNA coated surface) gave
rise to $K = 3.16 \times 10^5$ M$^{-1}$, with their self-assembled monolayer QCM/electrochemical
sensors. Association equilibrium constants were estimated by determining the best fit using
experimental data and Equation 3.2 and Equation 3.3.

$$k_{el} = \frac{RT}{n^2F^2AR_{ct}} \times \frac{1}{C_O^{* (1-\alpha)}C_R^{* \alpha}}$$

Equation 3.2
where the transfer coefficient $\alpha$ is assumed to be 0.5. $C_o^{(1-\alpha)} = \alpha C_\text{R} = C_0$, $C_0$ being the bulk concentration of hexacyanoferrate (II) ion and $A$ is the area of the electrode.\textsuperscript{22,82} The electrochemical rate constant, $k_{el}$ meanwhile, can be calculated using

$$
\frac{k_{el,\text{obsd},t\rightarrow\infty}}{k_{ss}} = k_{ss} - (k_{ss} - k_{ds})(Kc/(1 + Kc))
$$

Equation 3.3

where $K$ is the equilibrium constant for the association of ON at the electrode surface, $c$ is the concentration of the target DNA in the solution, $k_{ss}$ is the electrochemical rate constant for ferri/ferrocyanide reactions at the single strand (probe) coated polymer surface and $k_{ds}$ is the equivalent rate constant in a double strand (probe and target) coated surface. The dependence of $\Delta R_{ct}/R_{ct}^0$ on $c$ can be obtained as $R_{ct} \sim 1/k_{el,\text{obsd}}$.

It should be noted that at higher concentrations the data is well modelled; however at lower concentrations a deviation of fit is observed, Figure 3.19A.

Figure 3.19 Response curves showing different fittings for the EIS data collected using P(Py-co-PAA)/PSS films for different target DNA concentrations.
A) Data is fitted with a low equilibrium constant $2.0 \times 10^4 \text{ M}^{-1}$, and B) Data is fitted with a higher equilibrium constant $= 1 \times 10^7 \text{ M}^{-1}$.

High K values indicate strong binding between probe ON immobilised on the polymer surface and complementary target DNA in solution. The lower concentrations appear to have a somewhat linear increase in $\Delta R_{ct}/R_{ct}^0$. This could be because:
1) The increase in $\Delta R_{ct}/R_{ct}^0$ is mainly due to a change in the polymer film from incubation (as observed in the stability experiments indicating a small increase may be expected (Figure 3.5)).

2) The model described in Kannan et al. is insufficient to explain the behaviour of the sensor at lower concentrations.

Assuming reason 2 is the major contributor to the incomplete fitting, a satisfactory explanation for the insufficiency is that the equilibrium constant may be changing for different concentrations of target DNA. For example at a low concentration there is a higher association between the probe and target DNA, but once the concentration of target DNA increases, the equilibrium constant decreases. For further explanation, $K = k_{on}/k_{off}$, where $k_{on}$ and $k_{off}$ denote the rate constants of the complementary ON with the surface-bound ON to form surface-bound dsDNA, and for dissociation of the surface-bound dsDNA, respectively. Therefore, the decrease in $K$ could arise from a decrease in $k_{on}$ or an increase in $k_{off}$ or a combination of the two. As most variables such as the temperature or mixing conditions are not being changed during the incubations, $k_{off}$ should remain fairly constant.

The patch model used for fitting assumes that $k_{on}$ also remains constant over changing target concentrations. This would be true if the transport of target DNA to the surface is fast compared to the time taken for binding, and therefore has no influence on the binding kinetics. This is not the case for our system however. This can be explained by the fact that at higher target DNA concentrations more surface is covered by DNA, hindering target DNA in solution from approaching the polymer surface and lowering $K$ at higher concentrations. In this way, different values of $K$ are at play at different target concentrations which is not taken into account with the patch model used. Despite the deviation at low concentrations, the model successfully fits experimental data at higher concentrations and hence imparts valuable information about the system.

### 3.8 Effects of Redox Couple on Sensor Performance

The prepared P(Py-co-PAA) copolymer film based sensor has shown the ability to selectively detect target DNA sequences. Many variables have been examined for their effect on the sensing system, including dopant and post-growth treatment etc. Subsequently, questions as to what effects a different redox couple may have on the sensing platform were investigated. Many DNA sensors, including the one described earlier (section 3.5), employ a
ferri/ferrocyanide redox reporter to enhance and report surface hybridisation.\textsuperscript{35} The ferri/ferrocyanide redox couple shows rapid redox processes, high sensitivity, well-behaved electrochemistry and good indications of hybridisation in DNA sensing.\textsuperscript{35, 44, 46, 47, 56, 231} Though widely used, the ferri/ferrocyanide redox couple possesses characteristics which may be disadvantageous to sensing platforms. Although not investigated here, a cationic redox reporter such as a hexamineruthenium complex is of interest, aided with PNA as a probe for DNA sensing.\textsuperscript{232} Another redox reporter suitable for use with EIS is hydroquinone (H$_2$Q) and benzoquinone (BZQ).

Hydroquinone holds advantages over the typically used ferri/ferrocyanide redox couple including:

1) the neutral charge, making it a useful redox couple where charged species are present, e.g. whereas a negatively charged redox ion may be expelled from approaching a negatively charged electrode coating, the neutral H$_2$Q holds no such limitation.\textsuperscript{80} Research suggests the ability of H$_2$Q for partial diffusion into bulk PPy.\textsuperscript{233}

2) interactions may occur between solution species, such as those present in blood substitute, and the ferri/ferrocyanide couple which do not exist for the H$_2$Q/BZQ redox reporter.\textsuperscript{231}

3) the comparative cost of H$_2$Q,\textsuperscript{234}

4) the different “signal on” approach possible with such a redox system,\textsuperscript{76}

5) a larger range of DNA concentrations may be investigated as compared with ferri/ferrocyanide, as electrostatic repulsion does not come into play with H$_2$Q, merely steric hindrance,

6) PPy has been shown to favourably interact with H$_2$Q with a good catalytic effect.\textsuperscript{233}

7) Finally using a neutral redox reporter allows further understanding of the polymer/probe DNA surface, permitting investigation without the complication of electrostatic repulsion between the redox probe and the surface DNA.
Conversely, the main disadvantage of using H$_2$Q as a redox reporter exists with both redox couples in their inherent toxicity. Of the pair H$_2$Q possesses higher toxicity, though a smaller quantity of H$_2$Q is generally employed.$^{46, 235, 236}$

To this end, a systematic comparative study was performed on the effect of redox couple on developed electrochemical DNA sensors. To our knowledge few studies directly compare the redox couples H$_2$Q and ferri/ferrocyanide, with none describing the effect on a sensing system such as ours. Therefore such a study is interesting and can deepen the understanding of the sensor system.

3.8.1 **CV and EIS in H$_2$Q and Ferri/Ferrocyanide Redox Solutions**

CV and EIS measurements of the polymer coated electrodes allow analysis of the system electrochemically. Cyclic voltammograms in solution indicate a large oxidation peak of H$_2$Q at c.a. 0.225 V, and a reduction peak at -0.030 V, Figure 3.20. A decrease in peak separation (from 0.287 V to 0.259 V) and improved redox capability is observed after polymer growth, Figure 3.20A. This effect is also observed with EIS (section 3.3.4 for the K$_3$[FeCN$_6$]/K$_4$[FeCN$_6$] redox couple), suggested to arise from the increased surface area provided by the copolymer (Figure 3.7) as well as the acceleration of the electrochemical process kinetics.$^{237}$ EIS with H$_2$Q also shows a decreased charge transfer resistance (Figure 3.21) after polymer growth, as well as a similarly depressed semi-circle as seen with the ferri/ferrocyanide redox couple supporting this being an artefact of the polymer/electrode surface properties.

In terms of the bare electrode, characteristic CVs are displayed in Figure 3.20B in H$_2$Q solution and in K$_3$[FeCN$_6$]/K$_4$[FeCN$_6$] solution. Major differences can be observed in Table 3.2 from the summary of results from CV and EIS spectra fitting. A smaller peak separation of oxidation and reduction peaks is observed for K$_3$[FeCN$_6$]/K$_4$[FeCN$_6$] solution than H$_2$Q solution.$^{236, 238}$ This indicates the higher electrochemical reversibility of this redox reporter. The charge transfer resistance also shows a marked difference, as a combination of the concentration used and the inherent properties of the redox reporter.$^{238}$
Table 3.2 Table comparison of the ferri/ferrocyanide redox couple with the hydrquinone/benzoquinone redox reporter for results with a bare GCE.

<table>
<thead>
<tr>
<th></th>
<th>Ferricyanide/Ferrocyanide</th>
<th>Hydroquinone/benzoquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mmolL⁻¹)</td>
<td>10 total</td>
<td>1</td>
</tr>
<tr>
<td>ΔEp (V)</td>
<td>0.092</td>
<td>0.287</td>
</tr>
<tr>
<td>Solution resistance, Rₛ (Ohm)</td>
<td>327</td>
<td>440</td>
</tr>
<tr>
<td>Rₑₜ (kOhm)</td>
<td>1.34</td>
<td>27.2</td>
</tr>
</tbody>
</table>

Figure 3.20 Cyclic voltammograms of bare and copolymer coated electrodes. A) Cyclic voltammograms of a bare GCE (grey line) and the same electrode after growth of copolymer P(Py-co-PAA) (black dashed line) in 1 mM H₂Q in PBS. B) Cyclic voltammograms of a bare GCE in 1 mM H₂Q in PBS (grey line) and in 5mM 1:1 K₃[FeCN₆]/K₄[FeCN₆] in PBS (black dashed line). All voltammograms were taken at scan rate of 100 mVs⁻¹.

3.8.2 DNA Sensing with H₂Q

3.8.2.1 Electrical Impedance Spectroscopy and DNA Sensing in H₂Q

Following polymer growth and probe DNA immobilisation, synthesized sensors were exposed to a range of target DNA concentrations. A typical set of impedance spectra are displayed as Nyquist plots, Figure 3.21. EIS results can again be modelled by a modified Randels’ equivalent circuit (section 2.7.3)²⁸,³⁵,⁴⁶, inset Figure 3.21. Again the change in Rₑₜ can be represented by ΔRₑₜ, normalised against the value of Rₑₜ prior to target incubation (Rₑₜ⁰), giving ΔRₑₜ/Rₑₜ⁰.  

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Figure 3.21 Electrical impedance spectra presented as Nyquist plots as measured in PBS solution with 1 mM H$_2$Q:
(a) bare GCE, (b) P(Py-co-PAA) coated electrode prior to immobilisation of probe ONs, (c) after exposure to probe ONs, (d) after incubation with 1 µM complementary target ON. Inset: equivalent circuit model used for modelling the data. Experimental data are shown as symbols while the fitting data are shown as lines.

3.8.2.2 Effect of Probe Density on DNA Sensing with H$_2$Q

Probe density has been shown to be an important parameter for sensor design.$^{19}$ To this end, a range of probe densities were investigated for their effect on DNA sensing with H$_2$Q as the redox probe. The ratios are achieved by altering the amount of PAA monomer as compared to pyrrole monomer during copolymerisation. The ratios examined were 10:1 and 50:1, pyrrole:PAA. Details can be found in section 2.3.2. Altering the ratios produced films with a large immobilised probe DNA density and films with a sparser immobilisation of DNA. It should be noted that immobilisation efficiency with EDC chemistry is not commonly 100 %.\textsuperscript{194} While absolute probe densities were not measured here, the number of DNA binding groups (PAA carboxylate groups in our case) is assumed proportional to the amount of probe attached, as supported by other research.$^{46,99,239}$ As can be seen in Figure 3.22, there is different behaviour to target DNA with different probe densities. This can be schematically shown (Scheme 3.2) and explained as follows.

In the case where there are many probe DNAs, as with a 10:1 ratio, increasing the target DNA concentration causes a decrease in the charge transfer resistance. A decrease in $R_{ct}$ is an indication of an enhanced electron transfer between the solution and electrode surface. A proposed explanation for the negative correlation observed when the H$_2$Q redox reporter is used is given in Scheme 3.2A. Previous research by Reisberg et al. found that DNA
Conformational changes were determinant in transduction processes for their sensing system. Results demonstrated that the electrostatic charges present on DNA play only a minor role in the transduction process, with the major effect arising from the steric influence of DNA strands. In the case of H$_2$Q as a redox reporter this is supported, whereby the neutral redox reporter’s approach to the electrode surface is sterically hindered. Additionally, when a positive potential is applied to the electrode surface (as is the case during EIS measurements), where possible, flexible ssDNA physically blocks the surface as it is undergoes electrostatic attraction. Because of the surface probe density however, the single-stranded DNA (ssDNA) strands are forced to bear a semi-upright conformation as they accommodate their conformation to neighbouring probe DNA strands. The decreased gyration and other DNA motions are limited in this case, making the DNA coverage less vulnerable to electric forces. Upon hybridisation the DNA undergoes an increase in rigidity from double-stranded DNA (dsDNA) formation. A rearrangement of surface organisation occurs, explaining the decrease in charge transfer resistance. As the DNA strands hybridise the dsDNA no longer blocks as much of the polymer surface, allowing ions to pass through with less impedance to the electrode surface. In this case the redox couple and the DNA are interacting spatially.

Figure 3.22 Results of EIS for altering immobilised probe ratio from 50:1 to 10:1 Py:PAA monomer. A) Changes in charge transfer resistance ($\Delta R_c/R_c^0$) for P(Py-co-PAA)/PSS sensors, as measured in PBS solution with 1 mM H$_2$Q, where the ratio used to grow the copolymer film was 50:1 pyrrole:PAA (black circles) and 10:1 (grey squares). Error bars are standard deviations. B) Changes in sensor output as a function of increasing surface probe ssDNA coverage, controlled through PAA incorporation percentage, for target concentration 100 µM.
Scheme 3.2 Scheme describing the polymer surface interactions with H₂Q ions in 1 mM H₂Q solution before and after target DNA exposure for different probe immobilisation densities. A) a copolymer film where a ratio of pyrrole:PAA of 10:1 was used, and B) a copolymer film where a ratio of 50:1 (pyrrole:PAA) was used.

When a lower density of probe ssDNA is present on the surface, as is the case with a ratio of 50:1 a different trend is observed, whereby increasing the target concentration causes an increase in charge transfer resistance, Figure 3.22A. The redox probes in solution can thus be assumed to experience a stronger blocking from the electrode surface after hybridisation than before it, thereby increasing impedance, Scheme 3.2B. When few probe ssDNAs are present it can be assumed that any two strands upon the surface are unlikely to sterically interact. Low probe ssDNA coverage hence renders the probes more susceptible to the impact of the electric field. This electrostatic attraction of the DNA to the potential applied to the electrode thereby causes a blocking of the polymer surface, see Scheme 3.2B. With a combination of the high ionic gradient between the electrode surface and the solution, causing the high field strengths felt by the DNA, as well as the enhanced electric field within the ionic double layer, tilting of the dsDNA below a critical angle can occur, labelled the
This subsequently triggers electrostatic interactions to prevail over entropic effects (influenced by thermal motions) and the preferential DNA strand conformation becomes one where the dsDNAs and ssDNAs lie close to the electrode surface. As discussed above, the main factor influencing the charge transfer resistance is steric interactions. In this case the dsDNA, effectively blocks the polymer surface more than ssDNA, perhaps due to its rigid structure with persistence length ($P$) of $\approx 50$ nm, while ssDNA maintains $P$ of 1-2 nm in a compact conformation.

The relationship between amount of PAA incorporated in the copolymer (assumed to be a measure of probe ssDNA density) and the resulting signal output can be observed in Figure 3.22B with complementary target (100 µM). This shows an increase in charge transfer resistance observed after hybridisation when low probe density is used. Meanwhile, steadily decreasing charge transfer resistances are observed after hybridisation when higher probe densities are present (results from a mid-way probe density of 25:1 pyrrole:PAA is also shown). The interesting results observed here indicate the importance of surface probe ssDNA density.

### 3.8.2.3 Comparing DNA Sensor Sensitivity with $K_3[\text{FeCN}_6]/K_4[\text{FeCN}_6]$ and $H_2Q$

Sensors were examined with both redox couples and the results compared, Figure 3.23. The ferri/ferrocyanide redox couple indicates good sensitivity with a positive sigmoidal correlation between concentration and response. In contrast there is a less sensitive correlation for sensor response with $H_2Q$. It should be noted that conditions for these experiments were not identical – the applied potential differs (0.12 V for $H_2Q$ and 0.23 V for $\text{FeCN}_6^{3-/4-}$). The applied potential is selected based on redox probe behaviour, and is targeted in order to maximise redox reactions. However, despite this difference a comparison between the two is still feasible. Though the potential applied to the polymer differs, both potentials used ensure the polymer is in an oxidised state. The different sensitivities observed can therefore be explained by considering the species present at the polymer surface and interactions with the redox probe.

As previously discussed, in the case of $H_2Q$ as a redox probe with a copolymer surface with low probe ssDNA coverage, steric hindrance causes an increase in charge transfer resistance as more target DNA covers the copolymer surface. Only a small increase in charge transfer resistance is observed over the concentrations examined herein. On the contrary, the sensor
response observed for ferri/ferrocyanide is much larger. It is believed this is a result of the response being significantly influenced by the electrostatic negative charges the ferri/ferrocyanide couple possess. In this way there are not only steric hindrances to account for, but also electrostatic considerations. In this case although the steric hindrance may only slightly increase once the DNA is in the form of dsDNA, the increase in negative charges present from the additional DNA strand will electrostatically repel the negatively charged redox ions $^{35,46}$ giving rise to a much larger sensor response. Therefore higher concentrations of target DNA give rise to large increases in charge transfer resistance signals.

![Graph showing changes in charge transfer resistance](image)

Figure 3.23 Changes in charge transfer resistance ($\Delta R_{ct}/R_{ct}^0$) for $\text{P(Py-co-PAA)/PSS}$ sensors as measured in PBS solution with 5 mM $\text{Fe(CN)}_6^{3-/4-}$ (black circles), and with 1 mM $\text{H}_2\text{Q}$ (grey squares). Error bars are standard deviations.

### 3.8.2.4 Effect of Polymer Dopant on Sensor Response with the H$_2$Q Redox Couple

The way in which the H$_2$Q ions interact with the polymer surface is of interest for the understanding of the sensing system. For this reason a different polymer surface was generated through use of an alternative polymer dopant (LiClO$_4$), as previously compared for the ferri/ferrocyanide redox couple (section 3.6). Results are displayed in Figure 3.24. As described in sections 3.3.1 and 3.6, a change in dopant affects the generated polymer surface morphology (and therefore surface area) as well as surface properties. PSS as a dopant appears to provide a higher density of negative charges as compared to LiClO$_4$ as a dopant. This, coupled with the greater surface morphology achieved gives rise to higher sensitivity when the ferri/ferrocyanide is used as a redox couple.

Based on these findings, a similar experiment was performed with H$_2$Q as a redox reporter. As with ferri/ferrocyanide, a higher sensor response is achieved with PSS as the dopant than...
with LiClO$_4$. It is believed the major influencing factors on sensor response after dopant change to be: 1) the interaction between the probe ssDNA and the electrode surface (probe DNA orientation),$^{46}$ 2) the surface morphology of the polymer, and 3) the amount of probe DNA attached to the polymer surface.

![Graph showing changes in charge transfer resistance ($\Delta R_{ct}/R_{ct}^0$) for sensors doped with PSS (diagonally striped bars) and LiClO$_4$ (grey bars) after incubation with complementary ON target of $1\times10^{-4}$ M concentration.]

3.9 Summary of the Effects of the Chosen Redox Couple

The comparative study performed is aimed at shedding light on the effect of the redox reporter used on sensor response. The two redox couples of ferri/ferrocyanide and hydroquinone/benzoquinone being examined each possess pros and cons, very much dependent on the sensing platform being used. Results showed the previously used ferri/ferrocyanide redox couple gave higher sensitivity for this sensor platform. Interesting trends were observed and explored with the effect of probe density on sensor response. In explaining these results, a number of different effects must be considered (steric and electrostatic) for their influence on the sensor surface and hence response. This deepens the understanding of the sensor surface being used.

In summary, the redox couple of ferri/ferrocyanide is the more suitable reporter of hybridisation events with the DNA sensing system presented.
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3.10 Conclusions

The development of electrochemical PPy-based DNA sensors to detect target DNA sequences has been performed with the use of P(Py-co-PAA) films. These films have subsequently been characterised using CV, FTIR-ATR, XPS and EIS. Stability investigations showed that PSS as a dopant improved polymer stability, and also produced responsive sensors less prone to non-specific DNA adsorption as compared to LiClO$_4$ doped polymer sensors. Such resistance to non-specific DNA adsorption and increased stability can be highly beneficial for biosensing applications.

The response of developed sensors with different lengths of target DNA indicated that an increase in target length produces larger sensor responses, due to the extra charges these longer DNA strands carry. The results can be described by a model based on the Donnan exclusion principle, which expresses the effect the fixed charge concentration within the polymer pores has on the sensor signal. ON probe length also affects the signal of fabricated sensors, with a longer probe producing a larger response.

Meanwhile, the response of developed sensors with the neutral redox couple hydroquinone was examined and compared with the previously tested ferri/ferrocyanide redox couple. With increasing target DNA concentration, $R_{ct}$ increased for electrodes examined in ferri/ferrocyanide solution. This is a result of the inherent negative charges ONs possess, which results in an inhibition of interfacial electron transfer. Meanwhile, in solution with H$_2$Q ions, a smaller increase in $R_{ct}$ is observed for increasing target ON concentrations. In this case it is only steric hindrance influencing H$_2$Q’s approach to the polymer surface. Regardless of the redox reporter used, higher sensitivity is achieved with a PSS doped polymer than with a LiClO$_4$ doped polymer. It is likely that surface morphology plays a major role in this sensitivity. Altering the immobilised probe density highlights interesting trends in sensor response and helps to further understand the sensor surface. For the sensor platform described here it was found the ferri/ferrocyanide redox couple is better suited for high sensor response.

The research presented in this chapter is of importance in developing sensors for real sample DNA detection, where the length of target DNA sequences may well be larger than the synthesized lab strands often used in literature. An example of this is the original mRNA
strand targeted here which corresponds to much longer RNA (> 1000 bases)\textsuperscript{18} specific to the blood protein Glyco A. Additionally, results can guide the design and development of future electrochemical sensors based on this and similar materials. Understanding the sensor surface and the interactions with solution redox species helps to tailor the choice of redox reporters for novel sensing systems. Knowing that a larger response is observed for longer strands (more likely to be encountered in practical situations) encourages and improves the practicality of this sensor platform for the analysis of real samples.
Chapter 4

Metal-ion Implantation Of Polypyrrole and Subsequent DNA Sensor Development

Marsilea Booth
Chapter 4. Metal-ion Implantation of Polypyrrole and Subsequent DNA Sensor Development

Polypyrrole-based DNA sensors showed promise for DNA sensing. However, despite many beneficial properties possessed by PPy, long-term stability has room for improvement, particularly for practical uses. Meanwhile, interesting changes in material behaviour have been observed with the process of ion implantation, including increased stability. To this end two metals were chosen to be implanted in PPy films, a highly conductive material, platinum, and a lower conductivity metal, lead. Lead displays about half the conductivity of platinum and is a commonly used metal for purposes such as soldering, is corrosion resistant, and is more abundant than platinum. Characterisation of the films was performed with comparisons before and after implantation. Following a thorough investigation into the effect of ion implantation, Pt implanted films were used to create a DNA sensing platform with a novel anchor point between the Pt and thiolated probe DNA.

The objective of the following section is to:

- Investigate the effects of metal-ion implantation into PPy, in particular in view of polymer stability, followed by development of a novel DNA sensor.

4.1 Implantation of Polypyrrole

As mentioned in section 2.5, ion implantation offers the incorporation of one material into another often causing an interesting change in properties of the base material. In this case PPy is used as the base material while two metals are implanted, platinum and lead.

Following ion implantation the visual coloration of the films changed as a gradient function of ion dose, and a metallic sheen was observed for all implanted films, Figure 4.1.

Figure 4.1 Captured images of PPy films with increasing fluence from left to right.
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4.2 Characterisation

4.2.1 SEM-EDS, TEM and RBS Characterisation

The physical properties of the films such as polymer composition and implantation depth were investigated using SEM-EDS, TEM and RBS.

4.2.1.1 SEM-EDS and TEM of Platinum Implanted Films

The presence of Pt in the film is evidenced by EDS spectra, Figure 4.2, alongside SE and BSE images of a cross-section of PPy film implanted with $2 \times 10^{16}$ Pt at. cm$^{-2}$. Due to the large atomic number, Pt signal is observed with a brighter intensity and higher contrast than the PPy (mainly carbon, hydrogen, nitrogen and oxygen). The depth profile of Pt implantation can be clearly observed in the cross-section, where the maximum implanted Pt ion concentration is found very close to the polymer surface, as expected by the 21 nm depth target. This is corroborated by TEM images, as shown in Figure 4.3, where the implanted Pt is clearly visible as a highly contrasted layer just below the polymer surface.

Figure 4.2 SEM images and EDS results for a PPy film implanted with $2 \times 10^{16}$ Pt at. cm$^{-2}$. Cross-sectional images of A) and B) Implanted PPy film as a captured A) SE image, and B) BSE image. The BSE image shows the distribution of Pt (white areas) within the PPy film. C) EDS results of the Pt implanted film. In both SEM images, the arrows indicate the implanted side of the films.
4.2.1.2 TEM and RBS of Lead Implanted Films

Investigation of Pb implanted films using TEM images clearly show implanted particles directly under the polymer surface at concentrations in correlation with the applied implantation fluence, Figure 4.4. A more continuous spread of Pb along the PPy surface was observed for higher fluences of Pb implantation than for lower, Figure 4.4B and C. A closer look at the implanted layer revealed a series of particles when high implantation fluence was used, Figure 4.5. In general, when Pb has been implanted into PPy above its solubility limit Pb precipitation will occur. Eventually even a layer of Pb precipitate (with high enough fluence) may form which increases with increasing fluence. Pb precipitate particle size may affect the conducting properties of implanted PPy films, and is expected to increase with increasing fluence.\textsuperscript{103} At low implantation fluences, distinct particles were hard to identify, hence a detailed study of particle size and conductivity could not be performed.

Figure 4.4 Cross-sectional TEM images of PPy films of different fluences. A) A non-implanted PPy film, B) a PPy film implanted with $2 \times 10^{14}$ Pb at. cm$^{-2}$, and C) a PPy film implanted with $1 \times 10^{15}$ Pb at. cm$^{-2}$. The implanted lead can be observed as a high contrast dark region below the PPy surface. The arrows indicate the ion implanted side of the film.
Figure 4.5 Cross-sectional TEM images of a PPy film implanted with $2 \times 10^{15}$ Pb at. cm$^{-2}$. Black rectangles and magnified images show examples of two areas: the implanted area with individual particles observable, and control PPy with no such particles.

Rutherford Backscattering spectrometry (RBS) was used in order to verify the implanted fluence and for elemental analysis. Both RBS measurements and RUMP analysis were performed by Jérôme Leveneur and Dr. John Kennedy. Table 4.1 summarizes the Pb implanted concentration as retrieved from RUMP analysis. The calculated concentrations are consistent with the implanted fluences. However, the RBS measurements performed in this study display poor sensitivity to the lighter elements. Therefore, a comparison of the light element compositions between films was not advisable. Nevertheless, a relative elemental composition of C:3, H:3, N:0.8, O:0.5 and Cl:0.14 was retrieved using RUMP fitting. This is coherent with doped PPy, for which is expected a C$_3$H$_3$N stoichiometry. Displayed in Figure 4.6 are the RBS spectra for PPy films implanted with various fluences. As expected the peak observed for Pb increased with increasing fluence. Notably, chlorine is uniformly distributed throughout the sample, indicating constant doping.

Table 4.1. Results from RBS experiments

<table>
<thead>
<tr>
<th>Expected Pb fluence (at. cm$^{-2}$)</th>
<th>Fitted composition in the near surface region from RUMP</th>
<th>Pb (at.cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 $\times$ 10$^{14}$</td>
<td></td>
<td>0.004 (3.29 $\times$ 10$^{14}$)</td>
</tr>
<tr>
<td>5 $\times$ 10$^{14}$</td>
<td>3 0.8 0.5 0.14</td>
<td>0.009 (5.37 $\times$ 10$^{14}$)</td>
</tr>
<tr>
<td>2 $\times$ 10$^{15}$</td>
<td></td>
<td>0.020 (1.63 $\times$ 10$^{15}$)</td>
</tr>
<tr>
<td>5 $\times$ 10$^{15}$</td>
<td></td>
<td>0.470 (4.65 $\times$ 10$^{15}$)</td>
</tr>
</tbody>
</table>
4.2.2 Conductivity

The conductivity of PPy films were measured and compared both before and after implantation for different fluences, as seen in Figure 4.7. Calculations of conductivity were performed using the formula for a thin sheet. Freshly grown PPy films displayed conductivity on the order of $120 \pm 20 \text{ S cm}^{-1}$. Pristine PPy also undergoes a decrease in conductivity, presumably due to polymer degradation or other vacuum induced or conductivity limiting processes.

Interestingly, for both implanted metal types an initial decrease in conductivity is observed at implantations of above $1 \times 10^{15}$ Pt at. cm$^{-2}$ and $2 \times 10^{14}$ Pb at. cm$^{-2}$. It is believed this decrease in conductivity occurs from degradation to the polymer through processes such as disruption to the conjugated $\pi$ system. Radical formation from ion irradiation may be minimal at this fluence as free radicals and vacancies recombine to produce neutral species.
This decrease is followed by an increase in conductivity at implantations over $6 \times 10^{15}$ Pt at. cm$^{-2}$ and $2 \times 10^{15}$ Pb at. cm$^{-2}$ for Pt and Pb implanted films, respectively. At these fluences it is likely that the breaking of the bonds is either outweighed by the synergistic effect of the Pt precipitates and Pb-rich precipitates which are able to contribute to conductivity, or is no longer the main process occurring. The fluence at which this critical dose occurs is suggested to arise from an overlap of specific combination of energy and fluence, providing radical formation, as well as high enough Pt or Pb concentration to overcome the percolation threshold and enhance conductivity. It should be noted that Pb particles are not physically connected suggesting any aid in the conductivity of the PPy is not due to formation of a layer.

In these experiments relatively low energy ions are used (keV range), therefore the dominant process is expected to be elastic binary collisions, generally producing fragments, excited species and radicals. An increase in generated radicals explains the increase in conductivity at high ion fluence through the increased cross-linking within the polymer, the formation of unsaturated groups in the molecular structure and increased conductivity through Pt metal contribution.

Despite the different fluences examined, a similar pattern is observed for both Pt and Pb implanted films. Lead itself is a poor electrical conductor compared to platinum. Pt, as a good electrical conductor, is suspected to aid in electrical contact across the PPy film and hence conductivity. This is suggested as the reason for the high conductivity at a high fluence of implanted Pt. The difference in the conductivity profiles for Pt and Pb can be explained by the fact that PPy conductivity at specific fluences is dependent on a number of factors; implantation-related properties (particle connectivity and size), chemical changes to the PPy (e.g. degradation and density), micro-structural changes (e.g. surface smoothness), and more importantly intrinsic element properties (e.g. the bulk conductivity of the implanted material). The important difference observed in the evolution of conductivity between the Pb and Pt cannot be accounted for by the difference in the incident ions masses and resulting ballistic damage. Hence, these results suggest that the Pb-PPy and Pt-PPy chemical interactions and nanoparticle conductivity are likely to play an important role as they will lead to differences in the charge carrier concentration and conduction paths.

Nevertheless it is obvious from the conductivity versus concentration profile that the presence of Pt implanted at high fluences aids the conductivity of the PPy films, giving rise to the high conductivity observed after implantation of $2 \times 10^{16}$ Pt at. cm$^{-2}$. 
4.2.3 Raman Spectroscopy

To further investigate the effect of ion implantation on the conductivity of PPy films Raman spectroscopy was performed. Raman spectroscopy is able to provide molecular-level information which is highly sensitive to changes within the measured substrates. Spectra obtained for all PPy films indicate characteristic bands of oxidised PPy, Figure 4.8. As can be immediately observed, a striking increase in signal intensity arises from the implanted films as compared to that of pristine PPy, particularly for Pt implanted films. It is suggested this phenomenon is a feature of the surface enhanced Raman spectroscopy (SERS) effect, which is expected to result from the interaction between PPy nitrogen atoms and neighbouring Pt particles, hence suggesting the presence of Pt particles. The nucleation of a Pt precipitate is expected during ion implantation which increases with the implanted concentration.

There are three main areas of interest for comparison of the spectra, namely the band arising from the C=C backbone stretching of the PPy at 1580-1601 cm\(^{-1}\), bands at 1050-1083 cm\(^{-1}\) arising from the C-H in-plane deformation, and the band at 937-939 cm\(^{-1}\) indicating ring deformation. The latter two bands and their neighbouring bands can be considered composed of two adjacent bands, one arising from dication deformations (937-939 cm\(^{-1}\) and 1083 cm\(^{-1}\)), and one arising from radical cation deformations (~983 cm\(^{-1}\) and 1050 cm\(^{-1}\)).
By comparing the ratios of these two bands (Table 4.2 and Table 4.3) information can be gathered about the major species present in the PPy films.

The trend of the results is very similar for Pb implanted and Pt implanted PPy films. It should be noted however, that the Pt implanted films show a lower conductivity than the Pb implanted films. This decreased conductivity was also observed for the control pristine PPy sample in the ‘Pt implanted’ group (67 versus 117 S cm\(^{-1}\)). Therefore, it is likely this lower conductivity of the ‘Pt implanted group’ is an artefact of either the vacuum process or the atmosphere exposure and environmental conditions these films were subject to.

**Table 4.2. Band intensity ratios and positions for the different Pt ion implantation fluences**

<table>
<thead>
<tr>
<th>Pt implanted (at. cm(^{-2}))</th>
<th>(\frac{(I_{1083}+I_{937})}{(I_{1050}+I_{983})}) dication species</th>
<th>Raman band position of C=C stretch (cm(^{-1}))</th>
<th>Measured conductivity (S cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.17</td>
<td>1580</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>(1 \times 10^{15})</td>
<td>1.17</td>
<td>1582</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>(3 \times 10^{15})</td>
<td>1.09</td>
<td>1601</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>(6 \times 10^{15})</td>
<td>1.07</td>
<td>1596</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>(1.2 \times 10^{16})</td>
<td>1.17</td>
<td>1587</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>(2 \times 10^{16})</td>
<td>1.21</td>
<td>1601</td>
<td>110 ± 3</td>
</tr>
</tbody>
</table>

**Table 4.3. Band intensity ratios and positions for the different Pb ion implantation fluences**

<table>
<thead>
<tr>
<th>Pb implanted (at. cm(^{-2}))</th>
<th>(\frac{(I_{1083}+I_{937})}{(I_{1050}+I_{983})}) dication species</th>
<th>Raman band position of C=C stretch (cm(^{-1}))</th>
<th>Measured conductivity (S cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.22</td>
<td>1585</td>
<td>117 ± 2</td>
</tr>
<tr>
<td>(2 \times 10^{14})</td>
<td>1.27</td>
<td>1592</td>
<td>116 ± 1</td>
</tr>
<tr>
<td>(5 \times 10^{14})</td>
<td>1.18</td>
<td>1588</td>
<td>60 ± 8</td>
</tr>
<tr>
<td>(1 \times 10^{15})</td>
<td>1.14</td>
<td>1607</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>(2 \times 10^{15})</td>
<td>1.23</td>
<td>1587</td>
<td>129 ± 3</td>
</tr>
<tr>
<td>(5 \times 10^{15})</td>
<td>1.17</td>
<td>1596</td>
<td>100 ± 4</td>
</tr>
</tbody>
</table>
Within PPy films there is generally a combination of three species in different proportions; dications, radical cations and neutral species.\textsuperscript{9,248,249} Results suggest the sample implanted with the highest fluence of Pt and Pb have a higher proportion of dications (or bipolarons) than pristine PPy, which in turn has higher proportion of dications than mid-fluence implanted films (6 × 10\textsuperscript{15} at. cm\textsuperscript{-2} for Pt implanted films and 1 × 10\textsuperscript{15} at. cm\textsuperscript{-2} for Pb implanted films) correlating with conductivity measurements.

Despite the high number of dication species within the polymer film, implanted films appear to have low conjugation lengths. The band located at 1580-1601 cm\textsuperscript{-1} is able to provide information about the benzoid or quinoid structures, and hence conjugation length, within the film. It has been shown that the lower the wavenumber of this band, the higher the conjugation length within the polymer.\textsuperscript{246} From this it can be seen that highly implanted samples have a low conjugation length, while pristine PPy retains the highest conjugation length, a likely contributor to its higher conductivity over some implanted films. The low conjugation length in implanted films is explained by the collisions during ion implantation producing fragments within the polymer.\textsuperscript{245} It is inferred from the Raman spectra that although dication species are spaced intermittently within the highly Pt implanted PPy film, the increased concentration throughout the film provides high conductivity.

Figure 4.8 Raman spectra of control PPy pristine films and Pt and Pb implanted films. 
A) pristine PPy film (a), PPy film implanted with 6 × 10\textsuperscript{15} Pb at. cm\textsuperscript{-2} (b), and PPy film implanted with with 2 × 10\textsuperscript{16} Pt at. cm\textsuperscript{-2} (c). B) pristine PPy (a), PPy implanted with 1 × 10\textsuperscript{15} Pb at. cm\textsuperscript{-2} (b) and PPy film implanted with with 2 × 10\textsuperscript{15} Pb at. cm\textsuperscript{-2} (c).
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4.2.4 Electrochemical Characterisation

Following the increase in conductivity observed for implanted films, the electrochemical properties of Pt implanted films were investigated. Cyclic voltammetry (CV) of the polymer films indicate similar CV shape with very broad oxidation (between 0 and 0.3 V) and reduction peaks (between 0.3 to -0.4 V attributed to the ClO$_4^-$ dedoping of the film), Figure 4.9. An increase in charge is calculated for implanted PPy (158 mC cm$^{-2}$) as compared to non-implanted PPy (154 mC cm$^{-2}$), showing a beneficial effect of ion implantation on electrochemical activity perhaps due to fewer ohmic losses. Raman spectroscopy was performed on films after CV cycling (data not shown). Results indicate some degradation of both films by shifts in characteristic bands. Degradation can be explained by irreversible reactions to the PPy backbone that occur due to nucleophilic attack of species such as OH$^-$ ions. One degradation pathway can be monitored through analysis of the conjugation length in the films, estimated by the benzoid/quinoid band at ~1600 cm$^{-1}$, section 4.2.3, which shifted from 1580 to 1607 cm$^{-1}$ for the non-implanted PPy, and from 1601 to 1605 cm$^{-1}$ for the PPy implanted with $2 \times 10^{16}$ Pt at. cm$^{-2}$. This suggests conjugation length is better maintained by implanted PPy than by pristine PPy.

![Cyclic voltammograms of pristine PPy and implanted PPy films.](image)

**Figure 4.9** Cyclic voltammograms of pristine PPy and implanted PPy films. a) pristine PPy film, and b) PPy film implanted with $2 \times 10^{16}$ at. cm$^{-2}$. CVs are measured in 0.1 M LiClO$_4$ propylene carbonate solution, at 20 mVs$^{-1}$.

4.2.5 X-ray Photoelectron Spectroscopy (XPS)

In order to further characterise the Pt, Pb and polymer films, XPS was employed. Survey scans, depth profiles and core level scans were performed.
4.2.5.1 Survey Scan XPS

XPS survey spectra were performed on both groups. Survey scans of Pt implanted films indicate all expected elements present with no Pt contamination in non-implanted samples, Figure 4.10.

Figure 4.10 Survey XPS data of PPy films, both pristine and Pt implanted films. a) pristine PPy film b) PPy film with $6 \times 10^{15}$ at. cm$^{-2}$ implanted Pt ions, and c) PPy film implanted with $2 \times 10^{16}$ at. cm$^{-2}$. Written above is the assignment of elements. Surveys a) and b) are offset for ease of viewing.

Survey scans of Pb implanted films meanwhile, indicated some contamination for certain implantation fluences from components such as tin. This contamination may have arisen during the polymer growth or in the vacuum chamber during ion implantation.

Figure 4.11 Survey XPS data of PPy films, both pristine and Pb implanted films. a) pristine PPy film b) PPy film with $1 \times 10^{15}$ at. cm$^{-2}$ implanted Pb ions, and c) PPy film implanted with $5 \times 10^{15}$ at. cm$^{-2}$. Written above is the assignment of elements. Surveys a) and b) are offset for ease of viewing.
4.2.5.2 Depth Profiles with XPS

To examine the implantation of Pt and Pb ions, depth profiles of the highest implanted samples were performed. This was done by combining a sequence of ion gun etch cycles (argon ions were used), interspersed with XPS measurements from the unveiled surface. The concentrations for each element through the etched layer can then be calculated from peak areas. Survey spectra were captured for each cycle as core level measurements of PPy during sputtering are unadvisable due to the possibility of polymer structural changes during sputtering.\textsuperscript{115}

Results revealed for both samples (Pt and Pb implanted) that the PPy surface has a high oxide concentration as expected from atmosphere exposure and degradation. Focussing on the Pt implanted film, the Pt profile concentration begins low at the PPy surface but increases to a maximum at about 500 s of etch cycles to c.a. 18 at. % of the sample, complementing SEM results (Figure 4.12). The Pt profile is in good agreement with the profile expected from simulations, Figure 2.3A. The observed differences in the concentration are likely to originate from the presence of additional species in the film (such as silicon contamination) and corresponding changes in the PPy density. Ar sputtering may also affect the depth profile because of the different sputtering yields dependant on the different densities and structures in the film. The amount of chlorine remains stable throughout the depth investigated, suggesting that ClO$_4^-$ doping is fairly homogenous through the depth of the film. This data beautifully displays the distribution of the Pt through the PPy within the measured film.

![Figure 4.12 Depth profile of a PPy film implanted with $2 \times 10^{16}$ at. cm$^2$ after etching with argon. Concentrations are based on integrated survey spectra taken after every 30 s of sputtering.](image-url)
For the Pb implanted film, the implantation profile indicates a maximum concentration of implanted Pb of 6 at. % after the first etching cycle, Figure 4.13. This compares favourably with the calculated maximum concentration of 9.6 % (Figure 2.3B). As mentioned above, differences may be due to contaminants and density changes. Chlorine, arising from the LiClO₄ dopant, again appears stable throughout the depth investigated.

![Graph showing depth profile of a PPy film implanted with 5 \times 10^{15} Pb at. cm^{-2} after etching with Ar.](image)

Inset indicates a zoomed in view for ease of observing the Pb concentration profile. Concentrations are based on integrated survey spectra obtained after every 60 s of sputtering.

**4.2.5.3 Core Level Scans with XPS**

XPS core surveys allow in-depth analysis of the elemental state and composition of the films. Carbon core level deconvolution analyses of the PPy films describe the species present, namely C\(_\alpha\) bonds observed at 285 eV (C2), C\(_\beta\) bonds observed at 283 eV (C1), C-OH, C=N and C-N\(^+\) bonds at 286 eV (C3), C=O and C=N bonds at 287 eV (C4), and COOH, COO\(^-\) bonds at 289 eV (C5).\(^{107, 220, 250, 251}\) These are displayed for Pb implanted films in Figure 4.14. In comparing pristine films with implanted films (both Pt and Pb implanted) the major changes are: slight increases in C\(_\beta\) bond contributions for implanted films perhaps due to graphitic carbon\(^{107}\) and an increase in the COOH (carbonyl) signal at 289 eV (C5) for implanted films, shown in Table 4.4 for Pt implanted samples. The level of carbonyl defects within the polymer help to explain the observed conductivity measurement in which the lowest conductivity is for a film with \(6 \times 10^{15}\) at. cm\(^{-2}\) Pt implanted ions. The same can be observed for Pb implanted films, where C5 for the \(1 \times 10^{15}\) at. cm\(^{-2}\) Pb implanted samples is higher than for pristine PPy and PPy implanted with \(5 \times 10^{15}\) at. cm\(^{-2}\) Pb.
Figure 4.14 XPS spectra (C1s peaks) of A) Pristine PPy, B) PPy implanted with $1 \times 10^{15}$ Pb at. cm$^{-2}$ and C) PPy implanted with $5 \times 10^{15}$ Pb at. cm$^{-2}$.

Labels C1-C5 indicate the peak composition and fitting.

Table 4.4 XPS data from pristine PPy and PPy implanted with $6 \times 10^{15}$ and $2 \times 10^{16}$ Pt at. cm$^{-2}$.

<table>
<thead>
<tr>
<th>Pt impl. (at.cm$^{-2}$)</th>
<th>C2 285 eV</th>
<th>C1 283 eV</th>
<th>C3 286 eV</th>
<th>C4 287 eV</th>
<th>C5 289 eV</th>
<th>C=O 531 eV</th>
<th>C-OH 532-3 eV</th>
<th>Pt-O 530 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>69 ± 1</td>
<td>19 ± 1</td>
<td>8 ± 1</td>
<td>3 ± 1</td>
<td>1 ± 0.1</td>
<td>11 ± 1</td>
<td>86 ± 1</td>
<td>0.0</td>
</tr>
<tr>
<td>$6 \times 10^{15}$</td>
<td>72 ± 1</td>
<td>19 ± 1</td>
<td>2 ± 1</td>
<td>2 ± 2</td>
<td>4 ± 1</td>
<td>15 ± 1</td>
<td>71 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>$2 \times 10^{16}$</td>
<td>68 ± 1</td>
<td>22 ± 1</td>
<td>5 ± 1</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
<td>26 ± 3</td>
<td>63 ± 1</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

*Values are given as a percentage of the total C 1s or O 1s peak, with the error as % St Dev as calculated using Monte Carlo simulations.

In-depth analysis of Pt and Pb implanted films, in particular the metal core level scans, are discussed below.

**Platinum implanted Polypyrrole**

The Pt deconvoluted peaks, Figure 4.15, indicate the Pt species present in the implanted films. For the purposes of analysis, it was assumed that the doublet components were of equal half-widths, were positioned 3.33 eV in separation, and had an intensity ratio of 3:4 (5/2:7/2). The Pt 4f spectra reveal samples are composed of a mixture of Pt metal (Pt(0)) and Pt(II) species. For the highest implanted sample ($2 \times 10^{16}$ at. cm$^{-2}$), Figure 4.15A, peaks 1 and 2 at 72.1 and 75.4 eV, respectively, indicate 94.0 ± 0.3 % of the Pt exists as Pt metal, while peaks 3 and 4 at 74.13 and 77.46 eV respectively indicate the remaining 6.0 ± 0.3 % of the Pt is in the form of Pt(II). Meanwhile, the sample implanted with $6 \times 10^{15}$ at. cm$^{-2}$, Figure 4.15B, displays a Pt(0) concentration of 80 ± 3 % and a Pt (II) concentration of 20 ± 3 %.

The oxygen Pt-O signal at 530 eV also shows a higher contribution for a film with $6 \times 10^{15}$ at. cm$^{-2}$ than for a film with $2 \times 10^{16}$ at. cm$^{-2}$ implanted ions, Table 4.4. The majority of Pt present in both samples exists in metallic form, having been either precipitated as a result
of the energy of ion implantation and immiscibility of Pt in PPy or reduced by either the PPy film or through other pathways, such as environmental iron and silicon contamination. The Pt 4f<sub>7/2</sub> peaks (72.1 and 72.6 eV for films implanted with 2 × 10<sup>16</sup> at. cm<sup>-2</sup> and films implanted with 6 × 10<sup>15</sup> at. cm<sup>-2</sup>, respectively) are higher than that of bulk Pt (Pt 4f<sub>7/2</sub> = 71.2 eV),<sup>172,179</sup> though this can be explained by interactions between the Pt atoms and PPy, such as electron shifts causing decreased electronic charge density on the Pt.<sup>254</sup> Interactions between metals and ICPs have been observed, for example exhibited as metal/ICP complexes.<sup>255</sup> Such interactions may aid in the conductivity along the polymer as conducting links, therefore when present at high concentrations such as in a sample implanted with 2 × 10<sup>16</sup> Pt at. cm<sup>-2</sup>, can contribute to high conductivity. The presence of more Pt metal with increasing fluence is also in good agreement with the formation of metallic precipitates. Such precipitation during implantation has also been observed with gold metal nanoclusters in PDMS.<sup>103</sup> These precipitates are likely to stay in the form of particles as the implanted concentration is not high enough to lead to the formation of a Pt layer.

![Figure 4.15 XPS detailed spectrum and analysis of the Pt 4f region for a PPy film implanted with A) 2 × 10<sup>16</sup> Pt at. cm<sup>-2</sup>, and B) 6 × 10<sup>15</sup> Pt at. cm<sup>-2</sup>.](image)

**Lead implanted Polypyrrole**

The form of Pb present was found to have a 4f core level binding energy above the literature value of ~ 136.9 eV,<sup>256</sup> at about 138.6 eV. This suggests the presence of an oxidised form of lead. This value corresponds to lead-oxygen compounds such as PbO<sub>2</sub>,<sup>256</sup> and is confirmed by the core level Pb<sub>4f</sub> spectrum, Figure 4.16C. The changes observed in the O<sub>1s</sub> core level as depicted by XPS (Figure 4.16A and B)<sup>257</sup> confirm the presence of lead oxide. Indeed, the features around 528 and 530 eV increase significantly with lead implantation. The O<sub>1s</sub> XPS spectra of different lead oxides have previously shown peaks between 527.7 and 530.2 eV.<sup>256</sup>
The presence of PbO$_2$ may help to explain the increase in conductivity observed after implantation of a fluence of $2 \times 10^{15}$ Pb at. cm$^{-2}$, as PbO$_2$ is known to possess conductivity.$^{258, 259}$

![Figure 4.16 XPS spectra of A) O$_{1s}$ spectra for Pristine PPy, B) O$_{1s}$ spectra for PPy implanted with $1 \times 10^{15}$ Pb at. cm$^{-2}$, and C) Pb$_{4f}$ spectra for PPy implanted with $5 \times 10^{15}$ Pb at. cm$^{-2}$. Peak identifications based on literature.$^{220, 256, 257}$](image)

Furthermore, information regarding the doping level of the polymer can be measured using the ratio of charged (C=N$^+$ and C-N$^+$) and neutral nitrogen (NH and C=N) species.$^{260, 261}$ RBS results revealed a chlorine:nitrogen ratio of 1:5.7. The N$_{1s}$ core level scan (Figure 4.17) as depicted by XPS allows calculation of the ratio of charged to neutral nitrogen species. For pristine PPy this correlates well with the RBS results, with a charged nitrogen:neutral nitrogen ratio of 1:5. This lies close to typical literature values, in the range of 25-33 % for PPy after oxidative polymerisation.$^{261}$ It is suggested that most charged nitrogen species are involved in electrostatic interactions with chlorine species. Of interest is a change in the ratio of charged nitrogen:neutral nitrogen species after implantation. Following implantation of $5 \times 10^{15}$ Pb at. cm$^{-2}$ the ratio of charged to neutral species increases to 1:4, indicating that there are more charged nitrogen species after implantation. The charged species are polarons or bipolarons arising from radical cation and dication species thereby supporting observed Raman spectroscopy results, where implanted films exhibited higher proportions of radical cation and dication species. Note that care should be taken for interpretation of this data, as the noise level is quite high.
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Figure 4.17 XPS spectra (N$_{1s}$ peaks) of A) Pristine PPy, and B) PPy implanted with $5 \times 10^{15}$ Pb at. cm$^{-2}$.

4.3 Conductivity Stability

An important property of PPy exists in its inherent stability. Improvement to the stability of PPy films is keenly sought after. In this case the stability of PPy conductivity is investigated for PPy films, both pristine and implanted films. Although the observed conductivities are high for highly Pt implanted PPy films and certain fluences of Pb implanted PPy, the conductivity begins to decrease over time. This is presumably due to air exposure and polymer degradation,$^{104}$ and is a feature observed for all PPy samples, Figure 4.18 and Figure 4.19A showing Pt and Pb implanted films, respectively. After 15 days, however, the sample implanted with the highest Pt concentration still possesses the highest conductivity.

Figure 4.18 Graph showing the stability of sample conductivity for PPy films
a) PPy film with $6 \times 10^{15}$ at. cm$^{-2}$ implanted Pt, b) pristine PPy and c) with $2 \times 10^{16}$ Pt at. cm$^{-2}$ implanted.

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The same trend is observed with Pb implanted films, Figure 4.19A. Conductivity decay is most prevalent in films implanted with $5 \times 10^{14}$ Pb at. cm$^{-2}$ and $1 \times 10^{15}$ Pb at. cm$^{-2}$. This can be explained by defects present in these films resulting from implantation. After the initial decrease in conductivity, the decay rate appears fairly similar for all implanted films. Films implanted with other fluences, in particular $2 \times 10^{14}$ Pb at. cm$^{-2}$ and $2 \times 10^{15}$ Pb at. cm$^{-2}$, indicate higher conductivity and better stability than pristine PPy. This increase in stability is thought to be an effect of the increased number of charge-carriers, moreover the stability of these charge-carriers.\textsuperscript{105,109,262} Retained charge-carriers can be visualized by UV-vis spectroscopy by investigating the absorbance of the different PPy films. As can be seen in Figure 4.19B, the maximum absorption band is observed at about 1.5 eV (~822 nm) for all films, thought to arise from electronic transitions between or within the conjugated polycationic (polaron and bipolaron) segments (from valence to conduction).\textsuperscript{95,263-265} As was suggested by the Raman spectroscopy data, the radical cation and dication concentration inferred by the absorption band intensity\textsuperscript{105} shows good correlation with the measured conductivity of the films, see inset Figure 4.19B. The high concentration of radical cations and dication present is believed to be the major influence on the high conductivity exhibited by ion implanted films of fluence $2 \times 10^{14}$ Pb at. cm$^{-2}$ and $2 \times 10^{15}$ Pb at. cm$^{-2}$.

Figure 4.19 A) Graph showing the stability of sample conductivity for PPy films and B) UV-vis-absorption spectra of PPy films after 3 months of air exposure: where: a) Pristine PPy, b) PPy film with $2 \times 10^{14}$ at. cm$^{-2}$ implanted Pb ions, c) with $5 \times 10^{14}$ at. cm$^{-2}$ implanted Pb ions, d) with $1 \times 10^{15}$ at. cm$^{-2}$ implanted Pb ions, e) with $2 \times 10^{15}$ at. cm$^{-2}$ implanted Pb ions, and f) with $5 \times 10^{15}$ at. cm$^{-2}$ implanted Pb ions. B) inset: Graph displaying % Conductivity remaining after 3 months of air exposure versus absorbance at 1.5 eV. Signals in B) are normalised at 500 nm to the absorbance signal for pristine PPy (a) and have been smoothed using adjacent-averaging at 100 points for ease of band viewing.
4.4 Biosensing Ability of Films

Following incorporation of Pt into PPy films, and the observation that the Pt was present as precipitated metal as well as the increased conductivity observed, the biosensing ability of Pt implanted PPy films was investigated.

A sensor platform was developed to selectively detect target DNA sequences. The higher nominal and retained conductivity of PPy after Pt implantation serves to provide improved response when using electrochemical detection. Additionally, the simplicity of the sensor platform removed the need for complicated functionality of polypyrrole either before or following polymerisation, in contrast to most literature on biosensors using PPy. The DNA sensing ability of Pt implanted films was demonstrated using polymer films implanted with a fluence of $2 \times 10^{16}$ Pt at. cm$^{-2}$. A sensor was constructed using thiolated hairpin probe ON and thiolated mPEG attached to the Pt particles within the film, Scheme 4.1. Thiolated mPEG is used in order to modulate probe surface density. Electrical impedance spectroscopy (EIS) is used to detect target ON. Data was modelled as described in section 2.7.3 using a modified Randles’ equivalent circuit. With this system a different EIS spectrum is observed, easily realised in curve b) in the inset ii), whereupon a series of two semi-circular arcs are observed in place of the previously seen single arc. This behaviour may result from system differences in the two areas examined, the polymer/electrolyte and polymer/metal interfaces. In these experiments polymer films are used (thickness of approximately 5 µm), considerably thicker than the previously used thinly-coated electrodes (Chapter 3). A difference in spectra hence is expected, and despite the different spectra arising from the different systems, the data is still well modelled.

Impedance measurements were performed before and after incubation with target ON, both complementary ON and non-complementary. As can be seen in Figure 4.20 insets i) and ii), a significant decrease in the charge transfer resistance ($R_{ct}$) occurs after incubation of a probe modified PPy surface with complementary target ON, while only a small change occurs after incubation with non-complementary target ON, Figure 4.20 inset iii). Changes in charge transfer resistance can be normalized against the $R_{ct}$ value for the film prior to target ON incubation, section 2.9.2. The decrease in $R_{ct}$ after target incubation can be explained by the unfolding of hairpin probes upon hybridisation, Scheme 4.1B. Hairpin probes occupy a larger area as compared to single-stranded probes, hence following hybridisation and unfolding the
uncovered surface provides access to the $\text{Fe(CN)}_6^{3-/4-}$ redox couple, decreasing charge transfer resistance. In the case of non-complementary ON the slight decrease in $R_{ct}$ observed is suggested to arise from non-specific interactions. The large concentration of non-complementary target used (5 mM) should be noted and indicates the sensor possesses good selectivity.

Figure 4.20 Normalized changes in the charge transfer resistance for a probe ON incubated PPy film implanted with $2 \times 10^{16}$ Pt at. cm$^{-2}$ after incubation with different ON sequences and concentrations. A) 50 µM complementary target ON, B) 5 µM complementary target ON, and C) incubation with 5 mM non-complementary ON. Insets show the corresponding electrical impedance spectra measured in 5 mM $\text{Fe(CN)}_6^{4-/3-}$ redox couple in PBS for a) probe modified polymer, and b) incubation with i) and ii) complementary target ON, and iii) non-complementary ON.

Scheme 4.1 Depicting A) PPy film implanted with Pt ions with hairpin probe ON attached, and B) after hybridisation. Prior to hybridisation the probe ON conformation prevents some redox species from approaching the electrode surface. After hybridisation the double stranded ON formation allows redox species to approach the surface and hence redox reactions occur more readily.
4.5 Conclusions

The implantation of Pt and Pb ions within PPy conductive films causes interesting changes within the polymer behaviour and character. The implanted fluence affects the resulting properties. With the exact fluences dependent on the ion being implanted, general trends include that with mid-way-fluence implantations cause consequences such as disruption of the conducting π backbone and production of further defects (e.g. carbonyl functionalities) within the polymer. This in turn impinges on the conductivity of the film, whereby a decrease in conductivity is observed. When a high fluence is applied defects are still present, though their effect is overpowered by the synergistic effect of the increased Pt concentration, and to a lesser extent the Pb-rich particles, as well as the increase in charge carrier concentration, importantly resulting in increased conductivity. In this way, PPy conductivity can be influenced by a combination from the negative contribution of ion beam damage and the positive contributions of additional charge carriers and the conductive precipitations. In addition, the presence of Pt metal precipitates resulting from the large implanted concentration, evidenced through XPS analysis, can explain the increase in Raman signal through a SERS process. Furthermore, films implanted with certain fluences display and retain higher conductivity over time with air exposure as compared to pristine PPy. These enhanced properties for Pb implanted PPy and of PPy/Pt are observed with relatively small amounts of metal inclusion. For Pt implantation the optimum implantation fluence was observed at $2 \times 10^{16}$ Pt at. cm$^{-2}$, while Pb implantation showed optimal results at implantation fluences of $2 \times 10^{14}$ Pt at. cm$^{-2}$ and $2 \times 10^{15}$ Pt at. cm$^{-2}$.

In terms of Pt implantation, the Pt precipitates are advantageous for electrochemical biosensing applications where the implanted Pt provides means for excellent attachment of probe ON. Hence, a simple ON sensor was demonstrated which was able to selectively distinguish between complementary target ON and non-complementary ON using EIS as a detection technique.

The benefits of enhanced conductivity and the presence of Pt demonstrated after ion implantation of Pt into PPy are not limited to advances in DNA sensing. Likewise the improved properties exhibited by Pb implanted PPy may be beneficial for a vast scope of PPy applications, including energy storage systems, catalytic processes and chemical and biological sensing.
Chapter 5

Developing a DNA Sensor using Synthetic Nanopore Resistive Pulse Sensing

Marsilea Booth
Chapter 5. Developing a DNA Sensor using Synthetic Nanopore Resistive Pulse Sensing

In recent years resistive pulse sensing has become of increasing interest as technology is able to characterise the surface of both naturally occurring and synthetic nano-species. Scanning ion occlusion sensing (SIOS) possible with the qNano (IZON Ltd) holds great promise for 1) single particle analysis ensuring high sensitivity, 2) tuneable pore size ensuring the ability to adjust conditions to enhance signal output. Resistive pulse sensing as a research interest has a number of end applications, the most popular of which is currently detection of isolated single stranded DNA strands, holding capacity for DNA sequencing and identification. Another application, and of interest in this research, is the formation of a sensor able to selectively and sensitively detect target DNA in solution through particle surface hybridisation. A qNano is used to detect changes in particle surface properties in order to identify target hybridisation.

The objective of this body of work is to:

- Investigate the ability of detecting target hybridisation on particle surfaces using resistive pulse sensing.

5.1 Using the qNano to Measure Polystyrene Particles

IZON suggests the use of carboxylated and NIST traced polystyrene particles as a means to familiarise oneself with the instrument and to characterise unknown particle sets. The particles used were approximately 100 nm (NIST particles, labelled PS), 115 nm (PSCOOGH) and 200 nm (B200 COOH) in diameter. As per IZON suggestions, particle solutions are made to suggested concentrations and the particle solutions run with different instrumental conditions and the effects examined.

5.1.1 Altering the Applied Potential

As the applied potential is altered, there is a linear increase observed in the measured current, Figure 5.1A. As the potential difference between the electrodes increases, more current flows between them. At higher currents the signal to noise ratio is better (though the absolute noise
value is higher), so it is best to choose an applied potential where a high signal is observed without too much noise. Additionally as the potential is increased the blockade magnitude increases, due to each particle causing more current to be blocked from passing through the pore, Figure 5.1B. This allows the comparison of measurements of particles in solutions with differing conductivities performed at different potentials. Following normalization to an average current the particles can be directly compared.

5.1.2 Altering the Nanopore Stretch

Altering the stretch of the nanopore also has an effect on the response. The blockade magnitude decreases as the stretch is increased, explained by the fact that though the particle size remains constant, relative to the pore size it decreases. Additionally the particle rate increases, explained as more solution can pass through the stretched pore bringing with it more particles.

Figure 5.2 Graphs showing the effect of altered nanopore stretch on A) the blockade magnitude, and B) the particle rate. All other conditions remained constant, potential held at 0.44 V except for the highest stretch in which the potential was reduced to 0.34 V to ensure the instrument did not get overwhelmed.
Chapter 5

5.1.3 **Altering the Applied Pressure**

Increasing the applied pressure using the VPM increases the particle rate as more particles are pushed through the pore. When no pressure is applied there is still movement of particles through electrophoresis (if a charge is present on the particles) and through the inherent pressure arising from the 40 µL of liquid in the upper fluid cell.

![Graph showing the effect of applied pressure on particle rate. All other conditions are kept constant.](image)

**5.2 Trialling Different Particles**

A number of different particles were examined (Figure 5.4). Polystyrene particles and other commercial particles were coated with polypyrrole or the copolymer P(Py-co-PAA) through chemical oxidative polymerisation using either FeCl₃ or ammonium persulfate (APS). Different dopants for PPy were trialled, as well as varying the concentrations of pyrrole, oxidant and PAA monomer. Additionally Dr. Geoff Waterhouse synthesized polystyrene particles which were examined, coated with poly(N-vinylpyrrolidone) and subsequently coated with polypyrrole. Magnetic particles (Fe₃O₄) were synthesized and incorporated in the polymerisation solution with the idea that inclusion in the polypyrrole matrix coating would add magnetism to the particles. The copolymer and PPy coated particles were tested for their DNA sensing ability with fluorescently labelled target DNA and indicated a promising sensing platform, Figure 5.5.

Unfortunately all of these systems showed aggregation and non-uniformity so could not be further analysed with the qNano. For these reasons the particles used for the remaining experiments were commercially bought magnetic particles and polystyrene particles.
Figure 5.4 TEM images of a selection of particles that were examined but deemed unsuitable for qNano analysis.

A) Commercial bare PS particles, B) Particles in A after coating with polypyrrole doped with FeCl₃, C) P(Py-co-PAA) polymer coating polymerised chemically with APS, D) PPy coated particles doped with camphorsulfonic acid (CSA), E) PS particles synthesized by Dr. Geoff Waterhouse, F) The particles from E) after coating with a thin layer of PPy and synthesized Fe₃O₄ particles.

Figure 5.5 Images captured with the fluorescent microscope with constant exposure.

PAA coated and probe grafted particles after incubation with fluorescently-labelled target ON as A) a standard image, B) a fluorescence image (400x objective) indicating target ON, and C) a control image where PAA coated particles (no probe grafting) are incubated directly with fluorescently-labelled ON. The lack of fluorescence in C) indicates it is selective binding of target DNA causing the fluorescence in B).
Chapter 5

5.3 Finding Suitable qNano Conditions

5.3.1 Buffer Choice

A number of buffers were investigated for their current and noise level, namely SEB buffer, saline-sodium citrate (SSC) buffer, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer and PBS buffer. All were tested at two concentrations, 0.1 M and 0.01 M. All buffers displayed stable baselines, though SEB buffer showed a low noise level and as it is the default buffer for the qNano, it was used for qNano analysis.

5.3.2 Particle Choice

Commercial particles with elected surface moieties are available with a range of compositions, such as polystyrene, silica and dextran. Other commercially available particle specifications include fluorescence, biocompatibility and magnetic properties. Magnetic particles allow simple and selective washing (as most biological species are not magnetic), pre-analysis concentration of target, a high surface-to-volume ratio and have the advantage of already being used in established laboratory methodology e.g. DNA isolation for nucleic acid sequencing. Although polystyrene particles showed promise with measurement, magnetic particles were preferred and hence used for the DNA sensing system.

5.4 DNA Sensing using SIOS

5.4.1 Overall Scheme and Surface Derivitisation Efficiency

Displayed in Scheme 5.1 is our approach schematically. The labels are “Probe” for probe grafted particles, and “Target” for probe grafted particles after incubation with complementary target DNA. Discrimination between particle groups, in particular between “Probe” and “Target” is sought. The presence of chemically-accessible carboxylate species on the particle surface was confirmed by reaction with toluidine blue. The number of carboxylic acid groups was estimated at 20 (±1) × 10^3 per particle. Despite this likely to be an overestimate as non-specific binding of toluidine blue has been known to occur, it indicates that there are a large number of available carboxylate species present for probe grafting.
Scheme 5.1 Scheme depicting the overall DNA sensing scheme
a) carboxylated dextran particles, b) after grafting of probe DNA, using EDC and NHS, and c) after incubation with target DNA and hybridisation between the complementary strands.

Optimisation of both probe grafting and target hybridisation was performed. For probe grafting optimisation, particles were shaken in solution containing EDC, NHS and probe ON for varying times. At time periods solution was removed, the particles washed and diluted and qNano analysis performed. Results indicated that a time period of at least 4 h was required for maximum probe grafting, determined by stable and low baseline duration and FWHM values (Figure 5.6).

Figure 5.6 Graph showing mean (black squares) and mode (grey circles) baseline durations for particles versus probe incubation time.
Mean measurements can be skewed by aggregates, but mode measurements show a similar trend. Inset: FWHM versus time of incubation. Measurements are taken at a stretch of 47 mm and an applied potential of 0.54 V.
Chapter 5

For the optimisation of target ON incubation period a similar experiment was performed. Probe grafted particles were shaken for various times in fluorescently labelled target ON solution. At time periods solution was removed, the particles washed and diluted and fluorescence and qNano analysis performed. Overnight shaking was found sufficient to induce target hybridisation, consistent with other research.

Post incubations, complementary techniques were used to confirm particle modification. Probe grafting was confirmed by ATR-FTIR analysis (Figure 5.7A). The presence of a band at 1231 cm⁻¹ indicates the C-N stretch, and the appearance of band at 1634 (or shift from 1619) cm⁻¹ indicates an amide bond for C=O.²⁷¹ Both spectra possess bands at 3300 cm⁻¹ indicating hydroxyl stretching, bands at 2900 cm⁻¹ indicating C-H stretching and 1011 cm⁻¹ indicating C-O-C and C-OH linkages, presumably due to the dextran base of the particles.

Meanwhile, target hybridisation was deemed successful through using fluorescently labelled target DNA, whose fluorescence confirmed the validity of the resistive pulse sensor results (Figure 5.8). Additionally ATR-FTIR analysis of these particles with fluorescently labelled target indicated FITC bands present, Figure 5.7B. The spectrum can be directly compared to the FITC label standard spectrum, and involves characteristic bands at 3187 cm⁻¹ indicating a hydroxyl group, at 1631 cm⁻¹ representing the quinone-like stretch,²⁷² and bands at 1554 and 1462 cm⁻¹ representing aromatic C=C species.²⁷¹ Some features of the spectra in Figure 5.7 may be artifacts and distortions arising from the non-ideal sampling technique used (where particles were cast over the IR crystal).

Figure 5.7 ATR-FTIR spectra for probe and target incubated species. A) a) carboxylated dextran particles and b) Probe grafted particles and B) a) Probe grafted particles, b) Probe grafted particles after incubation with fluorescently labelled target (FITC), and c) a fluorescently labelled target standard (FITC).
5.4.2 Statistically Identifying Differences in Particle Group Signals

The overall aim is to distinguish between different particle groups, however visually a difference between the groups is hard to identify. Therefore statistical analysis able to identify differences was sought. The different intercepts model, section 2.10.2, was found to be an appropriate analysis technique. The identification of the groups are 1) the bare carboxylated dextran particles, 2) the “Probe” particles (probe grafted particles), 3) the “Target” particles (“Probe” particles incubated with complementary target DNA), and 4) Non-complementary incubated “Probe” particles, which should show a signal similar to “Probe” particles if non-specific DNA binding is not an issue.

As can be seen a difference in intercepts is observed between the carboxylated dextran particles and the “Probe” particles, supported by the calculated p-value of <0.0001 and with no overlap of confidence intervals. This was confirmed by multiple repeats. Similarly a difference is observed between the “Probe” and “Target” groups, with no overlap of confidence intervals and a p-value of <0.0001. With repeats it was seen that some experiments, while still indicating a p-value of <0.0001, showed a small amount of confidence interval overlap. This may be due to differing ‘extents’ of hybridisation (for example if not all the particles have hybridised target some will overlap with “Probe” particles). All showed significant p-values. Meanwhile, when comparing “Probe” and non-complementary DNA-incubated particles, only a very small difference is observed with a p-
value of $4 \times 10^{-4}$ suggesting a negligible effect of non-specific binding, perhaps occurring through electrostatic interactions.

It should be noted that if all particles have the same surface charge density, in theory FWHM duration should remain constant with particle size. A slight deviation of this is seen, presumably due to inhomogeneous particle surface charge density and/or deviation from the Smoluchowski approximation (relating to the diffusion and convection of the particles in solution). This method highlights the strengths of distinguishing between carboxylated dextran particles and “Probe” particles, as well as between “Probe” and “Target” particles.

Residual plots were examined for each analysis to confirm the validity of the assumptions made. As an example the plot shown in Figure 5.10 shows random residual scatter indicating the assumptions are valid.

Figure 5.9 Scatter plots with log(size) vs log(FWHM duration) for A) carboxylated dextran particles (black circles) and probe grafted particles – “Probe” (red circles), B) “Probe” particles (red circles) and “Target” particles – probe grafted particles after target incubation, $10 \times 10^6$ M (blue circles), and C) “Probe” particles (red circles) and probe grafted particles after incubation with non-complementary DNA, $50 \times 10^{10}$ M (green circles).

Figure 5.10 A residual plot as calculated in the program R. The plot indicates a fairly even spread of residuals.
5.4.3 Particle Properties and Use of the Variable Pressure Method

In this section a proof-of-concept for discrimination between “Probe” and “Target” particles is identified. As previously mentioned, a statistically significant difference is observed between “Probe” and “Target” particles. Subsequently this difference is further examined and other methods for distinction investigated.

As seen in Figure 5.11, incubation with target DNA causes a decrease in particle FWHM duration. Probe grafted particles display a mode FWHM duration of $0.95 \pm 0.10$ ms ($n = 4$), while target hybridised particles display a mode FWHM duration of $0.68 \pm 0.25$ ms ($n = 4$). When considering the duration parameter to be a combination of both driving and dominant counteracting forces (such as electrokinetics, convection and particle-nanopore wall interactions), the difference in signal duration can be explained. One influence on particles as they translocate the nanopore is particle surface charge. Inherent in DNA is a negatively charged phosphate backbone. As a positive potential bias is being applied across the pore (+0.48 V), the durations of “Target” particles are expected to be shorter than those for “Probe” particles, due to the increased DNA presence. Other factors may contribute to FWHM duration, such as drag force between particles and the nanopore wall, convection, and changes in the particle radius as dsDNA is known to have a longer persistence length than ssDNA. However, it is proposed that these are not the primary influences on the different FWHM durations observed for “Probe” and “Target” particles, rather that the key influencing factor is surface charge. Drag force is expected for both the single-strand and double-strand DNA coated particles, as is convection, and due to the flexibility of DNA in solution the blockade magnitude is not expected to differ greatly. Indeed, this is reflected in the mode particle size calculated for “Probe” particles being $109 \pm 11$ nm ($n = 8$), whilst $106 \pm 6$ nm ($n = 8$) for “Target” particles.
Figure 5.11 Comparisons of “Probe” and “Target” groups.
A) A histogram displaying the frequency vs. size for “Probe” and “Target” groups. B) Histogram of frequency of measured FWHM duration times for “Probe” and “Target” particles, and C) Single translocation events for “Probe” particles (red) and “Target” particles (blue). Current baseline has been shifted to 0 nA to simplify analysis. For all three graphs “Probe” is shown in red, while “Target” is shown in blue.

Because of the expected difference in charge between “Probe” and “Target”, the recently published method by Vogel and Roberts et al. for characterising nanoparticle surface charge using a variable pressure method was performed. Shown in Figure 5.12 is the mean blockade FWHM value versus time, while displayed in Figure 5.13 are the cumulative counts versus applied pressure for the particle groups, carboxylated dextran particles, “Probe”, “Target”, and PS-COOH (115 nm carboxylated polystyrene particles). A summary of results are shown in Table 5.1. As the applied pressure is gradually varied from positive pressure to negative pressure, particles begin by flowing from the top fluid cell through to the bottom fluid cell. As the pressure is reduced the duration of the particles increases, Figure 5.12, (up to 100 times longer in duration than blockade events when no pressure is applied) and the particle rate nears zero.

Figure 5.12 Graph showing the mean blockade FWHM duration versus time for four different data sets. Carboxylated dextran particles are shown in black, “Probe” particles in red, “Target” particles in blue and PS-COOH particles in green. Time is a measure of pressure (as every 30 s the pressure is adjusted by 50 Pa).
this case a positive potential is applied, and the pressure begins as positive pressure. In this way the longest time equates to the highest vacuum. In this way it can be seen that the order of inflection point (also where FWHM is the greatest) from lowest to highest is carboxylated dextran particles and “Probe” particles < “Target” particles < PSCOOH particles.

Eventually, as the net pressure on the top fluid cell is negative, particles will reverse and flow from the bottom fluid cell to the top fluid cell. The point at which this switching occurs is dependent on a number of parameters including the zeta potentials of the particles and pore-wall, the applied pressure and the electric field. The cumulative particle count during the VPM experiment is described by an S-curve for each particle type. Inflection points during the experiment were also estimated by the observed closest pressure where the particles swapped directions, Table 5.1. Meanwhile more accurate and detailed inflection points were calculated through fitting of the data with a third order polynomial, followed by differentiation of this fit to determine the inflection point, Table 5.1. For the conditions used here, a higher (more negative) inflection point arises from a more negatively charged particle.

As explained by Vogel et al. the observed inflection points are independent of particle concentration.\textsuperscript{52} It should be noted that the errors observed for these readings are quite high. This could be improved by using a continuously changing pressure application (as was used by Vogel et al.\textsuperscript{52}) in place of altering the pressure every 30 s. Additionally, the VPM used can lead to errors due to the difficulty in applying an accurate pressure, also improved by using a system similar to Vogel et al.

<table>
<thead>
<tr>
<th>Particle Description</th>
<th>Experiment</th>
<th>Experimental estimated inflection point (to the closest 50 Pa)</th>
<th>Calculated inflection point (from S-curve)</th>
<th>Average Calculated inflection point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylated dextran particles</td>
<td>1</td>
<td>-50</td>
<td>-64.8</td>
<td>-77 ± 17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-100</td>
<td>-88.9</td>
<td></td>
</tr>
<tr>
<td>“Probe” particles</td>
<td>1</td>
<td>-50</td>
<td>-45.5</td>
<td>-77 ± 21</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-50</td>
<td>-101.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-50</td>
<td>-83.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-50</td>
<td>-68.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-100</td>
<td>-87.09</td>
<td></td>
</tr>
<tr>
<td>Non-</td>
<td>1</td>
<td>0</td>
<td>-81.6</td>
<td>-75 ± 15</td>
</tr>
</tbody>
</table>
As can be seen, the experimentally estimated values of inflection point are not very accurate, however can provide an approximation to check the fitted inflection points. Overall these correlate sufficiently to support the fitted inflection points.

![Figure 5.13 S-curves and a bar graph indicating the inflection points for different groups of particles. A) S-curves for particle groups, carboxylated dextran particles (black squares), "Probe" particles (red squares), "Target" particles (blue circles), and carboxylated polystyrene particles (PSCOOH). Experimental data are shown as symbols, while the polynomial fitting data are shown as lines. Data is collected with 0.48 V applied potential. B) Applied pressure to reach inflection point for different groups of particles. Firstly of interest is the small pressure required for inflection of the dextran carboxylated particles, suggesting low surface charge. This is confirmed by the –COOH density (as presented in section 2.9.1), where the number of carboxylic acid groups was estimated at 20 \((\pm 1) \times 10^3\) per particle. PSCOOH particles on the other hand have a high inflection point pressure, as expected from the manufacturers claim of surface charge as 171 µequiv/g (as...
given by Bangs Laboratories using a conductometric titration, and where equiv is the equivalent moles of titrant per gram of sample required to reach the equivalence point, providing a –COOH density of 91 $\times$ 10$^3$ per particle. A comparison can be made whereby PSCOOH particles possess over 3 times more carboxylate groups per squared nanometer than carboxylated dextran particles.

By averaging multiple measurements (Table 5.1) the following applied potentials were required to reach S-curve inflection points: 77 ± 17 Pa, 77 ± 21 Pa, 75 ± 15 Pa, 129 ± 19 Pa and 269 ± 27 Pa for carboxylated dextran particles, “Probe” particles, “Probe” particles incubated with non-complementary DNA, “Target” particles and PSCOOH particles respectively. The expected relationship between the total inflection pressure opposing electrophoresis and blockade duration is that as the required pressure increases the blockade duration decreases. This can be investigated by examining the product of duration and total inflection pressure for various particle types which should remain constant. As discussed by Vogel et al., the product of total inflection pressure and blockade duration for different particle types should afford a constant value. Our results indeed show this, Table 5.2, indicating the expected relationship is present.

Table 5.2 Mean FWHM duration for events with electrophoresis as the dominant driving force, along with inflection pressure and the product of these two measures.*

<table>
<thead>
<tr>
<th>Sample</th>
<th>FWHM (ms)*</th>
<th>Inflection pressure (Pa)</th>
<th>FWHM × pressure (ms.Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare carboxylated dextran particles</td>
<td>0.95 (0.58)</td>
<td>77 (17)</td>
<td>73 (47)</td>
</tr>
<tr>
<td>“Probe” particles</td>
<td>0.95 (0.10)</td>
<td>77 (21)</td>
<td>73 (21)</td>
</tr>
<tr>
<td>“Target” particles</td>
<td>0.68 (0.25)</td>
<td>129 (19)</td>
<td>88 (35)</td>
</tr>
<tr>
<td>PSCOOH</td>
<td>0.3 (0.09)</td>
<td>269 (27)</td>
<td>86 (31)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent standard deviations.

* FWHM measurements are taken at a point where applied pressure is equal to the sum of inherent and electroosmotic pressures, therefore making electrophoresis the sole driving force of the particles through the pore. At least 45 particle traces were examined for each particle type to provide the mean FWHM values.

One important consideration is the large standard deviations associated with all measurements, likely to arise from aggregates and non-uniform particle modification. Additionally it is perhaps unexpected that no difference is observed between carboxylated dextran particles and “Probe” particles, considering a difference was observed using statistical analysis (section 5.4.2). It is believed that this is due to the high standard deviations.
observed for the FWHM and inflection pressure measurements. As discussed previously, improvements such as a continually changing pressure system would aid in the discrimination between these particle sets.

5.4.4 Particle Zeta Potentials

Particle zeta potentials were determined using a pore sensor method reported previously. In brief, this method is based on measuring the blockade rate while varying the applied pressure (as previously discussed in section 2.8.2.2 and above in section 5.4.3). The Nernst Planck equation was used to calculate electrokinetic (electrophoretic and electro-osmotic) and convective contributions to particle transport through a conical pore with particle zeta potentials being extracted. The conical pore geometry (small and large pore opening diameters and pore length) was estimated from evaluating baseline current and blockade (signal) magnitude of a calibration particle of known size. Errors were extracted from the pressure uncertainty arising from the repeat VPM measurements. Dr. Robert Vogel performed calculations for the particle zeta potentials.

These zeta potentials can then be compared with zeta potential measurements examined using PALS, Table 5.3. As can be seen, the zeta potentials support expectations where the highest charge is observed for “Target” particles, followed by “Probe” particles, while carboxylated dextran particles have the lowest zeta potential. All particles, but in particular probe grafted particles and bare carboxylated particles tended to aggregate, Figure 5.14, explained with general zeta potential theory with regards to colloid stability. At zeta potentials of higher than ±30 mV particles are considered strongly charged, and more likely to form stable colloidal solutions, while low magnitudes are likely to enhance aggregation. Despite the variation in results between the techniques, the overall trend is as expected.

Table 5.3 Comparison of zeta potentials for bare, probe grafted and target incubated particles as measured using PALS.

<table>
<thead>
<tr>
<th>Description</th>
<th>Zeta potential (variable pressure) (mV)*</th>
<th>Zeta potential (PALS) (mV)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylated dextran particles</td>
<td>-11 ± 2</td>
<td>-3.1 ± 0.50</td>
</tr>
<tr>
<td>“Probe” particles</td>
<td>-11 ± 2</td>
<td>-8.0 ± 1.7</td>
</tr>
<tr>
<td>“Target” incubation</td>
<td>-17 ± 2</td>
<td>-15 ± 2.0</td>
</tr>
</tbody>
</table>

*Measured in pH 8.0 SEB solution.
5.5 Distinction between “Probe” and “Target” Particles

The pressure sweep and zeta potential data show a promising differentiation between “Probe”, non-complementary DNA incubated probe grafted particles and “Target” particles. The change in inversion pressure upon target incubation (after being normalised to the “Probe” inversion pressure) is 68 %, while the normalised change in inversion pressure for non-complementary DNA incubated particles is -3 %. Moreover, the increase of 68 % observed for target DNA is for 10 µM complementary target, showing sensitivity to nanomoles (3) of 50 base pair target DNA. These results indicate promise in both the selectivity and sensitivity of this DNA sensing technique. Sensitivity of resistive pulse sensors, as was discussed by Saleh et al., can be limited by such variables as: 1) the concentration of DNA bound to each particle, 2) the dispersion within colloid size and surface modification (affecting FWHM duration), 3) geometry of the particles and off-axis translocations, 4) the formation of aggregates, 5) the concentration of particles used, and 6) the pore wall surface, where modifications may aid in reducing particle to pore wall interactions. Improvements in sensitivity may be achieved by altering any of these parameters. In particular for our purpose, the homogeneity of particle modification (probe grafting) could be improved producing less variation in FWHM duration. Nevertheless it has been demonstrated that nanopore resistive pulse sensing can be used to detect target DNA in solution.
5.6 Conclusions

A sensing platform is presented which is able to distinguish between carboxylated particles, particles which are probe ON grafted and those which have been subsequently incubated with target DNA. Resistive pulse sensing can be used to identify differences in the behaviour of the particle groups. In particular the relationship between FWHM duration of the translocation events and particle size for each particle group is compared, and together with a statistical model of different intercepts, has been used to demonstrate the difference between carboxylated particles and “Probe” particles, as well as between the latter and “Target” particles. Additionally using a variable pressure method a measure of surface charge can be calculated allowing discrimination between “Probe” and “Target” particles. “Target” particles have a higher surface negative charge when compared to “Probe” particles, due to the extra negatively charged DNA strands. The sensing platform is supported and confirmed by ATR-FTIR, fluorescence and zeta potential measurements.

The size of the DNA used in this study is much smaller than the previously researched DNA, antibodies or other species attached to particles; hence being able to detect these changes is an important achievement. The simplicity of the method, as well as the information rich output and individual particle measurement indicate the allure of such a sensing system. As these types of DNA sensors are quite novel many limitations are still to be overcome before a practical sensor may be developed. This work, however, demonstrates a significant step towards target DNA sensors using synthetic nanopore resistive pulse sensing.

5.7 Enhancement of Signal and Potential Future Work – Incorporating Quantum Dots

Enhancement of the sensor response will aid in the sensitivity of the platform. One way in which this may be performed is by altering the salt concentration of the electrolyte solution. A lowered salt concentration may cause less shielding of charges and subsequently enhanced signal. Interesting results were demonstrated by Smeets et al. and Steinbock et al. following lowered electrolyte salt concentration. Care must be taken however, as lowered salt concentration also produces decreased particle frequency and lowered (or even inverted) current drops.
In order to enhance the change in properties of the particle post target hybridisation, a sensing platform was proposed which involved a third ON strand, denoted the detection probe, Scheme 5.2. This detection probe is attached to a quantum dot. The idea was that the particle-DNA-QD complex would possess more negative charges as well as a larger size, therefore enhancing the changes observed with SIOS sensing. Moreover, the fluorescence of the QDs would provide another method for sensing, as well as the possibility of testing for multiple target DNA sequences where QDs of different emission wavelengths would be functionalised with different detection probe DNA sequences. Wang et al. successfully demonstrated a multiple target sensing QD system using electrochemistry as the DNA detection technique.\textsuperscript{71}

Scheme 5.2 Overall scheme for sensing enhancement with quantum dots. Firstly the particles are probe grafted, followed by target hybridisation (where the target is longer than the probe ON), and lastly incubation with detection probe modified quantum dots.

Modification of the QDs was performed using EDC/NHS chemistry (section 2.9.3.1). Testing was then performed for:

- Non-specific binding of non-complementary DNA to the QDs. This was done using Cy3 modified non-complementary DNA and using fluorescence spectroscopy to examine whether Förster resonance energy transfer (FRET) between the Cy3 (Ex
Chapter 5

512:550, Em 570:615) and the QDs (Ex 495, Em 525) occurred. If FRET occurs the two are in close vicinity, hence non-specific interactions are present.

- After incubating probe grafted QDs with target DNA incubate with ethidium bromide (EB, Ex 495 nm, Em 620 nm). Subsequently use fluorescence spectroscopy to excite the QDs at a lower wavelength (400 nm) and examining EB emission. EB is known to intercalate between double stranded DNA, hence if the signal is increased after target incubation then FRET is occurring and a double stranded DNA is present.

These tests were performed which indicated that QD modification had occurred and was not subject to non-specific binding of non-complementary DNA.

Unfortunately analysis with the qNano indicated no difference between “Target” particles and “Target” particles following incubation with modified QDs. The belief is that this is from incomplete modification of the QDs and or insufficient concentrations of QDs used. Although this part of the project could not be completed it exists as an opportunity for further research in this field.
Chapter 6

Investigations into Testing Real Samples of Forensic Importance

Marsilea Booth
Chapter 6. Investigations into Testing Real Samples of Forensic Importance

The object of this work is to investigate samples which may be of forensic interest, such as blood, saliva and other body fluids. The composition of such fluids means there are a number of species which may adhere, block or interfere with target DNA approaching immobilised probe DNA, giving rise to false positives and or false negatives. Blood is chosen as the fluid of interest for experiments due to the vast literature, known composition, ease of collection and forensic importance. The sensing system chosen is the electrochemical PPy-based DNA sensors discussed in Chapter 3.

Therefore, the objective of this section is to:

- Examine the interactions and effect of the blood matrix with and on developed PPy-based electrochemical DNA sensors, identifying issues with false positives and false negatives specifically.

6.1 Real Sample Testing

Biosensor technology has been of growing interest for research and development, particularly with technological progress in analytical chemistry and miniaturization. Under well-controlled conditions researchers have displayed a high performance of selectivity and sensitivity. Meanwhile, only a very small fraction of research articles dealing with DNA sensors have ventured beyond synthetic oligonucleotide strands and evaluated sensor performance in real sample matrices. The commercialisation of biosensor technology has been lagging behind the research output. The lack of application of developed sensors for commercial purposes is thought to arise from cost considerations, some technical barriers such as stability, detection sensitivity, reliability, and further real sample testing required.

Being able to detect target DNA sequences from within a complex contaminant-ridden real sample, without the use of numerous sample preparation techniques would be an ideal situation for developed sensors. The sensors developed in this research are aimed at analysis
within the complex matrix of blood. The complexity of real biological samples means there are many species which can interact negatively with the sensor surface causing either false negatives or false positives. False positives, also called “type I” errors, can be defined as accepting the “alternative hypothesis” (in this case that the target DNA sequence is present) when this is not the case. Conversely false negatives (“type II” errors) can be defined as accepting the “null hypothesis” (in this case that there is no target DNA present) when that is wrong. Differences between synthetic DNA testing and real sample testing are depicted in Scheme 6.1, where there are biological sample species which may block the immobilised probe DNA, interact with the DNA, degrade the target DNA, adsorb to the sensor surface etc.

Scheme 6.1 Schemes of A) synthetic DNA laboratory testing commonly performed in DNA sensor research, and B) real biological testing. Scheme is based on the reference by Tosar et al.\textsuperscript{150}

Research by Kuralay et al.\textsuperscript{276, 277} showed the ability to detect target DNA in undiluted human serum with ~ 50 % of the signal in the absence of serum. Although the DNA can be detected at high sensitivities (pM range) a surface passivation occurred through incubation. The matrix effects of urine were also examined, with target able to be detected down to 100 pM while also displaying some signal suppression. Meanwhile, Xia et al. were able to detect 1 µM of target DNA in 50 % serum,\textsuperscript{278} while Pei et al. could detect as low as 100 pM of target DNA in 50 % serum at near the value of the original signal.\textsuperscript{279} Very high sensitivity is reported by Zhao et al. at 5 pM in 50 % serum.\textsuperscript{280} Many of these examples use serum in place of whole
blood, as do most researchers. The research discussed here, however, is aimed at use with straight blood samples to be incorporated in practical on-scene procedures. For this reason the following experiments are performed in order to examine the interaction of probe immobilised copolymer P(Py-co-PAA) with blood matrix.

Inspiration may be taken from commercial sensors used for the testing of blood glucose. Commercial glucose sensors have membranes in order to prevent proteins fouling the electrode surfaces and for other purposes. The use of membranes here may limit non-specific interactions with blood components.

6.2 Blood Components

The blood components present in the blood matrix can be seen in Table 6.1.

<table>
<thead>
<tr>
<th>Blood component</th>
<th>Description</th>
<th>Typical concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>Liquid medium containing water, proteins and trace amounts of other materials</td>
<td>About 55 % v/v</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>Oxygen transport</td>
<td>About 45 % v/v</td>
</tr>
<tr>
<td>White Blood Cells</td>
<td>Defence against microorganisms</td>
<td>About 1-2 % v/v</td>
</tr>
<tr>
<td>Platelets</td>
<td>Blood-clotting</td>
<td>-</td>
</tr>
<tr>
<td>Other blood components</td>
<td>e.g. glucose, amino acids, fatty acids, serum albumin, proteins and electrolytes</td>
<td>-</td>
</tr>
</tbody>
</table>

6.3 Incubation with Whole Blood (False Positives)

Direct incubation (section 2.12.1) of the probe grafted P(Py-co-PAA) coated electrode with whole blood will provide express evidence of whether non-specific interactions are occurring. As can be seen in Figure 6.1 a large increase in charge transfer ($\Delta R_{ct}$) is observed after whole blood incubation, where $\Delta R_{ct}$ is the change in charge transfer resistance after incubation, normalized to the charge transfer resistance prior to incubation (typically $R_{ct}$ of the probe immobilised film). The $\Delta R_{ct}$ arising from blood incubation ($\Delta R_{ct} = 5.29$) is considerably higher than the typically examined non-specific binding of non-complementary DNA ($\Delta R_{ct} =$ 162
0.07). Moreover, it is much higher than the signal observed for synthetic target DNA ($\Delta R_{ct} = 0.66$, about $8 \times$ higher). This increase may be occurring for a number of reasons, but the overall consequence is limited redox couple oxidation and reduction at the electrode surface. Suggestions for why this could be are:

- Blood components (such as proteins) are being adsorbed onto the polymer surface, either through electrostatic interactions or through other attractive forces, essentially blocking the redox couple from approaching the surface.
- The blood is interacting with the polymer surface causing degradation (either the polymer itself or the immobilised probe DNA) or causing impedance to the flow of electrons through the polymer. Essentially a chemical change or significant changes to the polymer surface may be occurring.

This displays a limitation of the platform for use as a practical blood sensor due to the large false positive signal generated. It should be noted that although a blood-specific probe is being used in these sensors, it is specific to an mRNA sequence. Within whole blood, these mRNA strands are unlikely to be free as no cell lysing has occurred, so signal generated by blood mRNA binding to the probe ON is assumed to be negligible. No cell lysing has occurred in order to simplify the experiments. The concentration of the mRNA Glyco A sequence between cells may vary, and the exact concentration is hard to determine. For this reason only synthetic target DNA was investigated.

**Figure 6.1** Resulting signal from whole blood incubation for a probe grafted P(Py-co-PAA) coated electrode. A) Nyquist plot showing results of a probe immobilised polymer surface before and after whole blood incubation, and B) A bar graph showing signal arising from non-complementary DNA, synthetic target DNA ($1 \times 10^{-4}$ M) and whole blood incubation.
Assuming blood components contribute to the increased resistance, attempts to reduce non-specific binding were investigated. Additionally a barrier layer was investigated to prevent polymer surface and blood interactions.

6.4 Attempts to Reduce Non-Specific Binding of Blood Components

6.4.1 Attempts to Remove Non-Specifically Adsorbed Blood Species

As described in section 2.12.2, a number of different methods were trialled in an attempt to reduce non-specific binding. These include an application of potentials (a positive and a negative) in fresh solution, surfactant PBS washing of the electrode surface and soaking of the electrode in surfactant PBS solution. Results from these three attempts can be seen in Figure 6.2. It was hoped the application of charge may repel any electrostatically adsorbed species, while the surfactant solution may wash away any lightly bound species. Unfortunately none of these methods worked, and in fact increases in charge transfer were again observed. This is most pronounced after potential application and is presumably due to polymer degradation.

![Figure 6.2 A bar graph showing the changes in charge transfer resistance after attempts to remove non-specifically adsorbed species. Attempts using applied potentials and surfactant washes and soaks to remove non-specific binding were unsuccessful.](image-url)
6.4.2 Preventing the Adherence of Unwanted Blood Species to the Polymer Surface

As methods to remove adsorbed species were unsuccessful, the focus was shifted to prevent the initial adsorption. Research by Odaci et al. described the use of a membrane of dialysis tubing to cover the electrode surface to entrap cells for use in a bacterial biosensing platform.\(^{283, 284}\) The idea here was to use the dialysis tubing in the opposite manner, to prevent unwanted adhesion of blood species. As seen in Figure 6.3, an increase in charge transfer resistance is still observed, though it is much smaller than direct whole blood incubation.

Research by Scampicchio et al. describes using nanofibrous membranes (NFM), namely nylon mesh, to act as selective barriers to prevent unwanted interactions.\(^{205}\) They report that NFM coated electrodes have sustained activity as compared with uncoated electrodes after immersion in solutions containing contaminants.\(^{204, 205}\) A/Prof Paul Kilmartin, having visited the group mentioned, provided a sample of NFM. This was also used to try to prevent unwanted proximity between blood components and the probe immobilised P(Py-co-PAA) coated electrode surface. Results suggest that NFM membranes are less effective at preventing blood species adsorption than dialysis tubing, Figure 6.3. However, it should be noted that the NFM membrane is harder to manage than dialysis tubing, so incomplete electrode coverage may have contributed to the higher change in charge transfer resistance observed. The large variation in results using NFM coverage attests to this being an issue.

Dialysis tubing is suggested to reduce non-specific binding of blood components by its property of semi-permeability (blocking proteins of molecular weights higher than 12,000). Size exclusion of larger blood species means a number of unwanted interactions are prevented.
Figure 6.3 A bar graph showing the changes in charge transfer resistance after attempts to prevent unwanted interactions between the blood and the electrode surface. In this case it can be seen that dialysis tubing is a more efficient blocking membrane.

### 6.5 Combined Blood and Synthetic Target Testing

From the experiments performed it was determined that the most successful method for limiting the effects of non-specific interactions on the sensor performance was use of a dialysis tube membrane. Therefore further experiments used dialysis tube membranes.

#### 6.5.1 Synthetic Target DNA Incubation with and without a Dialysis Membrane (Positive Control)

It is important to examine whether a dialysis membrane limits the hybridisation of target DNA to the probe DNA immobilised on the polymer surface. An experiment was hence performed by incubating a dialysis membrane-covered probe immobilised polymer surface with synthetic target DNA. The results were then compared to previously-performed synthetic target DNA incubations (without the dialysis membrane). The results (Figure 6.4) show that dialysis tubing does not hinder the hybridisation of synthetic target DNA to the probe immobilised polymer surface, and hence is a viable membrane for this system.
6.5.2 Incubation of Synthetic Target DNA with and without Blood (False Negatives)

Interferences by blood species as well as false negatives also need to be examined. The blood species may prevent target hybridisation, either through degradation to the target DNA in solution or through interactions with the probe ON. To this cause an experiment was performed by incubating whole blood simultaneously with target DNA. Results, Figure 6.5, show that a response similar to that of just synthetic DNA is achieved with the combination. It can be seen that a considerable variation in results was observed. This could arise from changes in blood composition, ambient temperature, polymer surfaces and so on, and serves to highlight the complexity of the blood matrix itself as well as its polymer interactions. As all error bars overlap, no direct comparisons can be made. However, in looking specifically at experiments with a single electrode the following can be suggested: a slight increase in signal was observed after the concurrent incubation of blood and synthetic target DNA as compared to solely blood.

In order to confirm this, the incubation was broken into two steps. Firstly the probe immobilised P(Py-co-PAA) and dialysis membrane coated electrode was incubated with whole blood (then washed) and subsequently incubated with synthetic target DNA. An increase in response was observed, Figure 6.5, though again not statistically significant. This suggested that target DNA was still contributing to sensor response. This ‘extra’ response does not equate to the sensor response from only the target DNA, however, suggesting that blood species are interfering with the sensor and or that the response lies outside the linearity of the sensor detection.
Figure 6.5 Experiment showing sensor response to four different scenarios. A bar graph is shown above and a descriptive scheme below. The black dashed line represents dialysis tubing.

### 6.6 Discussion and Conclusions

The purpose of this chapter was to examine the effect of the blood matrix on developed DNA sensors. The preliminary study performed here has certainly highlighted issues with interactions between the blood matrix and sensor surface. Indeed blood generates a significant increase in the charge transfer resistance of the electrode. This can be reduced to
an extent by inclusion of a dialysis membrane between the whole blood sample and the electrode surface. The dialysis membrane was found not to affect the hybridisation of synthetic target DNA.

Investigation into incubations of both blood and target DNA together revealed that both may contribute to sensor response. The challenge then becomes whether we can separate the signal generated by the target DNA from that of the background blood matrix. A second baseline, background or compensating electrode may be included to this effect. On this electrode a non-blood-targeted probe may be immobilised which should show no specific binding with blood. Subsequently the difference between the background electrode and the working electrode (with blood specific probe) should equate to target signal. However, the signal generated by target DNA following blood incubation is not as high as the signal arising from solely target DNA. From this it can be seen that more research needs to be performed to minimise the non-specific interactions before a sensor of practical use can be developed.

Additionally there was quite a lot of variability in results, which could arise from changes in blood composition, changes in ambient temperature, changes in the polymer surface etc. Therefore it is believed that there is complex mixture of reactions going on at the electrode surface.

Deepened understanding of any interactions and reactions between the blood species and the electrode surface is required. This can be done by in-depth analysis of the polymer surface following incubation e.g. Raman spectroscopy, ATR-FTIR, elemental analysis etc. Indeed these techniques may also help to identify any changes to the polymer itself through degradation. Despite there being many problems still to address, this study helps to guide subsequent research and identifies areas for further investigation.
Chapter 7

Summary and Future Directions

Marsilea Booth
Chapter 7. Summary and Future Directions

7.1 Conclusions and Limitations

The objectives listed in Chapter 1 and at the beginning of each results chapter were set out as follows: 1) to develop, characterise and understand a stable, sensitive, selective, and robust DNA sensor platform, 2) to investigate the effects of metal-ion implantation into PPy, in particular with polymer stability, followed by development of a novel DNA sensor, 3) investigate the ability of detecting target hybridisation on particle surfaces using resistive pulse sensing, and lastly to 4) examine the interactions and effect of the blood matrix with and on developed PPy-based electrochemical DNA sensors, identifying issues with false positives and false negatives specifically.

The key contributions of the thesis are discussed below:

1) Electrochemical PPy-based DNA sensors were developed which showed stability, selectivity and adequate sensitivity. Optimisation of the polymer growth conditions was performed in order to increase stability and robustness, followed by investigations into factors affecting sensor response. Sensor response was measured using EIS. The dopant used influenced sensor response, with PSS providing the most stable copolymer as well as a higher sensor response. The length of the target DNA as well as the length of the immobilised probe affected sensor response in that longer DNA strands gave rise to increased sensor response. This data was explained using a patch model based on the Donnan exclusion principle, describing the influence on sensor response of the fixed charge concentration within polymer pores. It represents a step towards real sample analysis, as longer DNA strands were investigated instead of the shorter model genes often used in research. A second redox couple of hydroquinone and benzoquinone was investigated in place of ferri/ferrocyanide to examine the effects on sensor response. Results furthered the understanding of the sensor surface as well as highlighting the pros and cons of each redox couple. The ferri/ferrocyanide redox couple was found to be most appropriate for the sensing system used here. Analysis of this sensing platform with affecting parameters helped to better understand the sensor surface and interactions with solution species.
Indeed real sample analysis was performed, whereby developed sensors were investigated with the body fluid blood. This was performed directly and with a set of preliminary control experiments to try to examine blood and sensor interactions. A number of issues were highlighted with interactions between the blood matrix and sensor surface. Attempts to eradicate these interactions were unsuccessful, although reductions in the interactions were achieved. A dialysis membrane between the sample and electrode surface aided in limiting non-specific interactions. It was found that both the blood and target DNA were able to contribute to sensor response. Although only preliminary experiments could be performed and the identity of the interactions with blood species could not yet be confirmed, these experiments represent a step towards testing such DNA sensors with real samples. They also serve to highlight the areas where more research needs to be done before any sensors such as these can be used in practical applications.

2) Implantation of platinum and lead ions within PPy films was performed. Depending on the implanted fluence and the ion being implanted, changes such as altered DC conductivity, radical cation and dication concentrations, polymer degradation levels and electrochemical properties were observed with ion implantation. The optimal results were observed at a fluence of $2 \times 10^{16}$ Pt at. cm$^{-2}$ and $(2 \text{ and } 20) \times 10^{14}$ Pb at. cm$^{-2}$. At these fluences increased conductivity and retained conductivity over time were recorded. Additionally, a simple, selective and sensitive DNA sensor was developed using a Pt implanted film. As well as imparting increased conductivity, the platinum served as an anchor point from which to immobilise hairpin probe DNA strands. EIS was used to detect target DNA in solution as the two strands hybridised. A successful and selective DNA sensor was demonstrated.

3) A third DNA sensing platform was developed involving the use of resistive pulse sensing and modified particles. SIOS technology together with statistics as well as a variable pressure method was used to detect the differences between probe grafted particles and the same particles following target hybridisation. The validity of the sensing platform was confirmed through PALS zeta potential measurements and fluorescence measurements with fluorescently labelled target.

The research project described here shows the early stage development of a sensor ambitiously tasked with the identification of body fluids in forensically important samples. A number of limitations were experienced including stability issues, reproducibility issues and
aggregation issues amongst others. One of the key limitations of developed sensors here is the sensitivity. Unfortunately although three sensing platforms were developed, none were sensitive enough for real forensic sample testing for RNA or DNA. All three sensors showed promise in the micromolar detection range, which with the DNA used equates to approximately milligram per millilitre concentrations of DNA. In terms of mass of DNA in the 40 µL examined for the electrochemical DNA sensors, this equates to the working range of the sensor lying between approximately 10 and 100 micrograms of DNA. In a typical forensic stain the amount of RNA present may be considerably less than this. For whole human blood typical values collected using kits are 13-50 µg DNA mL$^{-1}$.\textsuperscript{282} This highlights a required improvement in the sensitivity of sensors before any practical working sensors can be developed.

### 7.2 Future Directions

As stated, a marked improvement in sensitivity is required before practical sensors for fluid identification in forensic samples can be developed. This could be achieved in a number of different ways. Miniaturisation of the sensing platform may aid in increasing the sensitivity of the sensors through the benefits hemispherical diffusion is able to provide over the planar diffusion of macroelectrodes, improved surface area to volume ratios, improved properties of polymer films grown on microelectrodes, and improved ratios for the size of the biological entities used (DNA and RNA) as compared to the polymer features. This serves to highlight the advantages possible with miniaturization.

Another option would be to increase the sample processing prior to DNA sensing. Following purification, the polymerase chain reaction (PCR) could be used to increase the concentration of target DNA or RNA until sufficient quantities are present for analysis. However, this requires both time, consumables and may introduce the problem of increased contamination (as although PCR increases target DNA concentrations it may also increase contaminant DNA concentrations). Additionally these sensors may not be applicable for on scene analysis due to the instruments required for PCR analysis, though recent advances in microfluidic PCR arrangements may solve this. Indeed, developed isothermal amplification technologies may be coupled to the sensors developed herein to produce useable sensors.
Stability improvements may also be required before practical sensors can be developed. Although stability has been examined here, a deeper understanding of the degradation and changes to PPy films may aid in stability improvement. Until stability issues are resolved, EIS measurements in supporting electrolyte solutions (in the absence of redox couples) would be favourable in order to characterise the conducting polymer surface for each experiment. This was not performed for every experiment here, unfortunately. Moreover, real sensors often require a defined shelf-life of months of stability in conditions such as room temperature and air exposure. Aging of the polymer film will affect sensor response. Therefore a conducting polymer such as poly(3,4-ethylenedioxythiophene) (PEDOT) may be targeted, which has shown improved stability over PPy.

In addition, further examination into real sample testing should be performed, where the exact interactions between the blood matrix and the sensor surface are examined in-depth. Techniques such as elemental analysis, Raman, ATR-FTIR, and X-ray Photoelectron spectroscopy may be used. Further or improved methods to prevent non-specific interactions between the surface and the blood species would be advantageous, with inspiration from commercial glucose sensors. Furthermore the baseline or background electrode used in commercial glucose sensors could be mirrored here allowing signal generated from the blood matrix to be separated from signal generated by true target.

For SIOS sensing, improvements need to be made with regards to aggregation and extracting suitable experimental and modification conditions. The blocking of pores can prove a significant problem, so ways of reducing aggregation would be highly beneficial. One of these could be to alter the chemistry of the nanopore thereby reducing interactions between particles and the pores. Another may be to improve the particle surface modification (increasing the charge on the particle and therefore ideally the zeta potential producing a more stable suspension), and also the homogeneity of the particle modification (allowing a lower spread of distribution for parameters). An enhancement technique such as the one suggested in Chapter 5 with quantum dots, would also develop the sensing platform. Another improvement would be to include the reference particles within the sample solution in a similar manner to internal standards used in analytical chemistry.

Additionally, ideal commercial DNA sensors are either reusable or disposable, the former requiring high stability and robustness and the latter requiring cost effectiveness in sensor
manufacturing. Therefore, investigating the reusability of the sensors described here would be useful, or alternatively the cost effectiveness of production examined. Surface regeneration experiments should be performed with in-depth analysis of the surfaces, including changes in morphology, polymer degradation, probe damage and orientation etc.

Finally, once a stable, selective, sensitive and robust sensor has been developed, introduction of an array system would stride towards achieving this thesis’ aim. The ability to, in parallel, test for multiple body fluids would be a valuable property, as would information about the concentration ratios between the body fluids. Such a DNA sensor is not limited to forensic sample analysis, and would no-doubt prove useful for many applications including medical diagnostics, food analysis, and pathogen analysis amongst others.
Appendix A
and References
Appendix A

Technical Descriptions of General Characterisation Techniques

**Nuclear Magnetic Resonance (NMR) Spectroscopy**

Nuclear magnetic resonance (NMR) spectroscopy involves investigation of the nuclei of atoms for their absorption abilities, by firstly placing them in an intense magnetic field. The assumption is that certain nuclei possess the property of spin. NMR relies on the induced transition of nuclear spin. Irradiation of the sample with energy corresponding to the exact spin state separation will cause transitions in the presence of a strong external magnetic field. When this field is applied electrons move in response to the field, generating local magnetic fields that oppose the applied field. The local fields then “shield” the proton from the applied magnetic field, which must therefore be increased in order to achieve resonance. The resonant frequency, energy of the absorption and intensity of the signal are proportional to the strength of the applied magnetic field. This allows analysis of the physical and chemical properties of atoms or the species they form.

**Scanning Electron Microscopy and Transmission Electron Microscopy**

Scanning electron microscopy (SEM) involves directing a beam of electrons in a raster pattern at a sample surface in order to capture images. Initially the electron beam is passed through electromagnetic lenses - namely a condenser lens, which serves to focus the beam onto the desired spot, and an objective lens which allows magnification. Interactions between the electron beam and the sample atoms cause further species’ (particles, X-rays, ion/atoms, photons and primary-, backscattered-, Auger- and secondary electrons) emission processes. The scattering of emitted electrons can either occur elastically (involving no energy losses) or in-elastically. Back-scattered electrons (BSE) which are elastically reflected from sample surfaces can be detected using a BSE detector. The intensity of the BSE signal is strongly related to the atomic number of the sample species; hence it can be useful for identifying the distribution of heavy atoms, such as platinum amongst carbon and nitrogen species. Secondary electrons that are in-elastically scattered can be detected using a secondary electron (SE) detector. Additionally, in an energy-dispersive spectrometry (EDS) SEM the photons emitted by the sample are collected and measured. In this way, the obtained signal is
proportional to both the energy and the intensity of the electron beam, allowing information regarding species’ identity and concentrations.

Transmission electron microscopy (TEM) involves a beam of electrons being transmitted through the sample, while interacting with said sample as it passes through. The interactions allow an image to be formed of the electrons transmitted through the specimen. As with SEM, the same electromagnetic lenses are involved in TEM set-up, as well as projector lenses. These serve to focus the beam onto a plane, which can be photographed or stored.

UV-Vis Spectroscopy
Ultraviolet-visible (UV-Vis) spectroscopy is a type of absorption spectroscopy in the ultraviolet to visible spectral region. The range of wavelengths examined typically varies from 190 to 210 nm to an upper wavelength extreme of 800 to 1000 nm. Typically bandwidths vary from 2 to 8 nm, while wavelength accuracies of ± 0.5 to 2 nm are reported. In this way, molecules are able to absorb energy from the incoming light, exciting electrons to higher anti-bonding molecular orbitals. The wavelengths absorbed provide information about the electrons, and hence the species present. The Lambert-Beer law (Equation 4) is used to provide quantitative analysis in UV-vis spectroscopy.

\[ A = \log \frac{P_0}{P} = \varepsilon bc \]

Equation 4

Where \( A \) is absorbance, \( P \) is the power of the transmitted radiation, \( \varepsilon \) is the extinction coefficient (litre mol\(^{-1}\) cm\(^{-1}\)), \( b \) is the beam path length (cm), and \( c \) is the concentration of the absorbing species (mol L\(^{-1}\)).

Fourier Transform Infrared Spectroscopy (FTIR) and Attenuated Total Reflection FTIR (ATR-FTIR) Spectroscopy
Infrared absorption, emission, and reflection spectra for molecular species can be explained by the assumption that various changes in energy brought about by transitions of molecules from one vibrational (or rotational energy) state to another will give rise to such spectra. These spectra can be used to identify the species present. The data is displayed typically as absorbance or transmission versus wavenumber (cm\(^{-1}\)). A linear wavenumber scale is used due to the direct proportionality between wavenumber and both energy and frequency. When
a molecule is irradiated with IR radiation, the molecule transitions from one vibrational energy state to another. The specific wavelengths of the absorptions to cause the transitions are characteristic to the species present. Therefore resulting FTIR spectra provide information about the functional groups present as well as the general configuration of the molecule. Despite the information-rich output, it should be noted that unambiguous establishment of species identification and structure can be elusive as uncertainties frequently arise from band overlaps, spectral variations, and instrumental limitations.

Attenuated total reflection FTIR (ATR-FTIR) allows analysis of more difficult samples, such as solids, films, pastes etc with less sample processing. The property of total internal reflection is used, whereby the incident beam enters a crystal or plate, which is in contact with the sample, and subsequently (due to the different refractive indices and densities between the two) is reflected internally between the sample and the crystal (if the angle of incidence is greater than the critical angle). During this reflection the radiation is able to penetrate the sample surface a small distance (then called the evanescent wave). If the less dense medium absorbs the evanescent wave, attenuation of the beam occurs at absorption band wavelengths. In this way the absorption bands of the sample can be collected.

Raman Spectroscopy
Raman spectroscopy relies on the fact that after irradiating a sample with a powerful laser source of visible or near-IR monochromatic radiation, scattered radiation can be collected at some angle (typically 90°) by a suitable spectrometer. The scattered radiation exhibits properties different to the irradiated source (inelastic scattering) caused by sample interactions. Therefore information about the surface species can be collected from the scattered radiation. The two techniques Raman spectroscopy and FTIR yield similar information but are complementary techniques. A major advantage of Raman spectroscopy over FTIR lies in the fact that water does not cause interference.

Surface-Enhanced Raman Spectroscopy (SERS) is also possible, allowing enhancement of Raman signal. Enhancement of Raman lines can be observed for samples that are adsorbed on the surface of colloidal metal particles or rough metal surfaces (e.g. silver or gold).

Rutherford Backscattering Spectrometry (RBS) Analysis
Rutherford backscattering spectrometry (RBS) is a technique well suited for thin films. The working principles of RBS involve the following: the energy of the elastically scattered beam
is proportional to the mass of the target nuclei, secondly the cross section (yield of backscattered particles) is proportional to the elemental composition of the target material, and lastly the incident beam and the backscattered particles lose energy as they pass through the sample.\textsuperscript{264} In this way, the scattering events can be matched for depth in the sample and energy level in the spectrum. This allows investigations of elemental concentrations of matrix components.

**Fluorescence Microscope and Fluorometry Experiments**

Photoluminescence is a term to explain both fluorescence and phosphorescence, which themselves are processes in which excitation is induced by the absorption of photons. Molecules absorb photons, raising them to an excited state. The molecules then re-emit photons as they return to ground state. Fluorescence is relatively short-lived ($<10^{-5}$ s) due to the fact that the electronic energy transition responsible for the fluorescence does not involve a change in electronic spin. Meanwhile, with phosphorescence emissions an electron spin change occurs, causing the radiation to endure for longer.\textsuperscript{178} The absorption of photons almost always involves excitation of electrons from the lowest vibrational level of the ground state, into a higher vibrational level of the first excited singlet state. Then as the molecule relaxes back to the ground state, photoluminescence may occur, as well as some non-radiative relaxation pathways such as dissipation of heat or neighbouring molecule interactions.

The majority of experiments involving fluorescence in this research involved the use of a fluorescent label, fluorescein isothiocyanate (FITC), Figure A.1.

![Chemical structure of the fluorescent label, fluorescein isothiocyanate.](image-url)
Elemental Analysis

Elemental analysis is performed based on a method involving complete and instantaneous oxidation of samples by “flash combustion”. In this way, all the organic and inorganic substances in the sample are converted to combustion gases. These are subsequently passed through a reduction furnace and swept into a column in which they are detected by the thermal conductivity detector, giving an output signal proportional to the concentration of the individual components of the mixture.
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