Conductive Nanocrystalline Cellulose Polymer Composite Film as a Novel Mediator in Biosensor Applications

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science in Forestry

Faculty of Forestry
University of Toronto

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Abstract

Recent biosensors using the glucose oxidase enzyme to detect glucose ("blood sugar") were made with intrinsic conducting polymers such as poly pyrrole (PPY) to mediate the reaction. PPY coated electrodes were difficult to employ via electropolymerization because PPY is only soluble in solvents that are potentially damaging to the enzyme. Nano crystalline cellulose – poly pyrrole (NCC-PPY) colloid circumvents this by forming natural, enzyme compatible and hydrophilic films that can be mechanically bound to the electrodes and the colloids are easy to disperse. NCC-PPY was used in this study as mediator to investigate its use in biosensor applications. Using the NCC-PPY film casted on microfabricated interdigitated electrodes, a glucose biosensor with sensitivity factor of 20 was achieved. As a mediator, NCC-PPY showed enhanced catalysis with no enzyme inactivation and a total current of 2mA. Enhanced sensitivity was attributed to the combination of resistance changes of doped PPY, redox mediation of the reaction and compatibility of the cellulose with the enzyme.
Acknowledgments

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Table of Contents

Acknowledgments .................................................................................................................. iii
Table of Contents .................................................................................................................. iv
List of Tables ........................................................................................................................ viii
List of Figures ....................................................................................................................... ix
List of Appendices ................................................................................................................ xii
Chapter 1 Literature Review ................................................................................................. 1
  1 Introduction ....................................................................................................................... 1
    1.1 Glucose Biosensors ....................................................................................................... 2
    1.2 Device Specifications and Performance ....................................................................... 3
2 Microelectronic Glucose Biosensors ................................................................................... 4
  2.1 Enzyme Layers ............................................................................................................. 6
    2.1.1 Enzyme Reaction Mechanism ................................................................................ 6
    2.1.2 Quantifying the Enzyme Reaction ......................................................................... 10
  2.2 Electrode Layer ........................................................................................................... 10
    2.2.1 Interdigitated Electrode Array .............................................................................. 11
    2.2.2 Fabrication of Interdigitated Electrode Array ...................................................... 14
    2.2.3 Electrode Micro/Nano Fabrication ...................................................................... 15
  2.3 Mediation Layer ......................................................................................................... 17
    2.3.1 Mediator Materials ............................................................................................... 18
3 Nanomaterials ................................................................................................................... 20
  3.1 Nano Crystalline Cellulose Conducting Polymer Composites .................................... 21
    3.1.1 Properties of NCC-PPY ...................................................................................... 21
Chapter 2 Thesis Objective and Approach ........................................................................... 27
Chapter 3 Experimental Methods ......................................................................................... 30
4 Materials and Apparatus ..................................................................................................................30
  4.1 NCC-PPY Synthesis and Enzyme Activity testing .................................................................30
  4.2 Micro/Nano-fabrication .............................................................................................................30
  4.3 NCC-PPY film characterization ...............................................................................................31
5 Enzyme Preparation ..........................................................................................................................32
  5.1 Compatibility and Activity Test for Enzyme Glucose Assay ..................................................32
    5.1.1 Enzyme Deposition on Solid Substrates ............................................................................33
  5.2 Nanocrystalline Cellulose – Poly Pyrrole Composite Characterization .................................33
  5.3 Inter-Digitated Array Electrode Fabrication ...........................................................................35
    5.3.1 Wafer Preparation .............................................................................................................35
    5.3.2 Photo/Electron Beam Lithography ..................................................................................35
    5.3.3 Lift-Off .............................................................................................................................36
  5.4 Current Voltage tests ...............................................................................................................38

Chapter 4 Results and Discussion ....................................................................................................61

6 Enzyme Compatibility with NCC-PPY ..........................................................................................61
  6.1 Material Compatibility ..............................................................................................................63
    6.1.1 Inhibition Verification .......................................................................................................63
  6.2 Enzyme Deposition Process .....................................................................................................68
    6.2.1 Effect of Drying Conditions and Enzyme Deposition Method on Enzyme Activity ........68
  6.3 Enzyme Immobilization Techniques .......................................................................................71
    6.3.1 Effect of Glutaraldehyde Fixing ....................................................................................72
  6.4 NCC-PPY Colloid vs PPY:PSS Colloid ....................................................................................74

7 NCC-PPY Film Deposition ..............................................................................................................75
  7.1 Film morphology ......................................................................................................................75
  7.2 Atomic Force Microscopy Images .............................................................................................75
10 Appendix A: List of Acronyms .................................................................117
11 Appendix B: Glossary of Terms ..............................................................118
12 Appendix C: Post-Bake and Processing ....................................................119
    1.1.1 Bi-Layer Photolithography ..........................................................124
List of Tables

Table 1  Comparison of advantages and disadvantages of enzymatic biosensors
Table 2  Property comparison between NCC, PPY, and NCC-PPY composite
Table 3  Effects of glycerol additives on sheet resistance
Table 4  Lift-Off strip techniques for gold removal on IDA electrodes
List of Figures

Fig 1. General Structure of Enzyme Glucose Sensor as a field effect transistor

Fig 2. Cyclical Redox reaction equation for Glucose Oxidase mediated electrodes

Fig 3. Photograph of Inderdigitated Array Electrode with 5mm digit width

Fig 4. Electric fields of Enzyme cyclical reactions diffusing across nano-IDA electrodes

Fig 5. Model of actual commercial IDA electrode by Layer Laboratories

Fig 6. Pictorial diagram of Lift-Off procedure in micro/nano fabrication

Fig 7. Process flow diagram of Lift-Off procedure in micro/nano fabrication

Fig 8. Cross section of poly pyrrole penetration in cellulose poly-pyrrole composites

Fig 9. Possible hydrogen bonding structure of conducting polymer cellulose composites

Fig 10. General structure of Glucose Biosensor electrode construction (side-view)

Fig 11. Thesis Research Strategy process flow diagram

Fig 12. Schematic example of Inderdigitated Array Electrode top view

Fig 13. Comparative graph of effect of enzyme activity and glucose concentration with NCC-PPY film

Fig 13. Possible fiber arrangements of NCC-PPY when deposited as a film across IDA electrodes

Fig 14. Microscope image of IDA electrode pattern 1 um in width after development

Fig 15. Pictorial diagram of bi-layer lift-off for micro/nano fabrication

Fig 16. Microscopic images of E-Beam exposure dose test with LOR-ZEP

Fig 17. Photo of developed wafer after E-Beam development revealing optical grating
Fig 18. Photo of gold coated wafer prior to lift-off

Fig 19. SEM photos of 100nm IDA lines after lift-off

Fig 20. UV exposure tests on S1811 photoresist for development

Fig 21. Image of developed LOR with 10 um width IDA electrode

Fig 22. Structure of ABTS and TMB

Fig 21. Image of overdeveloped IDA in lithography development

Fig 23. Enzyme activity as a function of glucose concentration

Fig 24. Comparison of drying conditions on enzyme activity

Fig 25. Enzyme activity with Glutaraldehyde immobilization in the presence of NCC-PPY

Fig 26. Comparing activity of enzyme with NCC-PPY and buffer vs. PPY:PSS

Fig 27. Photo of PPY:PSS deposited over silicon substrate

Fig 28. Atomic Force Microscopy images of NCC-PPY film over 100 um x 100 um area

Fig 29. Atomic Force Microscopy of 20 um x 20 um area with 3 dimensional modeling

Fig 30. Atomic force microscopy of NCC-PPY after image renders processing

Fig 31. Scanning Electron Microscopy of Glycerol added NCC-PPY film cross section

Fig 32. Electrical Resistance of NCC-PPY vs. NCC alone films

Fig 33. Graph of sheet resistance vs. concentration of NCC-PPY colloid

Fig 34. Graph of thickness and roughness of NCC-PPY surface relative to mass

Fig 35. Spin curve of cellulose Acetate over NCC-PPY film
Fig 36. Probe station setup used for I-V testing

Fig 37. Sensor current response to varying glucose concentration

Fig 38. Normalized resistance change comparing glucose response to control solution

Fig 39. Sensor current I-V characteristic with different thickness layers of NCC-PPY

Fig 40. Development of photoresist after hard baking showing resisted etching

Fig 41. Image of protective film growth on photoresist without hard baking

Fig 42. Photo of LOR development going through the cracks of impermeable photoresist

Fig 43. Image of Oxygen plasma treated

Fig 44. Pattern of overexposed IDA in LOR
List of Appendices

Appendix A: List of Acronyms

Appendix B: Glossary of Terms

Appendix C: Post-bake and Processing
Chapter 1
Literature Review

1 Introduction

Biosensors are commonly used to measure and detect biological chemicals. They can detect a wide range of analytes, such as glucose, urea, and estrogen found in human serum or tissues [1]. Glucose biosensors, for example, detect glucose (“blood sugar”) and are routinely used for medical laboratory testing, especially for diabetics who require continuous blood sugar monitoring. Dangerous blood sugar is at 3mg/mL. Fermentation detection can be higher. Glucose biosensors describe a class of devices that can turn a chemical glucose concentration into an electrical signal. Many types of glucose biosensors have been developed, such as transistors, electrochemical cells, screen printed electrodes, optical readers, and flow injection cells [2]. One of the most promising groups of emerging biosensors is microfabricated electrodes and transistors. Their smaller size allows the device to be integrated onto microchips. The device that couples biochemical sensing with microfluidic channels is known as a “Lab-on-a-Chip” device. Such devices can diagnose multiple diseases and measure several biological agents all on a microchip. This enables medical professionals to run hundreds of tests faster with smaller sample volumes [3]. This means more efficient testing diagnosis, less invasive techniques, and less technologist dependency. While there are types of tests, glucose testing is mostly wide used because of the large number of these tests performed every day. Glucose is tested on every standard blood test as well as is monitored continuously for diabetics [4]. The need for minimally invasive techniques performed at home is an important motivation behind glucose biosensors development activities.

There is significant amount of work has been done to develop glucose biosensors, particularly focused at the electrode [1]. Known as amperometric glucose biosensor electrodes, the electrode is the main feature as it is what interfaces glucose with the
electric current. Studies into electrode designs, such as interdigitated electrodes, are widely available. Material compositions using glassy carbon, gold and conducting polymers have also attracted much interest recently. The use of enzymes with electrodes to catalyze the chemical reaction was also looked at in depth. With growing technologies in micro fabrication such as electron beam lithography, there have been many improvements of lab-on-a-chip devices [5]. In addition, new nanomaterials present new frontiers for chemical interfaces. While original work on enzyme reactions with glucose and colorimetric detection methods were well established since the 1950s, new developments in material design and fabrication continue to push this technology bring faster, smaller and more efficient devices.

The following review will discuss the glucose biosensor technology to date, with a focus on integrated microfabricated devices on silicon based substrates, for lab-on-chip applications. Primarily, operating principles of glucose sensors will be discussed, followed by electrode material and design and then material interfaces, highlighting conducting polymers and bio-based cellulose nanocrystalline composites.

1.1 Glucose Biosensors

Glucose biosensors are devices designed to detect glucose. A sensor is defined as a device that converts a signal from a physical quantity to another form [6]. The eye, for example, is a sensor that converts an optical signal (light) into an electrical signal to be processed in the brain. Blood glucose levels are frequently used by medical professionals to help diagnose many diseases such as diabetes. As such, methods for the measurement of glucose received much attention from researchers and device designers. While many techniques to measure glucose exist, such as optical chemical measurements or assays, the scope of this review will be on amperometric glucose sensors; those that measure based on electric current [5]. Advances in microelectronic
devices will be highlighted because they are integrated with lab-on-a-chip” technology, which can make multiple readings on a single chip with microliters of human blood samples.

1.2 Device Specifications and Performance

There are key specifications and performance requirements to measure the effectiveness of a glucose biosensor. The main goal of a glucose biosensor microdevice is to measure glucose concentration in a drop of human blood or serum [7]. Mathematically it translates to an ideally linear monotonic function, correlating electrical signal (y-axis) to glucose concentration (x-axis). In reality the signal is non-linear and can be interpreted as a linear function with an added error term. In practice, the design needs to reduce this error. Key design benchmarks are signal to noise ratio, accuracy, and signal strength.

Signal to noise ratio (SNR) is a degree of measurement precision. The more the output signal increases due to the increase in glucose concentration itself, as opposed to random deviations from the system (defined as “noise”), the more statistically significant is the reading. Therefore, the bigger the SNR, the better is the device. Sensors are particularly vulnerable to low SNR because the output signal from micromolar chemical reactions is typically weak and can be easily overwhelmed by background noise [8]. Design of sensors to increase the SNR is vital, and the interdigitated array (IDA) electrode design is an approach to address this issue.

Accuracy is another measure of sensor response. Any measurement device needs to be accurate. In glucose biosensors, the straightforward approach is to compare the measured concentration with the actual concentration. In practice, one can compare the device measurement with a standard calibration test. Colorimetric enzyme assays, where the reactants change color, can be done and are akin to benchmark biosensor
devices at medical laboratories [9]. Naturally, the biosensor can be calibrated to match signal output with the expected output. To be successful, the sensor needs to produce reproducible outputs otherwise it would require calibration each time.

Output signal strength is another important parameter. Stronger signals use less costly equipment to detect the signal. Electric current signals on the order of milli amperes or micro amperes can be read by lower end “off-the-shelf” equipment and batteries. Signals in the nano or femto ampere range need advanced laboratory grade equipment that is both costly and large for portable household devices. Lower signal strength also decreases the SNR and decreases performance. Sensor designs that can maximize power output do much to improve the performance.

In summary, Micro glucose biosensors aim is to measure glucose concentration with microlitres of blood sample. Key performance benchmarks are signal-to-noise ratio, accuracy, and signal strength.

2 Microelectronic Glucose Biosensors

Microelectronic glucose sensors are devices that measure glucose and can be miniaturized onto the micron or even sub-micron level. In general they consist of at least three parts, the electrode, an active layer and a mediation layer. Electrode consists of a conducting material, typically metal, patterned to optimize collection and transfer of electrons resulting from a chemical reaction. It comprises a working and a counter electrode (cathode and anode) which interfaces with external measurement circuitry. Fabrication can be done with screen printing for large (greater than 0.1 mm) electrodes, or photo/electron beam lithography (below 10um) for smaller electrodes. The active layer is responsible for the chemical reaction that converts glucose into free electrons.
and is positioned over the electrode. Enzymes, biomolecular catalysts, are often used to react with glucose to generate charged molecules that can donate electrons, via an oxidation/reduction (Redox) reaction. Finally, a mediation layer is used to interface between the electrode and the active layer [10]. This is necessary to couple otherwise incompatible materials and/or to improve sensitivity, selectivity, accuracy, etc. An example of a glucose sensing transistor using conducting polymer mediation is shown in Fig 1.

Fig 1. [Source: [10]] Architecture of a glucose sensing transistor. Gold electrodes are patterned to connect to a cathode and an anode on an N+ silicon wafer; mediating material is PEDOT with Glucose Oxidase enzyme activating the reaction.

One example of a glucose sensitive transistor uses conducting polymer poly ethylene dioxythiophene (PEDOT) as a mediator and glucose oxidase (Gox) enzyme. In this configuration the enzyme catalyzes the glucose and oxidizes the PEDOT layer. PEDOT then changes conductivity proportionally and the drain current changes, much like a variable resistor. Applying a gate voltage, the total current is modulated so the device can be “tuned” [10]. On-off switching was not identical to a metal oxide semiconductor field effect transistor (MOSFET), however, it does demonstrate the general operation of glucose sensing as the principle application.
2.1 Enzyme Layers

Enzymes are often used as the active layer to react with glucose and direct current to the sensor electrodes. Enzymes are an important class of biomolecules found in living organisms. Glucose testing uses the enzyme glucose oxidase (GOX). Enzymes maintain homeostasis by catalyzing necessary biochemical reactions, and are highly selective and sensitive to environmental conditions such as temperature, pH, and can be particularly prone to “leeching” off of devices [11]. Nevertheless, because of their extreme selectivity and remarkable accuracy, they are often used in medical laboratory glucose tests, using in colorimetric analysis wherein the absorbance of a reaction product is measured against a calibrated sample. GOX is extracted from Aspergillus Niger and used as a standard reagent.

Enzymes in glucose measurements were extensively studied as far back as the nineteenth century, and well documented today. They are highly accurate and have been studied for over 100 years, thus have become the most attractive form of active layers to date. In micron sized glucose sensors, the challenge is using them on a small area. While "enzyme-less" electrodes were demonstrated with nickel electrodes, electrode degradation and poor selectivity were issues [12]. Hence enzyme use is still preferred in both medical lab chemical testing and in amperometric sensors.

2.1.1 Enzyme Reaction Mechanism

While enzymes can be used to react with substrates for chemical detection, they also interface with amperometric sensors. In amperometric glucose sensors, GOX catalyst breaks down glucose in the following reaction (Eqn. 1)
Glucose + O₂ + GOX\text{ox} \rightarrow H_2O_2 + D−Gluconolactone + GOX\text{red} \quad (1)

H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2 + 2e^- \quad (2)

Eqn. 1 Simplified redox reaction of enzyme breaking down glucose and generating electrons.

Glucose is broken into gluconolactone and hydrogen peroxide. At some electrodes, such as platinum electrodes, hydrogen peroxide is further oxidized into water with the release of an electron (Eqn. 2). This electron can be picked up at an electrode and detected as current [10]. In general, electrodes sensitive to equilibrium changes by the reaction produce an electric signal proportional to glucose concentration, enabling glucose detection. The efficiency of the sensor depends on the electrode design.

Fig 2. [Source: [10]] Diagram modeling the cyclic redox reactions using PEDOT:PSS mediated gold electrodes. Electrons are forwarded to the electrode via the redox reaction between glucose and glucose oxidase.
Sensor designs make use of enzyme reaction in several ways. The first is by detection of the product hydrogen peroxide. The electrode converts hydrogen peroxide to water with the release of an electron (eqn 2). Secondly, the enzyme becomes reduced at an electrode, such as one coated in PPY, to produce the required current [13]. One variation is to couple this with enzyme peroxidase which breaks down hydrogen peroxide and in the process becomes reduced as well. Lastly, a sensor electrode sensitive to oxygen concentration is placed in the solution. This electrode measures the concentration of oxygen and as it is consumed, registers it as a corresponding change in glucose. It is important to note that not all electrode materials can serve all purposes. Hydrogen peroxide oxidation reactions can only occur on some electrode materials without corrosion, such as platinum or gold. When electrode material becomes too costly or impractical, coatings of “mediator” materials are used to facilitate transfer. Analogous to coatings on decks or ohmic contacts, mediators couple enzymes with an otherwise incompatible electrode; this is discussed in more detail in a later section.
<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>- High Selectivity</td>
<td>- Requires calibration</td>
</tr>
<tr>
<td>- High Accuracy</td>
<td>- Sensitive to temperature, pH</td>
</tr>
<tr>
<td>- Well studied reagents in colorimetric studies</td>
<td>- Potential to “leech” out and cause errors if enzyme not immobilized</td>
</tr>
<tr>
<td>- Bio-compatible, biodegradable, “green reagents” naturally occurring in plant species</td>
<td>- Lifespan limited due to spontaneous deactivation and leeching</td>
</tr>
<tr>
<td>- Unlikely to interact with materials other than target substrate</td>
<td></td>
</tr>
<tr>
<td>- Can react non-destructively with many electrode materials and be reused.</td>
<td></td>
</tr>
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</table>

Table 1: Comparison of advantages and disadvantages of enzyme based sensors
2.1.2 Quantifying the Enzyme Reaction

Effectiveness of enzymatic reactions is quantified by measuring the output of its expected reaction. One method is known as colorimetry wherein the enzyme reacts with a color changing reagent, called chromogens that are detectable by optical means. Enzyme reactions and activity are dependent on several factors: analyte concentration, enzyme concentration, presence of inhibitors, temperature and pH [39]. Activity is directly proportional to the analyte concentration thus the activity can be used as an indicator of how much analyte is present, in this case glucose. This is because the overall rate of reaction is proportional to the glucose concentration and the electric current is proportional to the reaction. Hence, the resulting electrical signal in the sensor is rate-limited by the enzyme reaction. The overall reactivity of enzymes is known as its “activity”. Being the rate limiting step, it is imperative to prevent enzyme inhibition and control factors to optimize activity.

2.2 Electrode Layer

Electrodes for glucose sensors are used to collect electric current, to be read and analyzed. Design considerations include materials used and its geometric pattern. The basic requirement is that it participates in an electrochemical redox reaction. Design of electrochemical electrodes includes material, surface area and geometry. The main objective in designing an electrode for amperometric glucose sensing is to increase the overall sensitivity, change in current vs. change in glucose concentration and the overall current density. This is to increase the signal to noise ratio and require less precise, and less costly, measuring equipment. Rotating ring disk electrodes, planar, and cylindrical electrodes are examples of some electrode designs [14]. One design worth mentioning is the interdigitated array electrode because of its high current/glucose ratio particularly with enzyme layers.
2.2.1 Interdigitated Electrode Array

The interdigitated electrode array (IDA) is an efficient electrode and arguably one of the best for enzyme-based sensors [15]. It comprises two electrodes with evenly spaced, parallel “comb like” lines (digits) extending from it from each side. Digits from each electrode interlock, but never touch and alternate one after the other in an array, hence the name “interdigitated array” (Fig. 3). Critical design factors are digit width, digit spacing, and length [16].

Fig 3.[Source: http://www.ermicrosensor.com/prod01.htm] Interdigitated array from ER Microsensor. Large 5x5 mm electrode has ten pairs of interlocking fingers increasing effective surface area 10 times.
While electrochemically, any exposed reactive metal can produce electric current, IDA optimizes the current produced for sensing. Using a sample droplet for example, such as a drop of blood, the surface area is radial and confined in space. Electric currents generated by enzyme reactions are also very small, on the order of picoamperes, giving a low signal-to-noise ratio. For an IDA with 'n' digits, the effective sensor length and surface increases unit area. Secondly, the close proximity of electrodes increased the electric field density and decreases the diffusion distance for the enzyme. Zhou et al. demonstrated using IDAs significantly increases electric current generated by the electrode and increases response to glucose concentration [16]. A theory of enzyme recycling was proposed wherein, the enzyme, upon oxidization at one electrode, quickly becomes reduced again; this is because of fast migration to the nearby electrode [16]. Each one of the factors can be improved by reducing the width of the IDA digits; this in turn is improved by increasing resolution of advanced fabrication techniques.

Advantages and Disadvantages of IDA Electrodes

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>- better sensitivity per unit area</td>
<td>- more complex design</td>
</tr>
<tr>
<td>- higher currents and faster response time</td>
<td>- fabrication is more difficult at higher resolution such as nanometer lines</td>
</tr>
<tr>
<td>from enzyme recycling</td>
<td></td>
</tr>
<tr>
<td>- less power intensive</td>
<td></td>
</tr>
<tr>
<td>- better signal-to-noise ratio</td>
<td></td>
</tr>
<tr>
<td>- simple design</td>
<td></td>
</tr>
<tr>
<td>- can be printed using almost any printing or</td>
<td></td>
</tr>
<tr>
<td>lithographic technology</td>
<td></td>
</tr>
<tr>
<td>- can be used in lab-on-a-chip applications</td>
<td></td>
</tr>
<tr>
<td>like microfluidics</td>
<td></td>
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</tbody>
</table>
Fig 4. [Source: [16]]. a) Diagram of redox recycling phenomenon of enzymes on IDAs. b) Electric fields around closely spaced n-IDAs.

Fig 5. [Source: Layer Laboratories] Image of commercial IDA product from Layer Laboratories 2010. IDA spacing is 10um.
2.2.2 Fabrication of Interdigitated Electrode Array

Several methods for fabrication of IDAs exist and depend on their size. Very large patterns > 100um can be printed using inkjet or screen printing of metal layers. This is done on a variety of substrates including paper, such as a screen printed silver electrode for PPY mediated glucose sensors[17]. Bayer also employs this technique for their home glucose meters [18]. For electrodes 10 um - 100 um, photolithography can be used. This is limited to relatively more mechanically stable and uniform substrates, with polymer and semiconducting substrates like silicon wafers commonly used. 1 um features are fabricated using electron beam lithography [19, 20] or nano imprint [21].

2.2.2.1 Nano Interdigitated Electrode Array

Nano Interdigitated Electrodes (n-IDA) are IDA electrodes with digits on the nanometer scale and use special techniques for fabrication. n-IDAs are arguably more efficient because of decreased diffusion distance and higher density of electrodes per unit area however; unlike IDAs on the micron scale, they require more sophisticated nano lithography and lift-off techniques such as bi-layer lift-off. This is because the resist used in nano lithography is less thick and more difficult to strip off the substrate.
A technique known as bilayer lift-off was used to fabricate nano-lines of chromium on a silicon wafer. In this technique, a double resist layer is coated. First an E-beam insensitive layer, like poly methylglutarimide (PMGI) is coated. The second coating uses an E-beam sensitive resist such as ZEP or Poly Methyl Methacrylate (PMMA). The pattern is then written using E-beam and developed. Once developed, the first layer is developed and “etched” away to expose the substrate and produce a more pronounced undercut. This is followed by metal deposition. The bilayer stack is then stripped off to reveal the remaining pattern.

2.2.3 Electrode Micro/Nano Fabrication

General techniques in fabricating electrodes involve lift-off lithography. It is loosely analogous to spray painting; spray painting involves placing a plastic stencil with holes representing the pattern design. Paint is sprayed over the whole surface and the stencil removed. Lift-Off uses polymer film layers wherein the pattern design is carved into the polymer by exposing UV light or electron beams, via lithography. The exposed areas become soluble and only those areas dissolve in a developer solvent. Only the exposed design is dissolved. Metal is then deposited by evaporation or sputtering, coating the entire sample. Finally the whole polymer layer is stripped off with another solvent or O$_2$ plasma, revealing the final pattern (Fig 6); this final stage is known as “lift-off” (Fig 7).
Fig 6. Lift-off procedure: a) resist coat, b) expose/develop, c) metal evaporation, d) lift-off and mechanical scrub, e) final pattern left.
When it comes to IDA electrode fabrication, each step in the microfabrication process is complex with many factors. Biosensor in particular has specific requirements such as, gold electrodes to make ohmic contact with PPY (semiconducting with a work function ~6.1 eV [74]), no short circuiting and microscopic in size.

2.3 Mediation Layer

Mediation layers are chemical films that improve biosensor output by modifying the interface. While metal electrodes used in general electronics have some intrinsic glucose sensing potential, such as nickel [12], most suffer from incompatibility with enzymes or have poor electrical transfer characteristics that make them unsuitable.
alone. Like a composite material which enhances structural performance, adding polymers or materials can enhance sensing characteristics by modifying the interface to have special interfacial properties which can enable or greatly enhance the sensing characteristics of electrode materials. While some metals may be corrosion resistant, mediators can also act to improve electric current, stabilizing enzymes, filter unwanted species and make the electrode more compatible with other analytes. Mediators usually are coated on the electrode surface, between the electrode and the analyte. The type of material or chemical composition will depend on the desired property. For example, conducting polymers can enable an electrochemical redox reaction to an otherwise inert metal, such as gold. Mediators used with glucose biosensors will be discussed in the following sections.

2.3.1 Mediator Materials

There are many different materials used as mediators. The material/chemical composition depends on the desired function and specifications of the sensor. For glucose biosensors, new materials such as intrinsic conducting polymers [11], metallocenes [18], and nanomaterials [22] have been demonstrated. At times they are necessary to facilitate the reaction, while others enhance performance. The following will discuss recent developments using conducting polymers and nanomaterials.

2.3.1.1 Conducting polymers
Conducting polymers are a novel class of polymers with electrical properties and have been used as biosensor mediators. Conducting polymers, such as poly pyrrole (PPY), poly aniline (PANI) and poly ethylene dioxythiophene (PEDOT), have semiconducting properties to transfer electricity as well as the flexibility and coating properties of polymers. In biosensors, they are susceptible to electrochemical oxidation and reduction by enzymes and their bi-products are used as mediators to couple enzymes with the metallic electrode contacts. Conducting polymers generally coat the surface of a metallic electrode, which connect to detection currents.

2.3.1.1.1 Poly Pyrrole

PPY has been demonstrated as a mediator with glassy carbon electrodes. Using electropolymerization in the presence of GOX enzyme, enzyme entrapment was observed within the film. Immobilization of enzyme is important because it prevents the enzyme from leeching out, reducing its effectiveness. Thus, sensor response was increased along with lifetime, because the PPY film served as both a mediator and an enzyme immobilizer in a one-step fabrication process.

2.3.1.1.2 Poly Aniline

Poly Aniline (PANI) and its nanofibers have been used in biosensors, hydrogen peroxide and gas sensors. PANI is known as among the first discovered conducting polymers. It has three known states, depending on the polymerization procedure and
amine/imine group ratio: permagraniline, emeraldine, and leucoemeraldine; emeraldine is the most conductive and used in sensors. Hydrogen peroxide sensors were made using PANI coated electrodes in its emeraldine state. This can be used in sensors by changing their conductivity in the presence of reactive gases such as ammonia; an ammonia gas sensor made with PANI nanofibers was used on an IDA electrode where its increase in performance was attributed to the surface area to volume ratio of the nano fibers, which allowed the gas to easily penetrate and react, to increase the signal to noise ratio [8]. In glucose sensors using PANI mediators, GOX converts glucose into hydrogen peroxide to couple sensor activity.

3 Nanomaterials

Nanomaterials are materials and may exhibit new beneficial properties to sensor applications. A nanomaterial is defined as a material with dimensions on the nanometer scale [23]. Carbon nano tubes for example, are simply a rearrangement of carbon however which possess electrical conductivity and high tensile strength [24]. In sensor applications, materials such as PANI nanofibers, were used to produce novel ammonia gas sensors, which capitalize on the high surface area to volume ratio[25].

A cellulose based nanomaterial promises to create a sustainable and improved material, with potential in sensor applications. Microcrystalline cellulose is comprised of refined cellulose particles of size ~20 microns. They are used in drug delivery (i.e. pressed pellets capsules), as they are biologically inert, biodegradable and hydrophilic. It remains a powder when dry, is opaque, is not soluble in water and is used in drug delivery capsules [26]. On the other hand, nano crystalline cellulose (NCC) is comprised of “needle-like” cellulose of less than 100nm. It has high tensile strength and can form solid transparent films. It can be uniformly dispersed in water [27] while still being biodegradable. With the advent of NCC, composite materials using NCC emerged.
Notably NCC composite with PPY forms transparent semiconducting films and well dispersed colloids [Abthagir, Syed “Study of Conducting Nanocrystalline Cellulose Composites, Wood & Biofiber Plastic Composites and Cellulose Nanocomposites Symposium. Forest Products Society, 2010]. Since the NCC-PPY composites possess properties similar to PPY, in theory, it has the promise to be used as a glucose biosensor mediator.

3.1 Nano Crystalline Cellulose Conducting Polymer Composites

Nano Cellulose Conducting Polymer Composites are emerging green nanomaterials with potential to be glucose biosensor mediators. While conducting polymers such as PPY are insoluble in water and many solvents and hence, are difficult to be coated conventionally (dip-coating, spin-coating, or solution casting)[28], NCC conducting polymer composites are soluble in water. The following will review development of cellulose conducting polymer composites, for all fiber sizes including NCC.

3.1.1 Properties of NCC-PPY

As a composite material, cellulose-PPY fibers have added value together. The strengths of PPY are that they are semiconducting and have unique redox properties. An important development to note is the development of PPY Cellulose composites that have enhanced PPY itself. PPY itself is practically insoluble in common organic solvents and disperses poorly, as with many conducting polymers [29]. This is a problem for conventional coating methods such as dip coating or spin coating that rely on dissolved or evenly dispersed colloids of polymer for uniform coating. This relegates PPY thin film coatings to expensive and technician dependent techniques, like chemical vapor deposition and electro-polymerization. Furthermore, it is very structurally weak compared with common polymers like poly ethylene. Fortunately, PPY cellulose
composites overcome these problems because cellulose has high tensile strength and is hydrophilic so it will disperse easily in water [27]. Moreover, cellulose is a natural biodegradable and biologically inert polymer, which has added advantage in terms of meeting safety regulations [30]. Together as a composite, NCC-PPY composite has good potential to be used in biosensors.
<table>
<thead>
<tr>
<th>Property</th>
<th>Poly Pyrrole</th>
<th>Cellulose</th>
<th>Poly Pyrrole Cellulose Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric conductivity</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Enzyme redox compatible</td>
<td>X</td>
<td></td>
<td>X</td>
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<tr>
<td>Mechanical strength</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Water dispersible</td>
<td></td>
<td>X</td>
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Table 2: property comparison chart for materials: PPY, Cellulose, PPY-Cellulose Composite

3.1.1.1 Conducting Cellulose Fiber Synthesis

Cellulose conducting composites combine the structural advantages of biobased cellulose with conducting redox properties of conducting polymers for the next generation of mediating materials[32]. Cellulose is a natural material, found in all plant species from wood to algae. It is the most abundant polysaccharide in the world, made from glucose B-d glycosidic linkages and having a high tensile strength and hydrophillicity due to the large number of hydroxyl groups. Hydroxyl groups on cellulose readily react with amino groups of some conducting polymers and act as binding sites.
for conducting polymers. Once oxidized, the monomer forms polymer chains that entangle and penetrate the fibers, creating a strong bond between cellulose and conducting polymer. The end result is a tightly bound composite of conducting polymer and cellulose with the advantages of both.

Applications included fabrics that can discharge static electricity [34]. PPY cellulose coating on Cladophora algae was made and demonstrated mechanical actuation for use in artificial muscles [33, 34]. Because fibers also possess a high surface area to volume ratio, both gas and temperature sensors were made from conducting fabrics.

Common methods to prepare conducting polymer cellulose composites are formed by solution and chemical vapor polymerization (Fig 8). Monomer precursors are adsorbed by the cellulose with hydrogen bonding with the hydroxyl groups on cellulose. As such, monomer is required to hydrogen bond as well. Pyrrole and Aniline monomer contain exposed amine groups that hydrogen bond with cellulose (Fig 9); as such poly-Pyrrole (PPY) and Poly Aniline (PANI) are preferred. Layer-By-Layer Polyelectrolyte layers were also synthesized. Penetration studies on PPY with lyocell, cotton, and rayon, fibers show the polymer penetrates as well as coats the fibers. Composites are made by solution polymerization wherein adsorbed pyrrolle monomer is adsorbed onto the fiber via hydrogen bonding to cellulose hydroxyl groups. Upon addition of oxidants such as ferric chloride or ammonium persulfate, the monomer polymerizes on the cellulose to form the conducting composite. On some fibers such as regenerated cellulose, polymer was found to both coat the outside surface of fibers and penetrate in to the core [31].
Fig 8. [Source: [31]] PPY polymer penetrating cellulose fibers of cellulose acetate and lyocell.

Fig 9. [Source: [35]] Possible hydrogen bonding structure of polyaniline on cellulose.
3.1.1.2 Nano Crystalline Cellulose Conducting Composite Synthesis

Microcrystalline cellulose fibers still consist of highly crystalline and amorphous regions. Acid hydrolysis reacts first with the amorphous regions, leaving the less reactive crystalline structures behind. Adding concentrated sulfuric acid at 60% to microcrystalline cellulose yields nanometer sized “needle like” fibers that disperse in water. Longer hydrolysis times or concentrations can lower the aspect ratio of these needles [36]. Unlike micron sized crystalline cellulose, NCC is better dispersed in water, is semi-transparent, forms a gel in water, and makes films that are temperature and acid resistant.

Applying acid hydrolysis to microcrystalline cellulose and combining with polymerization of pyrrole monomer, one can obtain NCC-PPY colloid according to the method by Syed Abthagir in Canada Provisional Patent Application 2,703,454. It too shows needle like morphology and forms transparent films; in contrast to microcrystalline conducting polymer composites which are black in appearance and semiconducting.
Chapter 2
Thesis Objective and Approach

Glucose biosensors that use enzymes have three main sections, the electrode, the mediator, and enzyme catalyst. For each component, fabrication techniques can vary and need to be investigated. In this thesis, specifically, the effects of each of these techniques on glucose sensing performance metrics, such as sensitivity, output current, etc., is be considered. When applicable, optimization of techniques to increase efficiency is also examined. Once, the techniques are relatively refined for each component, the device is assembled and tested as a whole as depicted in (Fig 10) The overall thesis hypothesis is that a composite of nanocrystalline cellulose and polypyrrole (NCC-PPY) is an improved bio-based material over existing conducting polymer mediators. Specific objectives are as follows:

- Determine if NCC-PPY will have a redox response with enzymes GOX and HRP detectable by changes in conductivity

- Prove that NCC-PPY does not inactive enzymes GOX and HRP beyond 10% reduction in activity and therefore establish compatibility

- Fabricate and develop an optimized the techniques to fabricate micro and nano IDA electrodes using photo and electron beam lithography technologies upon which the first biosensor using NCC-PPY will be realized. This will establish that as nanofabrication technologies improve, so can NCC-PPY sensors. Also, to determine which techniques are preferable in both the design and fabrication stage of the electrodes to improve the signal to noise ratio and make it more accessible to cost-effective measurement equipment.

- Validate the advantage of using NCC-PPY on a sensor by prototyping by solution casting a water soluble film on sensor electrode without costly electro-polymerization or chemical vapor deposition.
• Demonstrate a proof-of-concept that this new method of solution casting PPY meets the base requirements for a suitable sensor prototype defined herein, as a sensor that shows a positively correlated statistically significant change in electrical response current when exposed to varying glucose concentrations. Measurement ranges will be between voltages up to 6 volts and currents greater than 1 microampere.

• Exhibit this new and novel device of enzymatic biosensor device that used solution casted NCC-PPY, it is possible to measure glucose concentrations up to the lesser of 44 mg/mL or saturation, to show its potential in blood sugar or fermentation sensor applications.

Fig 10. General Cross-sectional diagram of glucose biosensor. The bottom layer is a solid support; above the support is the metallic electrode; a mediator material coats the electrode to provide a chemical interface; the glucose solution is tested by adding a drop of solution to the sensor.

To answer this, the following will be examined on the impact of this material on each stage of fabrication, as well as on the final device in the construction of a glucose biosensor. The overall approach of the thesis study is given in Fig.11.
Fig 11. Research Strategy
Chapter 3
Experimental Methods

4 Materials and Apparatus

Several materials were used for the testing and fabrication steps. They are as follows:

4.1 NCC-PPY Synthesis and Enzyme Activity testing

Glucose Oxidase (GOX) enzyme from Aspergillus Niger, horseradish peroxidase (HRP) enzyme, o-dianisidine dihydrochloride, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Tetramethyl Benzidine (TMB) Pyrrole monomer, Aniline monomer, Microcrystalline Cellulose (MCC), Ammonium Peroxydisulfate, Ferric Chloride, Dodecyl Benzene Sulfonate (DBSA), Hydrogen Peroxide, Sulfuric Acid, and D-Glucose, were obtained from Sigma-Aldrich chemical company. Organic solvents of acetone, methanol, ethanol, isopropyl alcohol, and n-butanol were obtained from Fisher Scientific.

For Enzyme deposition and testing, UV-Vis spectrophotometer, plastic cuvettes 1cm², temperature controlled bath, micropipette capable of dispensing 20 uL accurately. Freeze dried samples using a low pressure Freeze Dryer.

4.2 Micro/Nano-fabrication

LOR liftoff-resists, CD-26 developer solution (Tetramethyl Ammonium hydroxide 2.4%) and Remover PG (N-methyl Pyrrolidone 99%) were obtained from MicroChem company. ZEP 520 E-beam resist, ZDN50 developer, and ZDMAC remover, were
obtained from Zeon Chemicals Japan. Shipley S1818 photoresist and Microposit MF-321 (tetramethyl ammonium hydroxide 1.9%) was obtained from Rohm and Haas.

Electron Beam Lithography tool EBPG 5000+ was obtained by Vistec Lithography Ltd. Design CAD for layout of E-Beam writing and photomasks were performed with N-Edit software by Tanner EDA, with E-Beam fracture using CATS software. 2 inch Photomasks were obtained from 4D Labs at Simon Fraser University and UV exposure using a vacuum contact UV exposure box. All resists (Photo, E-Beam, and Lift-Off) were coat using a spin coater and baked on a hot plate unless specified otherwise. Metal Evaporation was done using a thermal evaporator.

For imaging, SEM images were taken using a Hitachi S-5200 Scanning Electron Microscope. Visible microscopy images were obtained using a Nikkon LV Reflective microscope.

4.3 NCC-PPY film characterization

Conductivity tests were performed using a four-point probe analyzer by linear sheet resistance measurement. Thick film roughness and surface analysis was done with an Alpha Step Profilometer. Oven was baked at 60 degrees C in a convection oven.

Atomic Force Microscopy (AFM) images were obtained using a micro-thermal analysis (uTA) tool manufactured by TA Instruments.
5 Enzyme Preparation

Enzyme was prepared by dissolving dry enzyme into buffer solution. Glucose oxidase enzyme and horseradish peroxidase were dissolved in aqueous buffer solution of potassium phosphate monobasic at a pH of 6.8, set with drop-by-drop addition of potassium hydroxide base. Enzyme was stored in -4 degrees Celsius and thawed when needed.

5.1 Compatibility and Activity Test for Enzyme Glucose Assay

For compatibility studies of NCC-PPY in colorimetric studies, enzyme solution was deposited in a timed way into a solution of NCC-PPY and glucose. Test tube sets were prepared each with 1mL Glucose ranging from 0 to 20mg/mL at 5 mg/mL increments. 1 mL of Chromogen 1mg/mL of either o-dianisidine dihydrochloride, tetramethyl benzidine, or ABTS was added. Two sets were made, one with the addition of 1mL of NCC-PPY solution 1% (w/w) and the other with water. Test tubes sets were placed within a temperature controlled bath at 30 degrees Celsius. At time 0 s, Enzyme solution (GOX:HRP 1:1 ratio in buffer) was deposited using an adjustable micro-pipette capable of accurately dispensing 20 uL of into each tube sequentially at 30 s intervals and allowed to react. Following time 600 s, 2mL of 3M sulfuric acid was added to each tube sequentially in identical order as enzyme addition at 30 s intervals to stop the reaction and agitated until a stable color was produced.

Next, the contents were filtered and measured for absorbance. Supernatant was filtered using a nylon 0.2 um pore HPLC filter tip on syringe into plastic cuvettes for absorbance measurements. Absorbance was measured in the visible light spectrum. Peak absorbance wavelength was determined by scanning a test sample between 400-
600nm wavelengths. The peak was used for subsequent measures of samples. When measuring the activity of enzyme after coating, the exact procedure as outlined above was used save for the addition of liquid enzyme. Instead the enzyme coated substrate was directly added into the test tube and dropped in at the same specified time intervals and removed manually when the reactions stopped.

5.1.1 Enzyme Deposition on Solid Substrates

Enzyme was deposited in two ways: room temperature using drop casting was implemented and freeze drying. Room temperature deposition method used solution casting method. 20 uL of enzyme stock solution was dropped onto the solid substrate of SiO2 on Silicon or NCC-PPY film. Freeze drying was done by freezing the aqueous solution and sublimated in a low pressure freeze drier. The substrate upon which the freeze dried enzyme was prepared was Silicon dioxide coated silicon wafer layered with NCC-PPY film over top. Silicon wafers were 1mm thick and the silicon dioxide layer was 100nm. 100uL GOX and HRP enzyme, both at 100 U/mL, were deposited on the NCC-PPY film after it was dried and solidified. Samples were frozen prior in a freezer at -4 degrees Celsius and left overnight. When required, an additional cellulose acetate layer was coated.

5.2 Nanocrystalline Cellulose – Poly Pyrrole Composite Characterization

Microcrystalline Cellulose – PPY Composite Film

NCC-PPy colloids were prepared according to the method developed by Syed Abthagir. The detailed procedure can be found in [Canadian Patent application 2,703,454]. NCC-
PPY was deposited on a silicon wafer using solution casting. 100uL of NCC-PPY 0.5%(w/w) in water was dropped on a 1x1cm silicon wafer piece and left to dry in air. NCC-PANI was prepared in same fashion. AFM and profilometer were used to measure surface topology, roughness, and film thickness.

AFM scanning was done over an area of 100 x 100 um at a scan rate of 1 um/s rate over the surface using the contact mode AFM with a pyramidal tip. Film thickness and roughness of the films were measured across 1mm random locations using the Alpha-Step profilometer.

5.2.1.1 Preparation of Glycerol Plasticized NCC-PPY

To examine the effects of plasticizers, glycerol was added to the original NCC-PPY aqueous colloid and measured. Glycerol plasticized NCC-PPY film was prepared by adding various concentrations of glycerol to the NCC-PPY colloidal suspension and solution casting onto SiO$_2$ coated wafers. Glycerol/NCC-PPY content was varied between 5 and 25 % (w/w) dried weight. NCC-PPY plasticized with glycerol films were prepared by droplet casting a 100uL droplet on a 1x1 cm chunk of silica wafer. Conductivity was measured using the linear 4 point probe method.

5.2.1.2 Cellulose Acetate Coating and Film Measurement

Effects of Cellulose Acetate encapsulation of enzyme techniques were observed by dip coating Cellulose Acetate film. Cellulose Acetate thin films were produced through dip-coating onto wafers over enzyme and NCC-PPY layer. Powdered Cellulose Acetate
was dissolved in acetone 8% (w/w). Wafers were then dipped into the solution and slowly withdrawn at a rate of approximately 0.2 cm/s and hung to dry in room temperature. Resultant film roughness and thicknesses were measured using the Alpha Step Profilometer.

5.3 Inter-Digitated Array Electrode Fabrication

Interdigitated Array (IDA) Electrodes were fabricated by wafer preparation, lithography, and lift-off techniques.

5.3.1 Wafer Preparation

The substrate used was silicon wafers, single side polished, obtained from University Wafers. 100 nm silicon dioxide layer was thermally grown in a BTU Bruce furnace to create a transistor grade SiO2 layer. Prior to thermal oxide growth, cleaning was done with standard SC1 and SC2 wafer cleaning solutions.

5.3.2 Photo/Electron Beam Lithography

After cleaning, a polymer resist was coated to produce the printed electrode image on the wafer. Photolithography was done using a photomask and UV exposure box for the 5 um features and the contact pads. The photomask was a 4 inch soda-lime glass plate coated in 100nm chrome layer, and was laser etched. The minimum feature size was 5 um, the width of the IDA digit. The photoresist used was S1818 and spun at 6000 rpm for 1 minute and baked at 100±1 degrees Celsius on a hot plate for 30s for a thickness
of 1 um. A UV exposure dose test was performed at 30 s intervals for 120s. The optimum dose was found at 90 s, and subsequently used. Microposit MF-321 (tetramethyl ammonium hydroxide 1.9% with 1% surfactant undiluted was used for photoresist development and was developed for 180s. Features were verified under reflective microscope. Electron Beam Lithography (E-Beam) was done for features less than or equal to 1 um. E-Beam resist ZEP520 1A 1:3 dilution in Anisole was coated on the substrate through spin coating at 2500 rpm, and then baked at 200 degrees Celsius. E-Beam exposure dose test was performed from 200 uC/cm$^2$ – 400 uC/cm$^2$, with the optimum dose found at 280 uC/cm$^2$. Development was done with methyl isobutyl ketone:isopropyl alcohol 9:1 ratio and developed for 60 s. This was the case for monolayer E-Beam lithography.

For bilayer lithography, prior to coating ZEP, a layer of LOR was coated. LOR 1A was spun at 2500 rpm for 60s and baked at 200 degrees Celsius on a hotplate. E-Beam dose test was repeated, this time at 280 uC/cm$^2$ to 780 uC/cm$^2$ at 20 uC/cm$^2$ increments. The optimum dose was found at 740 uC/cm$^2$. After E-Beam image writing and development, as above, LOR was etched using CD-26 60% dilution in DI water for 60 s. Undercut was verified by a faint shadow appearing under microscope observation.100nm and 1000 nm lines were written.

5.3.3 Lift-Off

It was found that if the layer was too thin, the case with E-Beam lithography, it was difficult to perform liftoff due to the layer coating. This was especially true for smaller lines sub-micron level because the thickness of the resist needs to be similar or thinner than the lines being patterned to get a good solid definition. Although ZEP does produce an undercut, it does have poor adhesion. Nevertheless it was observed that on a silica coated substrate, liftoff was not well with. Henceforth the following solvents were tested
for lift-off: acetone followed by IPA rinse, ZDMAC resist remover, and Remover PG. Sonication was done after to introduce mechanical agitation.

When lift-off with one layer was stymied, bilayer removal was studied. The use of a bilayer resist was suggested and studied. With a bilayer, an E-Beam insensitive coating is placed on the substrate first, followed by the resist. After exposure and development, the sacrificial layer is etched with a developer solution to produce an undercut. Once cleared, the final result is a more pronounced undercut and higher lift-off thickness for better lift-off profile.

LOR etching was critical to control un bilayer lift-off. LOR coating was etched using of CD-26 at 60% for more controlled develop time. To further decrease the etch rate of LOR, because LOR has the greatest effect on dissolution rate is the bake temperature, it was pre-baked at 200 degrees Celsius on a hotplate for ten minutes. LOR Strip with Remover PG, an N-Methyl Pyrrolidone (NMP) based solvent was used followed by finish with Oxygen Plasma to remove residuals.
5.4 Current Voltage tests

Current-voltage tests were run using a 4 probe station with voltage source and pA range ammeter by Keithly Instruments. A sensor coated with a layer of NCC-PPy, GOX enzyme, and cellulose acetate, had electrical contact pads exposed to contact the probes. A voltage sweep was done repeatedly between -6 to 6V and the current was measured. This was done with a dry device. Voltage sweeps were repeated for sensors exposed to DI water, and subsequent increases in glucose solution concentration.
6 Electrode Fabrication

6.1 Interdigitated Electrodes Array Fabrication

For enzyme based biosensors, the electric current from the chemical reaction can be so low that it becomes critical to use an electrode design that maximizes electrical output to wit, the interdigitated electrode array (IDA) (Fig 12). Fabrication can be done in a microfabrication facility. As a basic requirement, such a facility will need to be able to pattern metal electrodes onto a planar surface. Depending on the size and dimensions of the electrode, this can be a simple task (e.g. screen printing) or a highly complex task (e.g. photo-lithography). Generally, the smaller the device feature size is, the more time complex and time intensive are. On the other hand, the benefit for biosensor technologies is greater response current, higher sensitivities, more linear responses, and overall better performance [69].

Fig 12. [Source: http://electrochem.cwru.edu/encycl/art-s02-sensor.htm] an interdigitated array electrode design and example
Biosensors can directly benefit from advancements in microfabrication technology because it makes better IDA electrodes. To date, the advancement of nano-fabrication technology has advanced on par with computer chip applications, and has become a virtually endless frontier. Even 3D transistors for electronics, were recently achieved [70]. Electrodes, micro electro mechanical systems and nanowires are just a few examples of the plethora of possibilities enabled by new nanofabrication processes [35-37]. It is highly desirable for biosensors because performance increases as device features get smaller. When studying NCC-PPY nanomaterial on electrodes, it is important to understand the effects of changing the electrode size and dimensions when in contact with the NCC-PPY. Its effects on conductivity can be measured by comparing the electric current of the enzyme electrode with that of NCC-PPY coated electrodes. Because film thickness can potentially decrease the permeability of glucose or even adsorb some in cases, it may be necessary to vary the film thickness on the electrodes. These design characteristics will help determine to what degree the effects of NCC-PPY mediation are on the redox reaction itself.

Another point of interest is whether the needle-like structures will have any effect on the electrode conduction paths, and if so, what kind. For macroscopic electrodes, digit widths greater than 0.1 millimeter, the nanosized needles positioned along their lengthwise axis may form an ordinary homogeneous film. In that case, its effect would be akin to any typical film formation. At smaller depths when the spacing and size of electrodes approach the size of the particles themselves, interactions may be more pronounced. There are two likely arrangements (Fig 13):

1) the particles will overlay the gaps leaving pure solvent space in between, like a bridge forming over the electrodes;

2) the particles can fit randomly between and above the spacing.
Fig 13. Possible fiber arrangements across electrodes, arranged overtop (left) and aligned between (right)

These scenarios may have potential effects on the conductance of the sensor and sensitivity to glucose as they can route the conduction paths in different ways, affecting the diffusion of enzyme and analytes or disrupt some conduction paths. It can potentially affect the permeability of solvent and glucose particles through the cellulose layer to the electrodes and conductance across the electrode in a native sense or conversely create extra conduction paths as they aligned well enough to form a secondary electrode array with an increased surface area. There are a variety of possibilities so; it is vital to control the electrode fabrication techniques, especially with NCC-PPY.

6.2 Patterning techniques for 1 um IDA Electrodes

To pattern IDA electrodes under 1 to sub-micron resolution, it is necessary to use electron beam (E-Beam) lithography techniques. E-Beam is used to fabricate features accurately between one micron to ten nanometers. While photolithography has been demonstrated to develop lines about 1 um [75], anything smaller begins to suffer from problems with diffraction of UV at those wavelengths. For such small feature sizes,
options include using E-Beam, extreme UV, and nano-imprint lithography. While E-beam is costly to mass produce, it is sufficient for prototyping; thus, E-Beam lithography was selected in this study.

E-Beam lithography is typically used to write features less than one micron in size. Main difference between photolithography and E-Beam lithography is the exposure tool; E-Beam uses electron beams whereas photolithography uses UV light to expose the sample. A Tungsten filament atop a vacuum column is heated and thermal electrons are emitted. These electrons are then, accelerated down the column by strong electric fields and focused to the sample upon which the pattern is written. Computer controlled electromagnetic lenses guide the beams to draw the pre-programmed pattern line-by-line as the sample is exposed. It is important to note that since the E-Beam writes line by line, the exposure time (and cost) increases as the pattern size increases; this makes it good for prototyping but costly for large scale production.

There were several specific parameters used for this electrode fabrication. The desired pattern was designed with general computer aided design software called L-Edit and loaded onto an E-Beam specific file format by a transcription software called CATS. Only some polymers are sensitive to this type of E-Beam, such as poly methyl methacrylate or ZEP (trade name). ZEP from Zeon chemicals is known for its high resolution and development properties, and thus, they were used.

6.2.1 E-Beam Dose Testing

Before one writes an image with E-Beam, a dose test needs to be performed. “Dose” is the term used to describe the ratio between the amount of electrons emitted and the surface area exposed; dose units are in Coulombs per centimeter squared $\text{C/cm}^2$. While
there are some expected values, the right dose varies for each design, sample, E-Beam writer and even laboratory conditions. Too low a dose will underexpose the resist and fail to penetrate it down to the substrate underneath; too high can cause electrons to scatter laterally, interfering and potentially damaging other unwanted areas. Assuming all conditions are precisely controlled, it suffices to expose the same pattern at varying dose levels to determine an optimum dose. Once this optimum is found, it can be used subsequently within a margin of error in reproducibility. This set of tests wherein the dose is incremented stepwise within a range for each design run is called a “dose-test”, (Fig 16).

For this sensor electrode a dose test was performed and an optimum was found (Fig 14). Dose testing was done with an electrically insulating silicon dioxide coated wafer; ZEP 1A was used as the polymer resist. Two sets were done: first with a single layer of ZEP, second with a bilayer stack of LOR resist and then ZEP on top. The bilayer situation was tested because it can potentially have better lift-off results. Doses started at 180 uC/cm$^2$ and incremented by a 20uC/cm$^2$ step. For the ZEP layer alone of thickness 100nm, the optimal dose was found at 230 uC/cm$^2$; same result for thinner ZEP layers between 50-60 nm of ZEP. When using the LOR as a base layer, the optimal dose was much higher at 720 uC/cm$^2$. This can be due to electrons being absorbed by the LOR polymer which reduces the residual impact from reflections and electron scattering, to require more electrons to be exposed than without LOR present.
Fig 14. Image of developed IDA electrode. A single layer 100nm of ZEP was used over a silicon dioxide substrate.

Once the pattern is etched in polymer, gold metal was evaporated to make the electrode and contacts. Gold was chosen because it has a high work function. With evaporation, the sample is placed inverted above a gold source; a vacuum pump reduces the chamber pressure to $10^{-6}$ Torr; an electric current is run through the metal to heat the source, causing the metal to evaporate; the vapors condense onto the sample to form a coating of metal of controllable thickness. There was strong adhesion and the polymer was resistant to the final stage lift-off. This is typical when there is not a strong discontinuity of metal at the boundary edge of polymer with metals such as gold. To create a better discontinuity the bilayer method was used.
6.3 Bilayer Lift-Off With PMGI Resists

Bilayer lift-off is a procedure used when lift-off with a single layer proves insufficient. A typical problem with lift-off is metal layer being continuous at boundary between polymer and substrate. Generally this happens when there is no undercut at the boundary. This can be affected by the resist type, thickness, the exposure amount, metal type and metal thickness. This is of particular challenge with gold. In these cases when it is challenging or impossible to produce the needed discontinuity, a bilayer system can be used to improve lift-off. With the bilayer technique, a polymer layer inert to the E-Beam sacrificial layer is coated first, upon which the E-Beam sensitive resist (image layer) is coated second. The image is exposed using E-Beam lithography and developed. This removes only the exposed pattern on the top layer, leaving the bottom intact. After image development, the sample is placed in solution that dissolves the sacrificial layer. The solution makes contact with the sacrificial polymer only through the holes left by the E-Beam exposed pattern and etches the image into the sacrificial layer, however, because of the chemical etching process, some of the sacrificial polymer is etched deeper than the image boundaries, to create the undercut. This undercut introduces the needed discontinuity between the deposited metal film and the rest, making it easier for the final remover solvent to strip away the excess polymer.
Fig 15. Process flow of gold bi-layer lift-off used to microfabricate patterns. a) Bilayer stack of two polymer resists, imaging resist and lift-off resist on top and bottom respectively. b) Pattern area is exposed (light center) with UV light or E-Beam to solubilize the polymer. c) chemical etchants dissolve exposed areas, bottom layer forms an undercut. d) Gold metal is evaporated over the whole sample. e) Remaining polymer is stripped to leave only the metal in the shape of the design.

For this biosensor bilayer lift-off was then used with specific criteria. The sacrificial layer used was a poly glutarimide based resist under trade name LOR (for “lift-off resist”). ZEP was again used for the image layer. Spin coating was used to coat both the LOR and ZEP layers. When cross-linking the LOR however, the temperature of 200 °C was used; this is known to slow the etch rate of the LOR to make the undercut more controllable. After spinning both sacrificial and image layers, a dose test was repeated to discern any influence the LOR layer might have on the of E-Beam dose (Fig 15). It was expected that the dose would be the same because the image resist was on top
and other conditions were the same and sacrificial layer was known to be inert to E-Beam exposure. We found this was not the case.

The optimal dose for LOR-ZEP bilayer stack was more than twice that of the ZEP only. At first glance, using a microscope to observe large patterned areas for discontinuities, it appeared that the clearing dose (dose at which the E-Beam resist is removed completely within the pattern area boundaries) was 300 uC/cm² however; proceeding with etching of the sacrificial layer stray polymer became much more visible to reveal it had not been adequately cleared. In fact, it was found that the optimal dose was 780 uC/cm², over two and a half times higher than without LOR on silicon dioxide. Reasons for this may be due to the penetration of electrons into the LOR layer, providing an escape path for the electrons or absorbing them completely away from the ZEP. Another is limiting backscatter electrons. Backscattered electrons reflected off the SiO₂ substrate may be “reused” to react with additional ZEP polymer, whereas with LOR, they are reabsorbed; this would require more electrons from E-Beam to produce the same result, thereby increasing the dose. This increase in exposure dose requires more time and higher current from the E-Beam writer. In turn this can increase the writing time, thereby increase the cost. Knowing this can make users aware of another trade-off between cost and quality when choosing the bilayer technique to improve lift-off for biosensor arrays.
Fig 16. Microscope images of dose test of E-beam write of ZEP 1A on LOR 1A. a) and b) are underdosed. c) the optimum dose at 780 uC/cm² and d) an overdosed image. Development of ZEP was done in MIBK:IPA ratio 9:10 for 60s and LOR was done in CD-26 60% for 60s.
6.4 Development and Post Processing

ZEP layer was developed after E-beam exposure and LOR etched. ZEP was developed in ZDN50 (proprietary developer) and reaction stopped with a 9:1 solution of methyl isobutyl ketone: isopropyl alcohol (MIBK:IPA 9:1). Development time needed to be controlled precisely with 60±1s immersion in ZDN50 to give a reasonably reproducible result. Afterwards the LOR layer, which is soluble in alkaline solutions, was etched with a tetramethyl ammonium hydroxide preparation solution (trade name CD-26) at a 60% dilution in DI water. The dilution was necessary because the etch rate of pure CD-26 was too aggressive, it began bleeding into the IDA lines. An undercut was visible under microscope, appearing as a shaded region. For IDAs, the design of the distance between fingers required a slower more controlled etch rate because otherwise the whole array could be stripped off prematurely. A top view of the fabricated wafer is shown on Fig 17.

Fig 17. IDA electrodes shown after development of ZEP. Lines of 1 um act as an optical grating, emitting color when reflecting white light, like a prism.
6.5 Metal Deposition

Adhesion of gold to silicon was found to be poor (it came off when immersed in solution) so a 10nm layer of chrome was first deposited, following 100nm of gold layer. Evaporation of both metals was done in the same vacuum session to avoid contamination from ambient air. The result was an even metal coating on the wafer (Fig 18).

Metal layers were deposited using thermal evaporation. First a ten nanometer layer of chrome was deposited for adhesion of gold to the silicon. Unless stated otherwise, deposition was done under pressure of $8 \times 10^{-6}$ to $2 \times 10^{-5}$ Torr. This pressure was achieved using a vacuum pump. A chrome coated tungsten rod was used as the metal source and thermally evaporated at a rate of $1 \ \text{Å/s}$. Following, a gold source placed in a Tungsten boat was used for the next metal layer immediately after thickness of gold was set at 100nm with deposition rate of $1 \text{Å/s}$.
Fig 18. IDA electrodes of 1 micron spacing after gold deposition. Features appear identical with a gold hue indicating the metal layer.

6.6 Lift-Off and Resist Removal

The whole polymer stack was removed to reveal the finished electrode in a process called “lift-off.” For lift-off, the polymer is removed either chemically with solvent or piranha etching, or using O₂ plasma etching to remove the organic molecules. It was expected that standard methods would be sufficient. Surprisingly for the NCC-PPY sensor design many were inadequate. Several methods to lift-off the polymer was attempted but only the use of “remover PG” solvent supplied by MicroChem had promising results. The methods are summarized in the following table:
Table 4: Different lift-off methods used for the IDA. Only soak in Remover PG showed best results.

Of all methods, using Remover PG solution was the only method which showed practical promise. At first, for the single layer of ZEP using a resist stripper ZDMAC (a solvent 99% N,N dimethyl acetamide) overnight. While the contacts and other non-critical features were properly uncovered, between the IDA fingers, the metal was not adequately removed, even with ultrasonic agitation. This was likely due to the thin polymer layer whose thickness (60nm) was comparable to that of the metal layer (50nm). This suggests there was no undercut to disconnect metal along the polymer edges. Increasing the thickness of ZEP was impractical because a requirement to write submicron features is to have reasonably thin resist thickness. It was on this basis that the bilayer method using LOR was used. Even still, when using ZDMAC, the metal between the IDA digits did not resolve. Ultrasonic agitation did help in removing some of the inner layers however, tended to damage or strip away large areas of the pattern, particularly the digits, confirming the observations of other scientists [76]. Of all the hundreds or thousands of IDA digits in the electrode comb, it only takes one unresolved section of metal to short circuit the entire device rendering it useless. This was the case with using ZDMAC only. Piranha etching had the opposite effect. Piranha solution in a 3:1 mixture of sulfuric acid and hydrogen peroxide used to aggressively remove all
organic contaminants. The use of piranha solution however appeared to remove all the metal, including the desired pattern, right off the wafer. Theoretically piranha is not expected to react with the gold or chromium adhesive. Hydrochloric acid however does etch chrome. Practically it is possible there were trace chloride ions contaminating the sample, which in conjunction with the large hydronium concentration, caused etching of the chromium adhesive layer, detaching the gold along with it. Soaking in Remover PG, an N-methyl pyrrolidone-based solvent, showed the most rapid and effective removal.

To observe lift-off for sub-micron features, scanning electron microscopy (SEM) is needed. Due to diffraction of light, optical microscopes cannot see images at the nanometer level. For the 100nm electrode lines, SEM pictures confirmed that these lines were achieved (Fig 19). Gold, which has the tendency to stick and is more difficult to deposit and lift-off, compared to chromium, was naturally more challenging. As the SEM images show, the challenge of removing the inner layers between the IDA digits remains at that small feature size. It was observed that the areas at the digit extremities were the first to be removed.
Fig 19. Lift-off of LOR from 100nm line dose test is shown after metal deposition. a) stray non fully detached polymer is seen beginning to lift off at the extremities as it works its way inward b) identical pattern with fully lifted off metal lines only are shown
6.7 Lift-off With Photoresist

Fabrication techniques for sensors using photolithography were explored. While E-Beam lithography represents an advancement in fabrication techniques, it is much more costly than photolithography because the cost is proportional to the write time (i.e. each additional sensor bears an additional cost per wafer); in contrast with photolithography, the same photo mask can produce repeated copies at much lower cost much like a stencil can make copies of images. Photolithography has been extensively used and is the current technology behind fabrication of microdevices [76]. Sensors fabricated using the IDA pattern used photolithography for both contacts and sensing area [77] [76]. To increase sensitivity sometimes a combination of E-Beam and photolithography is used, the latter for the large non-critical areas and contacts, the former for the more sensitive parts [78]. For features larger than 1 micron, photolithography is deemed sufficient and has been used for IDA fabrication. Bilayer techniques are very useful to produce a desired undercut for lift-off [77]. While some reported to have feature sizes of 1 micron with specific conditions, it was found with UV flooding at higher wavelengths, the recipes did not provide reproducible results. In addition, some unwanted blending of the lines appeared with other defects. The myriad of other factors like development time and developer concentration and radiation dose add to the complexity and finding the right parameters to achieve the IDA digits becomes an increasing challenge for the sensor development. In this work, fabrication parameters are explored with the aim to optimize the production of the sensor reproducibly using standard equipment only. The trade-off is the loss of precision which, complicates the process and calls for optimization; in order to take advantage of the cost efficiency of photolithography, microfabrication-techniques specific to the sensor are discussed.
Photo lithography uses a photosensitive polymer resist coated onto a support substrate. UV light exposes the resist in the shape of the desired pattern; the pattern is transferred by filtering the UV light through a predesigned photomask. A photomask can be a glass plate coated with chromium with the designed pattern etched into it; Finally the resist is removed by another solvent that strips away the exposed polymer for positive resists, leaving the metalized image of the electrode patterned on the surface.

### 6.8 Effect of UV Exposure Time

In photolithography, it is critical to determine the amount of UV light to expose onto the substrate, especially for biosensors. One of the challenges of biosensor fabrication is obtaining an acceptable digit width so that it meets the design specifications. The point of using microfabrication is to pack as many electrode digits within the given area, without the electrodes touching one another. Diffraction of UV light, distance, and underexposure from the substrate can lead to the image transferred onto the resist layer to be much thicker than the one planned on the photomask. If the resist is overexposed, the widths can expand causing the digits to blend into one another; this would mean a short circuit and cause malfunction. On the other hand, if it is underexposed, the resist will not clear and no metal will be printed at all.

The UV sensitive resist used was S1811 supplied by Rohm and Haas. Upon exposure to UV light, the exposed area reacts and becomes soluble in alkaline solutions. Ideally only that area is soluble. In practice, unexposed areas are still soluble but dissolve at a much slower rate [80]. This can be influenced by the UV exposure. UV exposure test was done using a Hg UV lamp and by incrementally increase the exposure time at intervals of 15 s from 0 to 5 min. It was found that the optimal exposure time in this case was 90 s UV dose for clearing 5-10 micron features however rounding of edges was observed (Fig 20). This was expected because diffraction of UV light becomes more significant closer to the micron range as it approaches the Hg lamp's wavelength of ~400nm.
Fig 20. Photoresist S1811 was exposed at different UV exposure. Exposure times are 1m30s, 2m30s, 3m30s, 4m30s, 5m30s, from a) to f) respectively.
6.8.1.1 Bilayer One – step Development Technique

Developing both LOR and photoresist in one step was explored and found to be preferable. The issue is that both LOR and photoresist are soluble by the same TMAH based developers. In addition, while exposure to UV light makes the exposed resist more soluble, it doesn't make the unexposed resist insoluble. Even unexposed resist, given enough time will dissolve in the alkaline developer, just at slower rates. The extra time it takes for the LOR to develop will be added to the exposure time of the photoresist and patterned features will widen. The extent of this error is dependent on the each polymer's respective etch rates. For example, if the unexposed resist etching speed exceeds that of the LOR, the pattern will widen even further and prematurely. On the other hand, if the LOR etches at a speed greater than the exposed photoresist, then sagging will occur, as the underlying LOR will disappear long before the top layer can finish etching. The ideal would thus be if the LOR etched faster than unexposed resist but slower than the exposed resist. Fortunately etch rates can be controlled with soft-bake temperatures.

Controlling the etch rates of each polymer are critical. Etch rates on the polymer are slowed by increasing soft-bake temperature. For LOR, the etch rate is considerably decreased by soft-baking at a temperature of 230 degrees. Using HMDS the development was clearer. At a developer dilution of 50% CD-26, the removal of the top photoresist was at ~ 4 min. Subsequently the LOR began to etch faster than that and was removed in approximately 1 min furthermore. It was clear that the etch rates of LOR were still faster than that of the exposed S1811 series resist and needed close monitoring throughout. Fortunately the LOR layer stayed stable as the top layer was etched slower than the unexposed resist. Once the contact and exposed areas were removed, the etching of LOR in the undercut was substantially slower allowing for a more controlled undercut.
Fig 21. Image of sensor taken from one sample pattern at edge of wafer. From spin coating the edge may have been slighter thicker in photoresist. The pattern is not completely etched away but close to it and thus demonstrating the sensitivity of reaction time at that concentration of MF321 developer 100%.

Overall the ultimate pattern came out successfully (Fig 21). Though the original photomask had a 5 micron critical feature size, the width of the digits expanded likely from diffraction of UV light and imperfections in surface contact with the mask. Still there was no short circuiting so the device was produced within acceptable limits. It was found increasing exposure times produced a sharper sidewall at the polymer edges. These techniques can be extended to other forms of photolithography including extreme UV as a suitable alternative for cost effective practical biosensor fabrication.
6.9 Lift-Off and Resist Removal

Due to the bottom layer comprising LOR, it was sufficient to strip the LOR layer and of all lift-off methods, soaking in Remover PG had the best results. Remover PG was used as it demonstrated an ability to rapidly remove the LOR and S1811 resists layers. Initial tests began with chromium lines of 50 nm on S1811 alone without LOR, the pattern appeared completely resolved. Gentle swabbing with acetone was enough to remove all the residues proved sufficient. This was done as a precursor test for the gold electrodes. When gold was coated (Cr:Au 10nm:40nm) the situation was different. The larger non-critical features like contacts and taper arm were lifted off with acetone soak however; they had rough edges dangling that needed swab scrubbing to remove them. Ultrasonic agitation only damaged critical features. Once the LOR bilayer stack was used and Remover PG was employed for the lift-off strip, results were more promising and successful lift off of the IDA electrode for the biosensor was created.
Chapter 5
Results and Discussion

7 Enzyme Compatibility with NCC-PPY

To break down Glucose, the enzyme Glucose Oxidase (GOX) is used. GOX breaks down D-glucose into D-Gluconolactone in a redox reaction that transfers two electrons per molecule to the enzyme cofactor. For GOX, the enzyme redox cofactor is Nicotinamide Adenine Dinucleotide (NAD) which reduces to NADH$_2$. Typically when reacted in water, the NADH$_2$ reacts with water to return to NAD producing hydrogen peroxide [see eqn1]. In the case of the former, hydrogen peroxide can be further broken down by another coupled enzyme called Horseradish Peroxidase; this in turn will transfer electrons by the same process while protecting the electrode from possible degradation by hydrogen peroxide[40].

Electrodes will reduce the enzymes again for future use and collect the remaining electrons. This is a critical mechanism which differentiates the sensor from being a glucose sensor to any other type, so is vital to test[37]. There can be complications however; because GOX enzyme, as any enzyme is prone to deactivation under non-ideal conditions and sensitive to temperature, pH, and the presence of inhibitors.

Enzyme activity can be reduced in the presence of incompatible substances, known as inhibitors, so for a new material, such as NCC-PPY, it is important to verify its compatibility with the enzymes. There are two classes of inhibitors, competitive and non-competitive inhibitors [41]. Non-Competitive inhibition occurs due to the similarity of the chemical with the substrate, in this case glucose chemical binds to the enzyme reactive sites where otherwise glucose would be. This is reversible so varies with the concentration of the inhibitor. NCC-PPY is based on a cellulose material. While
cellulose is not known to inhibit enzymes, by virtue of being a polysaccharide, it shares many structural similarities to a glucose molecule [40]. This may seem obvious since both cellulose and enzymes co-exist in plants. Modification with PPY has the potential to trigger a non-competitive reaction thus, it is important to test for its inhibition. Non-competitive inhibitors bind with the enzyme to reduce their activity. Heating can also inhibit enzymes by denaturing the proteins and extreme pH can alter the hydrogen bond and active sites. It is predicted that the trace reactants used in synthesis, such as sulfuric acid, ferric chloride/ammonium persulfate oxidants, etc. will trigger potential non-competitive inactivation. Moreover, glutaraldehyde adhesives were known be used as a fixative for immobilization of enzymes onto solid supports and proteins [40]. Glutaraldehyde use at certain concentrations has been known to cause enzyme inhibition, so it will need to be measured. In general, NCC-PPY and all new agents need to be tested for enzyme inhibition.

Competitive inhibition happens when an inert but deceptively similar molecule replaces the substrate, in this case glucose, on the active site [6-8]. Like a bag on a bus seat, it takes up the spot which was meant for use. Molecules similar in shape of glucose will sit on the enzyme active region and prevent glucose from going there, to inhibit the reaction. Just like bags on bus seats can be removed, competitive inhibitor can also be removed so the inhibition is non-permanent and a function of inhibitor concentration. NCC-PPY is a polysaccharide and has many chemical groups similar to glucose. As such tests need to be done to ensure there is neither competitive inhibition.

Testing for non-competitive inhibition is the same for competitive and is done by measuring the enzyme activity in the presence of the potential inhibitor. In this study, the use of colorimetric detection is used. An enzyme is set to react with a color changing substrate o-dianisidine di-hydrochloride; the absorbance of color is measured [7, 9]. This reaction is done both with the samples with NCC-PPY and without, the latter set to be used as a control.
Enzyme performance saturates based on the limiting reagent. When the rate of reaction approaches its saturation point, further reaction rate increase is stymied at a maximum in spite of adding more glucose. Here its Michaelis-Menten constant (a measure of enzyme reaction rates) $K_m$ remains [46]. In competitive inhibition, the saturation point is the same and the $K_m$ shifts [47]. In either case, the net effect of inhibition is a noticeable decrease in activity relative to a pure measured sample. In this case, absorbance readings took into account deviations in a two sample comparison over varying concentrations of glucose; glucose concentration range was chosen to be between 0 and 10mg/mL, well above the expected threshold of dangerously elevated blood sugar concentrations [48].

Comparing the NCC-PPY activity with the control and monitoring any changes in activity or shifting of $K_m$ will show the presence of any inhibition and the type. Any premature saturation may be evidence of inhibition and this will be indispensable in monitoring the enzyme effectiveness important to sensor performance.

7.1 Material Compatibility

7.1.1 Inhibition Verification

Enzyme activity is very sensitive to environmental conditions so it is crucial to monitor interactions with other chemicals. When the presence of a chemical reduces the activity of an enzyme and some chemicals involved will deactivate the enzyme. Enzyme structures are extremely complex and any new material has the potential to interfere with the enzyme function because binding site geometry is crucial [2, 13]. In this case, the interaction of NCC-PPY in film form and as a colloid with the enzyme will be
assessed. This is done by performing an activity assay while exposing the enzyme to NCC-PPY, and comparing it to one not exposed to the mediator. Though its primary use will be as a film, NCC-PPY was also left in colloidal suspension during testing.

In order to test what effect the presence of NCC-PPY will have on the enzyme, colorimetric enzyme assay studies were done. For GOX enzyme this is done by coupling with another enzyme horseradish peroxidase, which reacts with a color changing substrate (chromogen). Chromogen concentrations are detected using a UV-Visible spectrophotometer and are proportional to glucose concentration. It is not necessary however, as tests using paper and micro-channels were used[50]. The reaction is timed and is stopped by adding a corresponding stop reagent or low pH acid. This reading is measured as absorbance.

\[
Glucose + GOx \rightarrow \text{d-glucolactone} + H_2O_2
\]
\[
H_2O_2 + HRP + \text{Chromogen}_{(ox)} \rightarrow \text{Chromogen}_{(red)} + H_2O + \frac{1}{2}O_2
\]

Eqn. 1 shows reaction of glucose with enzyme glucose oxidase. Secondary reaction produces the product hydrogen peroxide and reduces chromogen to reveal color.

Because the rate of reaction is also linearly proportional to the concentration of “available” and active enzyme, the absorbance reading of the spectrophotometer can indicate the relative activity or inactivity of an enzyme in question. Traditionally speaking, an enzyme calibration measurement must be done for every transported batch of enzyme stored because deactivation can randomly occur within each storage unit, hence the measures are all relative and variation can occur[51]. In principle, the
same technique can be used to measure the interactions of any species however; with NCC-PPY there were a few complications observed.

NCC-PPY is black in color; the mere presence of the nanomaterial interferes with the readings from the visible spectrophotometer since it occupies the entire visible spectrum. This can be avoided by filtering out of the particles. NCC-PPY comprises of sub-micron sized particles so it was difficult to filter out NCC-PPY on standard filter paper. In order to prevent molecular damage to the enzymes, a 200 nm HPLC nylon filter is used to separate the particles from solution; it was assumed that the remaining particulates less than 200 nm was negligible.

It was observed that NCC-PPY was unsuitable for use with some commonly used chromogens for enzyme assays. Common substrates for GOX include o-dianisidine dihydrochloride, tetra methyl benzidine (TMB), and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), the latter being less toxic. It was observed that after completing the enzyme chromogenic reaction after filtration, the visible color was removed, leaving the solution colorless. To be certain it was due to some adsorption of chromogen bi-product on the NCC-PPY particles rather than an indication of enzyme inactivation, a fully reacted solution without NCC-PPY was exposed after the reaction completed and then filtered. The result was the same, indicating the NCC-PPY was adsorbing or absorbing the reduced chromogen products. This was consistent with both TMB and ABTS. The activity measurements are relative, the theoretical error from stray particulates in solution may be neglected. The adsorption may be due to the structural similarities of cellulose and PPY to ABTS and TMB (Fig 22); another possible explanation is the doped dihydrochloride, which created a positive surface charge, was repelled by the similarly charged PPY particles, preventing adsorption. Still, to minimize error, filtration was employed to obtain readings. It was found that o-dianisidine dihydrochloride was able to retain most of its original color in both samples, therefore, it was chosen for subsequent studies.
Chromogen absorbance peak ranged between 525 and 560 nm for o-dianisidine dihydrochloride.

Fig 23. Enzyme activity as a function of glucose concentration. Higher absorbance indicates a higher enzyme activity.
When comparing the activation level of glucose oxidase and NCC-PPY, there appears to be little effect of inactivation of enzyme from NCC-PPY (Fig 23). This was expected because cellulose is known to be compatible with enzymes because NCC is bio-based and PPY is unreactive and with a low toxicity. Cellulose films are used as substrates in many enzyme based filtration systems. PPY is also known to be unreactive and virtually non-toxic compared to its monomer pyrrole [52]. It is possible that chemicals used during the process such as trace oxidants of FeCl$_3$ or ammonium persulfate or from sulfate ions via acid hydrolysis has an impact. Observed effects from the impurities however, look negligible. This can be because of the copious washing steps prior to the reaction and the buffer solution controlling the pH and removing other contaminants.

Furthermore, enzyme entrapment techniques were demonstrated using electropolymerization of PPY to develop glucose sensing electrode [40]. It was of little surprise that activity remained similar.

Interestingly in this case, the NCC-PPY version shows an increase in activity over the original enzyme. One explanation can simply be error associated with the presence of stray NCC-PPY nano particulates that bypassed the filter. Another explanation may be due to an interaction with the enzyme and NCC.

The curve of NCC-PPY absorbance was slightly greater than that of enzyme alone. This can be attributed to error, with stray PPY particles, originally black in color, absorbing additional amounts of enzyme. Another possibility is that the NCC-PPY is improving in efficiency upon redox reaction of enzyme. NCC-PPY does not alone catalyze the reaction so any possible improvement must be due to the interaction. One possible explanation is an intra-molecular conduction paths created by an NCC-PPY conducting particle attached to two enzymes. As one enzyme becomes oxidized, rather than returning to reduced state by migrating toward another reducing particle, the reaction can occur right away because the NCC-PPY provides an electrical conduction path to
that other particle. Thus the PPY conduction path and accelerate the process as it reduces the time it takes for the reaction to occur. Another explanation can be reduced surface tension between cellulose particles and glucose, bringing the enzyme molecule closer to the glucose molecule as it is adsorbed onto the cellulose.

One question concerning the use of NCC-PPY mediator is which immobilization method is best suited and how it will affect enzyme activity and stability for long term use.

### 7.2 Enzyme Deposition Process

#### 7.2.1 Effect of Drying Conditions and Enzyme Deposition Method on Enzyme Activity

Under high temperatures, enzymes can inactivate over time so it was important to assess temperature effects during fabrication. Time based inactivation is attributed to interactions in water as hydrogen bonds and other weak bonds within the enzyme structure rupture to deactivate over time [53]. As time progresses the more it inactivates [54]. After dispersion in water the enzyme was stored no longer than two months at 4 degrees Celsius or 6 months at – 4 degrees Celsius. On planar enzymatic sensors, as in this case, a layer of enzyme needs to be coated and dried on the substrate. During this time, water needs to evaporate to leave only the enzyme film. In contrast to polymer films which have smoother and more stable morphology when baked at higher temperatures during the film deposition process, high temperature can inactivate or destroy the enzyme activity. Beyond 60 degrees Celsius for example, GOX begins to denature and inactivate permanently [53]. Hence, evaporating water in a 60 degree
oven is not an acceptable option since lower temperatures are necessary. This tradeoff between temperature and time however is common in enzyme based device: the lower the temperature, the longer time the enzyme has to evaporate. An alternative to heating is freeze drying.

Drying enzyme comes with associated risk of inactivation due to both temperature and time [53]. In order to subvert the decay of the enzyme over time and temperature, a deposition process using freeze drying on NCC-PPY is adopted in this work. Freeze drying consists of evaporating water at low temperatures and pressures, such that solid ice skips the liquid phase and evaporates to gas phase. This procedure has potential to allow enzyme to be deposited quickly and in at low temperatures and prevent enzyme decay. The effects of low pressure evaporation on the properties of the enzyme are evaluated. A hypothesis is proposed that freeze drying will demonstrate better activity over solution cast evaporation and that film morphology will be generally unaffected. To determine this, two methods are presented and compared: one using drop casting at 4 degrees Celsius and another prepared frozen at –4 degrees C and then placed in a freeze dryer. Both were dried overnight. Enzyme activity was determined using colorimetric assay (see method chapter and Appendix ).
Fig 24. Comparison of Enzyme Activity using two deposition processes: Freeze drying and “fridge drying” (solution casted and dried at 4 degrees C in air)

Results in Figure 14 shows that there is very little difference about the mean values of the two conditions; however the variation is much higher for the enzymes dried in the fridge. It suggests that the main enzyme activity is on average unaffected and can be used as a baseline regardless of the fabrication method used. But because of the lower variations, freeze drying is a better technique for depositing enzyme on the film.
7.3 Enzyme Immobilization Techniques

When working with enzymes in sensors, enzyme immobilization is an important issue. It is not a problem for one-time use methods, such as colorimetric concentration measures or ELISA techniques [55], long term repeated use systems like reusable biosensors or filtration membranes need ways to immobilize the enzymes. Enzyme immobilization refers to fabrication considerations that prevent the enzyme molecules from leeching away, reducing their activity, thus reducing their effectiveness. Techniques used to immobilize enzymes are adsorption, absorption, covalent bonding, entrapping in between films or using chemical markers [56].

A simple technique is to use a cellulose acetate film to entrap the enzyme. Cellulose acetate allows for the passage of water and smaller molecules like glucose, while larger molecules like enzymes are retained. For large electrodes such as centimeter sized rods dipped in solution, it is sufficient for prepared cellulose acetate sheets to wrap around the exposed area dipped in solution. For micro device such as biosensors or planar systems like filtration membranes a cellulose acetate film coating may be preferred. GOX enzyme is approximately 50 angstroms [57] in diameter so pore sizes of cellulose acetate need to be smaller than that. Dissolution of cellulose acetate in acetone in controlled concentrations was determined [58].
7.3.1 Effect of Glutaraldehyde Fixing

Glutaraldehyde fixative added to NCC-PPY GOX enzyme complex was studied. Glutaraldehyde is a common cross-linking agent and is used in enzyme fixation and immobilization by producing covalent bond between amine groups of the enzyme with the cellulose for immobilization fixative substrates by nucleophilic reaction. Glutaraldehyde is insoluble and also appears to have potential to decrease leeching, normally associated with enzymes. The effects of NCC-PPY and glutaraldehyde on enzyme activity are still unknown. Activity testing was done to determine whether there is any enzyme inactivation (Fig 25).

![Effect of NCC-PPY on Insolubilized Glutaraldehyde](image)

Fig 25. Activity testing with glutaraldehyde insoluble enzyme with NCC shows slight inhibition. Linearity is established in the lower concentration however, it appears to begin to saturate at 5 mg/ml. Higher absorbance indicate higher enzyme activity.
For comparison purposes, synthesized poly pyrrole: poly styrene sulfonate (PPY:PSS) was tested under similar conditions. PPY is known to be highly insoluble in most common organic solvents [59]. One of the principle advantages of the NCC-PPY colloid is that it disperses readily in water to enable its use in conventional coating techniques such as dip coating and spin coating. PPY:PSS was synthesized by solution polymerization [60] as a PPY colloid that disperses in ethanol. Coating as thin films is difficult and shown to be incoherent on a silicon dioxide surface with heavy patches of agglomerates and poor adhesion. It was used as a control representing PPY colloidal dispersions alone without cellulose functionalization.

![Diagram](image)

**Fig 26.** Enzyme stability on glutaraldehyde immobilized NCC-PPY film on silicon substrate. Each iteration level refers to the number of subsequent tests with the same film re-used each time.
7.4 NCC-PPY Colloid vs. PPY:PSS Colloid

Comparatively when enzymes tested in the presence of PPY:PSS, there is dramatic loss in activity compared to NCC-PPY (Fig 26). Since PPY:PSS films were left to evaporate, it is reasonable to assert that most of the ethanol solvent has evaporated. The anionic presence of PSS in those concentrations as well as trace amounts of preservative may have persisted within the tight agglomerates of polymer. Any of those may have at some point leached out during testing. NCC-PPY however, maintains its integrity with mechanical and hydrogen bonding to cellulose nanoparticles. Thus the dispersability of NCC-PPY is due only to forces natural to the enzyme to preserve and possibly enhance performance. In contrast, dispersion properties of PPY:PSS is heavily dependent on surfactants and other synthetic agents for both intra-molecular bonding and surface modification, which can interfere with enzyme kinetics (Fig 27). Thus, for both film integrity and reaction activity, NCC-PPY is the better choice for biosensor films.

Fig. 27 PPY:PSS film deposited over silicon substrate. Poor adhesion and presence of enzyme inhibiting chemicals make it a questionable choice for sensors.
8 NCC-PPY Film Deposition

8.1 Film morphology

The surface of the NCC-PPY film can affect the diffusion of glucose or other reactants. Unlike conventional polymer films, NCC-PPY is composed of needle shaped nano-particles with distinct aspect ratio. Film morphology, roughness and thickness were obtained using atomic force microscopy (AFM), scanning electron microscopy (SEM), and profilometry.

8.2 Atomic Force Microscopy Images

AFM pictures of the surface of the dried NCC-PPY film were made. It was observed in the first solution casted films that there was significant cracking in thick films of both NCC-PPY and regular NCC. This led to suspicion of agglomeration or discontinuities in the drying process. Qualitatively the films were hard and quite brittle. AFM images can capture both the surface morphology and map out the three dimensional profile.
Fig 28. A 100 μm by 100 μm area of NCC-PPY solution cast film is shown. Lighter areas indicate higher altitudes. The agglomerations appear linear, indicating possible alignment of the needle shaped particles.
Fig 29. Close-up of the peak agglomeration on top right corner of Fig. 8. A 20 um x 20 um square is shown (top) and a tilted mesh mapping is shown as the 3 D morphology (below).
Fig 30. The same close-up image rendered with texture to show the possible visual appearance of the film surface. Linear regions appear clearer and easier to distinguish indicating the source of alignment and formation of particles.
From a macroscopic view, large peaks in roughness and uneven distribution in morphology is present (Fig 30). Large variances suggest a large degree of agglomeration within particles given the sizes of the distortions. Such agglomeration may be due to surface tension upon the local evaporation of water.

Due to the rigid and rough film surface, it was worth investigating the effects of film plasticizers. Glycerol is a known plasticizer of cellulose fibers and films. As an added benefit, it also occurs naturally so is unlikely to interfere with enzyme activity. Hence, glycerol was chosen as a plasticizing additive. The films with the plasticizer were comparatively smoother by the naked eye and had a softer feel. However, the film mechanical strength decreased so much that both the profilometer needle and AFM tip scratched the surface severely, making readings uncertain. As such, to observe film roughness and morphology, images of the film cross section were taken using SEM (Fig 31). For sensing purposes, the overall mechanical strength of the film is not a critical specification since it is placed above an already rigid silicon substrate. Should a future design need flexible substrates, as some digital microfluidics [62], this might become an issue.
Fig 31. SEM images show better defined uniformity with a roughness of less than 1 micron.

Occasionally unusual peak tops can be observed on the NCC-PPY film. This may be due to contamination. Another possibility is the agglomeration of NCC particulates, or agglomerates of conducting polymer. Overall, both SEM and AFM images show local degree of relative uniformity. Film thickness is approximated at 1 micron range. Because the thickness is mostly uniform and the film itself is much greater than the
height of the electrodes (30-50nm), small variations of film thickness are not expected to contribute significantly to sensitivity of the sensor.

8.2.1 Film Conductivity

NCC has shown some degree of conductivity. It is important to discern this for two reasons.

1) While common knowledge suggests cellulose is an insulating material, it was found to conduct very low currents in aqueous conditions [63]. Any contribution due to cellulose must be noted and studied as a control.

2) While the conductivity of cellulose may be rather small, for thin semiconducting films, they can be more influential.

It is thus premature to attribute all conductivity of the cellulose composite film to the conducting polymer coating. Mechanism of cellulose electrical conduction has been debated; it was determined that conduction is ultimately not solely due to migration of ions, yet a unique dipolar interaction by cellulose's surrounding hydroxyl groups. This additional conduction is expected because all test analytes are exposed to water from serum and enzyme buffers. NCC-PPY is a composite material of both cellulose and PPY. When performing readings the cellulose will absorb water, to make it conductive; this gives two sources of conduction, from the cellulose and the conducting polymer. To determine if the combination of PPY is a significant contributor, the conductivity of NCC-PPY was compared with NCC only (control).
Fig 32. Electrical sheet resistance of NCC-PPY compared to NCC alone. The addition of PPY clearly shows a significant decrease in resistance, hence increase in conductivity.

It is clear the PPY significantly adds to the conductivity of the film. This is not surprising since conducting polymers, while they are still in the semiconducting but are much more...
8.3 Effect of NCC-PPY Concentration on Electrical Conductivity

Since the solution cast film begins from an NCC-PPY colloidal dispersion in water, the effect of colloidal concentration, in turn thickness, was determined. It is well known that solution casting of polymer films are start off as liquid solutions or colloids. Generally it was expected that film conductivity increase proportionally, because of the increase in conducting particles; interestingly this was not the case. While initially it increased as expected, conductivity appeared to decrease over time. In this study, the concentration of NCC-PPY colloid in water was increased from 0.5 to 2.5 % by weight and its resistance (the inverse of conductivity) was measure by a linear four point probe method.
This unexpected decrease in conductivity suggests, for thicker films, disorder of fibers and insulating regions may play a more dominant role than the conductivity of PPY itself. Conventional conductors, such as metals, have conductivity proportional to the thickness of the layer [65]. For NCC-PPY films however, after 2% the less conductivity stayed the same. The chemistry was unchanged so it is plausible to rule out any chemical interactions and rather it is likely due to physical changes. Visual observation of slight cracking and uneven agglomerated regions confirmed such fiber disorder. At higher thicknesses, some areas were noticeably thinner and even began to peel. These disconnects created an electrical “bottleneck” which limited electrical conduction.
Another theory is that the fibers themselves are somehow interfering with connection pathways of the coated PPY; as the agglomeration occurs during drying, hydrogen bonding between cellulose hydroxyl groups may fuse together forcing themselves between the thin PPY coatings to disrupt the connection. It was already shown that in-situ polymerization of cellulose fibers, as done in this case, resulted in more fragmented attachments compared to chemical vapor deposition [66]. It is a possible mechanical disruptions of nanocrystalline cellulose fusing together may have exacerbated the fragmentation of loosely adhered PPY particles. These contributions of fiber disarray may explain the decrease in conductivity with additional particles. Work with conducting plasticizers or additional nanoparticles like silver nanoparticles presents potential for improving the conductivity and structural stability of NCC-PPY films.
8.3.1 Film Deposition

As morphology was found to play a key role in conductivity of NCC-PPY, the effects of NCC-PPY surface density on roughness was studied (Fig 34). Mass density is controllable by changing the concentration of colloid to be casted to film. By increasing the NCC-PPY concentration in water, one can increase the film thickness and sheet density. In this study, thick films of increasing amounts of NCC-PPY were prepared with their roughness and thickness measured using a profilometer.
Fig 34. Surface of NCC-PPY film shows roughness and thickness varied with increasing amounts. Thickness increases as NCC-PPY is added but compresses after 200mg/cm$^2$. 

The roughness tended to increase steadily but independent of the thickness. The presence of roughness was expected as the films were uneven to begin with. The fact that the roughness is increasing however suggests progressive agglomeration of particles, producing more disorder as more particles stack upon the surface. Thickness appears to increase in the beginning, as expected however, later decreases. This non-linearity contrasts from the prediction that it is proportional. It suggests the presence of attractive forces pressing the film and supports the theory that, past a critical point, the
cellulose crystals fuse together to form tight bonds. This behavior is similar to how cellulose fibers bind as paper when dried. Hence, these bonds are likely hydrogen bonds, over van der Waals forces that will reinforce the structure.

8.3.2 Impact of Cellulose Acetate Encapsulation Coating
A cellulose acetate encapsulation coating can be used for enzyme immobilization and its impact on NCC-PPY morphology was worth studying. Cellulose acetate can be coated by dissolving it in acetone and using standard coating methods: spin coating, dip coating, solution casting, spray coating, etc. In particular, used with GOX enzyme immobilization as the pore sizes can be made smaller than 50 angstroms (approximate diameter of the GOX enzyme), and its ability to transport water and glucose through its membrane [67]. Being a passive layer, it is not as crucial as the enzyme, however; because the analytes pass through the layer, it can affect the rates of reaction as it can potentially slow diffusion. In conjunction with NCC-PPY, it was established that its surface morphology was uniquely needle-like, even in agglomerate form so may potentially have surface changes. Cellulose acetate will be coated using spin coating film alone to get a better understanding of film morphology.
Fig 35. Spin curve of cellulose acetate on NCC-PPY. Roughness of NCC-PPY is so large, increase spin rate does not affect the cellulose acetate coating.

It appears the thickness of the film is independent of the spin speed (Fig 35). This is interesting because for typical spin coatings, the film thickness is inversely proportional to the spin speed, hence is expected to appear thinner. This is likely due to the large non-uniformities of the NCC-PPY surface. The roughness is quite high. A likely explanation is that the cellulose acetate polymer is filling up the cracks left by the NCC-PPY film instead of progressively thinning out over the surface. Propagation of glucose through cellulose acetate is another factor that can influence the reaction rate of the sensor and the thickness of cellulose acetate can affect it. It is useful to note this because the variations in cellulose acetate thicknesses can vary the reaction speed if it is used for immobilization.

8.3.3 Deposition Additives

With such non-uniformity of tightly drying cellulose, the effects of plasticizers are explored. Of particular interest is to what effect glycerol, a cellulose plasticizer [68], will have on electrical conductivity. Glycerol itself is insulating so it is not expected to contribute significantly. Any potential increase in conductivity will likely be due to creating more uniform films to better interconnect the conducting fibers. Increase in conductivity will be achieved through increased electrical percolations. It may also go the other way and disrupt critical connection paths, needed for conduction, to interfere with overall conductance. To observe this effect, films were created with varying amounts of glycerol added to the NCC-PPY colloid and its conductivity measured.
<table>
<thead>
<tr>
<th>Concentration Glycerol/NCC (w/w)</th>
<th>Sheet Resistance (Ω/□x10^6)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>0.45</td>
<td>0.2</td>
</tr>
<tr>
<td>10%</td>
<td>0.44</td>
<td>0.37</td>
</tr>
<tr>
<td>20%</td>
<td>0.65</td>
<td>0.21</td>
</tr>
<tr>
<td>25%</td>
<td>0.44</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 3. Sheet resistance of NCC-PPY films containing glycerol plasticizer. Glycerol does not appear to disrupt the conductivity significantly.

Overall there is little impact on conductivity due to the addition of glycerol. After 25% concentration by weight, films became noticeably uniform to the naked eye and substantially weaker to the point of being easily scratched with little force. Based on that, 25% was chosen as an upper limit. Even at extreme values, it was not noticeably different.

9 Glucose Sensing

9.1 Sensor Response Current

Upon fabrication of the electrodes, the main measure is its ability to sense changes in glucose concentration directly as electrical current. The principle aim is to generate an electrical signal output that is a function of glucose concentration and to do so predictably. Ideally the curve generated around the region of interest, being 0 – 10mg/mL glucose in blood or serum. In practice there may be deviations and non-linear characteristics. For simplicity, the curve can be modeled linearly as the sum of a straight
line with error; this is approximated using linear regression and calculating the mean error is done with statistics. The smaller the error within the range of operation, the better the sensor precision. Sensors fabricated on small chip sensing areas was tested by adding compounds like metalloccenes and conducting polymers to interface with them [81]. NCC-PPY is be added instead to test its effectiveness as a mediator.

Fig 36. Probe station setup used for biosensor testing. Probes are guided to sensor contacts by microscope.

9.1.1 Test considerations and sources of error

Another issue is selectivity. Since the output is a single electrical signal, it is imperative to ensure it is caused by glucose alone. Human serum contains many bio-analyses other than glucose such as urea, electrolytes, etc. For the sensor output signals to be
accurate, they need to read only the effects of glucose and no other substances. Enzymes-less electrodes suffer from this and require filtration coatings. For example, the nickel plated electrode has demonstrated an ability to recognize this without any added mediators. The disadvantage is that it was prone to interference and needed filtration layers for each unwanted substance [82]. One advantage of using glucose oxidase enzyme is that is extremely selective; it will only react to glucose and no other molecule. This is important because interference from other reactions can lead to error which can be difficult if not impossible to distinguish on its own. For this reason, enzyme based electrodes were chosen over the multilayer filtration design. It is also a reason to carefully study the control factors.

While it is impossible to reduce all interfering analytes, preparations were done to minimize them. One source was the overall electrode surface area. The sensor electrode is a combination of enzyme active layer and current collecting electrode. As with electrochemical devices, the electrode exposed surface area can affect the net current. The contact pads interface the sensing area to multimeter are over 100 times larger than the actual sensing part (Fig 36). Hence it was necessary to encapsulate the non-contact areas with a polymer coating and to keep the measured solution within that area. This was to reduce errors between samples, and keep measures accurate. To test whether it had any apparent change with respect to glucose and no other naturally occurring solvents or ions present in the PPY layer, a controls used were using buffer alone, and enzyme in buffer solution.

The sensor design being tested is a two terminal device, one for the cathode and the other at the anode. Three terminal devices exist where a reference electrode is used, analogous to electrochemical potentiometer. Typically the reference electrode is used and consists of or is coated with silver-silver chloride for reversible redox reactions [83]. In this work, only a two terminal electrode IDA system is used: one for the reference electrode (cathode) and one for the working electrode (anode). This is done for the following reasons: principles of two electrode systems can be extended to three
electrode without much complexity, by setting an open circuit with the third reference electrode; this is optional. Secondly, the reference electrode, when coated with silver chloride adds an additional redox reagent into the system, which can potentially interfere with the prototype system. While previous work demonstrated conductivity to be not significantly affected in enzyme systems, it is still another ionic species that can influence the electrode and complicate or frustrate the initial system. Chloride ions for example, are doped electrochemically in PPY. NCC-PPY is prone to give potentially erroneous results showing improvement. PPY films are also suspected of changing conductivity in their doped state similar to PEDOT [84] and PANI [85]. Any improvement in conductivity or sensor sensitivity may be falsely attributed to the glucose sensing when it is in fact the influence of NCC-PPY electrode charging which is affecting the conductivity. Even though test can be done to account for this, like observing the charging effect and subtracting it from the overall electrode current generated, for simplicity and to stay within the scope of the work, varying ionic strength was not studied.

9.2 Current vs. Concentration of Glucose

To determine the sensor response to glucose, a curve generated for glucose concentration is needed. A calibration curve is a plot that measures the sensor output to sensory input. For a glucose sensor, the input is glucose concentration and the output is electric current. Ideally this relationship is linear. In practice, the sensor is linearized with regression analysis and then the deviations are considered error. The slope represents the sensitivity of the sensor [69].

Sensor output is measured by placing a sample droplet over the surface of the electrode. This can be done with any droplet generating device such as a simple pipette, with potential to be integrated in microfluidic systems both digital microfluidics
and non-digital, because of its planar nature and its coating over a silicon dioxide insulating surface. Droplet delivery is another issue and beyond the scope of the topic. It is noteworthy to remark however that the fabrication techniques and physical layout of the sensor are used in microfabrication facilities not unlike those used to develop microfluidic technologies on planar surfaces, meaning they have the potential to couple with an enable microfluidic delivery systems to on-board real time analysis for full lab-on-chip applications. Once delivery is assumed, the test method becomes straightforward, to expose a fixed volume of aqueous glucose solution on a fixed surface area of the electrode and measure the electrode current at a given voltage.
Fig 37. Current-Voltage (I-V) sweep when sensor is exposed to increasing glucose amounts. As glucose concentration increases, the current becomes larger, satisfying the main criteria of a sensor.

For completeness and to determine the optimal voltage for a given response, one can run a voltage sweep across several voltages. This has two advantages: one, it lays a complete profile of optimal voltage conditions that is essential to understanding the trade-off between performance and power consumption that plagues any electronic system; two, it can determine what the minimum and possibly maximum voltages to apply and the presence of possible semi-conductor junction voltages that need to be
overcome or possible overdrive voltages; three, it can detect any non-linearities present as a function of voltage, to see if it behaves more like a resistor or other phenomenon.

Sensor output shows that NCC-PPY delivers a sensor current. Final currents generated are over 1000 times that of the intrinsic control, as indicated by the 0 concentration sample (Fig 37). The ability to deliver such large currents is crucial to detecting the presence of glucose over other analytes and distinguish it from other sources of error. It is also important to note that while it is a feature of the PPY conducting polymer, compared to PEDOT:PSS, the result is over 1000 times higher than the nano and micro amperes of current found in prior work [50, 51]. This represents a significant improvement over current conducting polymer mediators. This improvement may come from the use of the IDA design as well as the larger surface-area-to-volume ratio of the NCC needles. The enzyme redox reaction with the electrode occurs at the surface and by electrochemical principle is dependent on the surface area. Increasing the surface area to volume ratio will have the effect of increasing possible reactive sites, thereby allowing a faster reaction to generate more electric current.

9.3 Conduction Mechanics

The redox reaction needed to be verified and the effect of NCC-PPY on glucose/GOX reaction. Electropolymerized PPY was shown to mediate the redox behavior with enzyme immobilized. NCC-PPY differs in that it is not electropolymerized and the enzyme is deposited on the surface. In addition, the bulk conductance may be hindered. The principle benefit of using PPY materials is the lower oxidation potential that arises when you have the reaction proceeding without H2O2 [52, 53]. Instead the PPY itself oxidizes the FADH2 cofactor in the enzyme immediately. Because the reduction potential of FAD2+ to FADH2 is ~0.3V [90], it is comparably lower than that of H2O2, at ~1.8V. This offers an additional promise of lower power required and stronger sensitivity.
It is also preferable to inhibit the generation of hydrogen peroxide because of its degradation effects of PPY linkages.

\[
\text{Glucose} + \text{GOX}_{\text{ox}} \left( FAD^{2+} \right) \rightarrow \text{GOX}_{\text{red}} \left( FADH_2 \right) + \text{gluconolactone} \\
\text{PPY}_\text{red} + FADH_2 \rightarrow \text{PPY}_{\text{ox}} + FAD^{2+} + 2e^- \\
\text{instead of} \\
FADH_2 + O_2 \rightarrow FAD^{2+} + H_2O_2
\]

Eqn 2. Pathways for glucose oxidase enzyme reactions with NCC-PPY mediator.

These alternative conduction pathways allow the preservation of PPY as it consumes electrons prior to reaction with water to form hydrogen peroxide. This improves stability in addition to providing the extra electrons to the sensory response.
Fig 38. Normalized changes of electric current compared to control solution. Linearity suggests that sensor mechanism is due to NCC-PPY changing resistance instead of simple redirection of reaction current, which would be seen as a constant line. This leads to greater levels of amplification.

To test the electrode, it is to be exposed to varying levels of glucose concentration. While it appears straightforward, because factors such as total electrode surface area and impurities can vary the results. In the design each electrode was limited to a
specific surface area with all the electrode digits of the IDA restricted to a 1 mm x 1 mm area. For each grade, 5 um, and 1 um, the total surface area was constant, as only the line width was changed. All spacing between the lines was to scale, exactly 3 times the width of the electrode. For example, in a 5 um digit width, the spacing between lines was 15 microns while in a 1 um digit, it was 3 um. Naturally the total number of digits and electrodes are increased as digit width decreases, tending to favor the smaller sized digits by default, when fixing the surface area (eqn. 3) however; in this scenario, it was deemed to be a more realistic view of the advantages in using smaller scale digits as a way of promoting and justifying the additional investment; such promotion is akin to that used in the microelectronics industry by what is known as “Moore’s' Law.

9.3.1 Theoretical performance increase by miniaturization

It is well known that the miniaturization of electronic components can lead to performance increases in sensors. Often however, the degree at which miniaturization occurs is not standardized and designs are taken as a guess based on equipment constraints. In this section a theoretical model is used to predict the performance increase of the electrode solely by contribution of the electrode design itself. Specifically we will demonstrate how \[ n = \frac{W}{w} \left( \frac{1}{1 + a} \right) \], models the number of \( n \) IDA digits producible by a rectangular array. \( W \) representing the total width, \( w \) be digit width, and \( a \) being the ratio of digit spacing to digit width.
For a rectangular evenly spaced IDA pattern,

Let

\[ W := \text{total width of IDA pattern} \]

\[ L := \text{total length of IDA pattern} \]

\[ w := \text{total width of digit} \]

\[ l := \text{total length of digit} \]

\[ n := \text{total number of digits} \]

\[ g := \text{gap distance between digit extremity and opposing electrode edge} \]

\[ s := \text{spacing between digits} \]
$$SA = LW$$

$$L = l + g$$

$$n = \frac{W}{w + s}$$

$$W = \frac{SA}{L}$$

$$= \frac{SA}{l + g}$$

$$n = \frac{SA}{l + g} \left( \frac{1}{w + s} \right)$$

For $l' \gg g$ (minimal gap), $l \approx L$

$$n = \frac{SA}{L} \left( \frac{1}{w + s} \right)$$

Let $a = \frac{s}{w}$ denoting the ratio of $s$ to $w$

$$n = \frac{SA}{L} \left( \frac{1}{w + aw} \right)$$

$$= \frac{SA}{Lw} \left( \frac{1}{1 + a} \right)$$

Since $SA = LW,$

$$= \frac{LW}{Lw} \left( \frac{1}{1 + a} \right)$$

$$n = \frac{W}{w} \left( \frac{1}{1 + a} \right)$$

Eqn 3. Derivation showing the number of IDA lines is a function of lithography resolution. This indicates a proportionality of performance with smaller sized features.

It is shown that for a rectangular array of IDA digits, fixing the surface area and ratio between finger width and spacing makes the total number of electrodes inversely
proportional to the electrode widths. Using more advanced technologies to decrease the width of the electrodes will increase the number of digits one can place on the IDA, thereby increasing performance. This can be used to justify the additional cost and research incentive into advanced microfabrication techniques. An interesting study would be to what effect changing the length of the array would be, in order to frugally optimize the surface area usage on chip. For this particular work, the design was set such that the gap distance of 5 um is much smaller than the length 1000 microns.

In addition, fixing the surface area, and varying 'n' the number of lines, has the added benefit of allowing us to observe any cumulative and potentially non-linear effects of IDAs from redox cycling while eliminating the known relationship between surface area, which is linear, thus simplifying interpretation of the data by isolating the most relevant factors.

The next consideration is the solution application method, be it complete immersion or droplet testing. While both have their merits, the former has more controlled and constant concentration gradients of the former with the more realistic scenario mirrored in the latter; the droplet application was used in this scenario. In this case the decision was based on the potential for application with both digital and conventional microfluidics where sample analytes may be as small as a few microliters. This allows couple with later research as it takes into account the scenarios in which there is limited sample analytes. For these tests droplets of glucose solution were administered using a micropipette onto the exposed sensing area while time based readings of current and voltage were read before and after.
9.4 Effect of NCC-PPY Thickness

Film thickness is an important parameter and was studied. Thickness determines the quantity of NCC-PPY and also is a separation distance between the analyte and electrode. In turn this can affect the reaction time and changes in electrical resistance. As the layer between the electrode and the analyte, it is the interface with the chemical reaction and the electrical response. In that regard, it is likely to affect the end performance of the biosensor. On the lower extreme without sufficient amounts of NCC-PPY it will not work at all. Too much can slow or stymie the diffusion of electrons rendering it ineffective as well. Thus understanding what effects will potentially arise by changing thickness is of practical importance.

The following study will determine the effect of NCC-PPY thickness on the sensory response. Since the layers are solution casted and agglomeration issues of NCC, thickness is increased by adding multiple coating layers in sequence on top of one another: the more layers added, the thicker the film.
Fig 39. Normalized electric current comparing the ratios of current with glucose added and without by voltage. Peak current is obtained at a thickness of 2 layers or 0.5 g/mm\(^2\) and holds steady past 6V.

Peak current was achieved at 2 layers of NCC-PPY (Fig 39). One layer produced a response that was barely noticeable. A sudden jump appeared at 2 layers and subsequent layers had a noticeable but not comparable difference. This is likely attributed to the morphology of the films. After 3 layers, films appeared visually thicker with some surface cracking and discontinuities. At 5 layers, it may have been too thick for the glucose and enzyme to diffuse through in time. 2 Layers appeared to have the...
right combination of thickness and amount of conducting polymer to deliver maximum current in time for the electrode. This allows films to be made to optimize performance and minimize costs.
Chapter 6
Conclusions and Recommendations

Nanocrystalline cellulose-polypyrrole (NCC-ppy) composites as emerging bio-based nanoparticles have good promise for biosensor applications. Coupled with the biocompatibility and process and performance advantages of nano-crystalline cellulose films together with the novel redox properties of poly pyrrole conducting polymer, they have good potential to be used for enzyme based biosensors. While PPY alone is insoluble in water and difficult to solubilize without potentially toxic chemicals, it is known to bond mechanically to cellulose, a natural and abundant material, and this provides an effective solution. The hypothesis that NCC-ppy can be suitable for glucose biosensor application was demonstrated with the suitable sensory response. This is the first time NCC-ppy has ever been demonstrated in biosensors. In fact, the current level was improved by over 100 times in strength compared with some other conducting polymers. Enzyme compatibility tests confirmed this is likely attributed to compatibility of NCC-ppy with enzymes and layering on electrode. Fabrication techniques of interdigitated electrodes were carefully scrutinized and adapted to deliver the most suitable electrode for this biosensor application; fabrication was achieved using latest technologies of Electron Beam and photolithography, both commercially available today. With NCC-ppy biosensors, it is possible to take the next step in innovating sensors with biocompatible cellulose nanomaterials. The following conclusions were established:

- NCC-ppy did have a redox response with enzymes GOX and HRP and the mechanism is a change in conduction
- It was proved that NCC-ppy does not significantly inactive enzymes GOX and HRP beyond 10%. In fact, evidence suggests an improvement
- IDA electrodes were successfully fabricated with photo and e-beam lithography specifically optimized for this biosensor. Techniques to develop and optimize the
fabrication were done and bilayer lift-off was the most effective in both cases. Lines small as 100 nm were achieved using gold electrodes; this design in theory and practice was established to have potential improvements as nanofabrication technologies improve. Using this design a signal-to-noise ratio was reduced by virtue of higher output currents which showed a 1000 times increase in current compared to that of previous work in glucose sensors with polymeric mediators reported by [10]. This improvement in efficiency offers promise of reducing cost.

- The advantage of using NCC-PPY on a sensor was clearly demonstrated by the ease at which a water dispersed solution casted film of PPY was made into a sensor. No costly or time consuming electro-polymerization or chemical vapor deposition needed.

- A device scale proof-of-concept was demonstrated that this new method of solution casting PPY meets the base requirements for a suitable sensor prototype defined herein. Current response was positively correlated with a normalized response of 20 times the base current when exposed to varying glucose concentration 0 – 44 mg/mL. Measurement ranges was up to 12mA, well above 1 micro amp, across a voltage sweep of 0 to 6 Volts.

- Overall, this new and novel enzymatic biosensor device combined with water solution casted NCC-PPY, demonstrates the possibility to measure glucose concentrations with a conducting polymer mediator in an easy to fabricate high performance sensing device. This shows good potential to be an integral core to a fully functional blood sugar or fermentation sensor.

The possibilities are endless and there are many questions to be answered in the future. NCC-PPY has potential to measure other analytes, such as Urea with Urease enzyme. The sensor can be optimized by using emerging techniques such as using biotinylated NCC-PPY for enzymes or nanoinprint lithography for cost-effective nano-IDA electrodes. Observing conductivity enhancements by adding other conducting nanoparticles such as carbon nanotubes or gold nanoparticles can lead to higher
current throughputs. There is much room for further study of the NCC-PPY itself such as effect of average fiber length or penetration rates in cellulose acetate films. It has yet to be developed as a stand-alone technology both for large scale applications, like fermentation detection, and small scale devices like flexible electronics or digital microfluidics. There is much ahead in both research and development of NCC-PPY biosensors.
References


[14] University of Southampton., Instrumental methods in electrochemistry, Chichester
New York: E. Horwood ;


[70] G. Lim, S. H. Lee, P. K. Kim et al., “Fabrication and characterization of 3-
Dimensional MOS transistor tip integrated micro cantilever,” Microsystem
Technologies-Micro-and Nanosystems-Information Storage and Processing

[71] S. Pauliac-Vaujour, C. Comboroure, C. Vizioz et al., “Hybrid high resolution
lithography (e-beam/deep ultraviolet) and etch process for the fabrication of
stacked nanowire metal oxide semiconductor field effect transistors,” Journal of


during self-magnetic-pinch e-beam diode operation,” Ieee Transactions on

Schottky barriers,” Journal of Applied Polymer Science, vol. 81, no. 9, pp. 2127-

[75] V. La Ferrara, B. Alfano, E. Massera et al., “Fabrication and characterization of a
hydrogen sensor based on palladium nanowires,” Transducers '07 &
Eurosensors Xxi, Digest of Technical Papers, Vols 1 and 2, pp. U1279-U1280,
2007.

[76] V. Reboud, P. Lovera, N. Kehagias et al., “Two-dimensional polymer photonic
crystal band-edge lasers fabricated by nanoimprint lithography,” Applied Physics

technology for Nano scale patterning,” Journal of Vacuum Science & Technology


[79] C. S. Yoo, Semiconductor manufacturing technology, Singapore ; Hackensack,

[80] B. Baylav, M. Zhao, R. Yin et al., “Alternatives to Chemical Amplification for 193
nm Lithography,” Advances in Resist Materials and Processing Technology Xxvii,

[81] X. Zhang, H. Ju, and J. Wang, "Electrochemical sensors, biosensors, and their

[82] J. P. Li, and J. G. Yu, “Novel Enzymeless Glucose Sensor Based on
Overoxidized Polypyrrole Modified Nickel Microelectrode,” Chinese Journal of

[83] V. S. Bagofskii, and Wiley InterScience (Online service), "Fundamentals of
electrochemistry," The Electrochemical Society series, Wiley-Interscience, 2006,
pp. 1 online resource (xxviii, 722 p.).


Appendices

10 Appendix A: List of Acronyms

AFM  Atomic Force Microscopy
IC   Integrated Circuit
GOX  Glucose Oxidase Enzyme
HRP  Horseradish Peroxidase Enzyme
IDA  Interdigitated Electrode Array
n-IDA Nano Interdigitated Electrode Array
ISFET Ion Sensitive Field Effect Transistor
MOSFET Metal Oxide Semiconductor Field Effect Transistor
OTFT Organic Thin Film Transistor
PANI Poly Analine
PPY  Poly Pyrrole
PEDOT Poly Ethylene-Dioxythiphene
PSS  Poly Styrene Sulfonate
NCC  Nano Crystalline Cellulose
NCC-PPY Nano Crystalline Cellulose and Poly Pyrrole Composite fibers
MCC  Micro Crystalline Cellulose
SEM  Scanning Electron Microscope
REDOX Reduction-Oxidation Reaction
### Appendix B: Glossary of Terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tr>
<td>Amperometric Sensor</td>
<td>Sensor that operates by generating electric current and collecting it directly at an electrode</td>
</tr>
<tr>
<td>Signal-to-Noise Ratio</td>
<td>A measure of desired signal divided by random fluctuations, or “Noise”; it is a measure of measurement precision, with high signal-to-noise ratio indicating a very precise and reliable signal,</td>
</tr>
<tr>
<td>Signal</td>
<td>A one dimensional function. Examples include electrical, optical, and acoustic waveforms.</td>
</tr>
</tbody>
</table>
To help make more precise lift-off hard-baking was investigated. One of the challenges of using photoresist S1811 is developer MDF-312 or CD-26 also etches LOR. This means that, unlike E-Beam where the developer ZDN50 did not react with the LOR layer, both the LOR and photoresist are etched simultaneously. With both reactions occurring at the same time, the etch rates of both are more difficult to control because the development of the top layer can prematurely affect the layer beneath. To help mitigate this, hard-baking (baking after development of the photoresist) at 135°C was done to remove the remaining solvent from the S1811 resist and protect it from further decay. This was investigated with hard baking and exposing it to different developer times. It was observed that hard-baking caused significant cracking in the surfaces and rendered it impractical.
Fig 40. Sample developed in 50% MF321 for 10 min. Etch rate was slowed considerably with photoresist still remaining. Very little to no etching of the LOR layer beneath save for some hairline cracks. Even at this develop time, there is still trace photoresist remaining, as seen from the paired lines appearing at each digit; the center of the digit is not yet developed.

Although it is not immediately apparent, the resulting interaction between photoresist and LOR becomes clear after hard-baking. At the interface the photoresist begins to crack along the LOR sample after hard-baking if even a small film remains. The sample was not moved at the time cracks began appearing so it is likely due to changes in the interfacial tension from thermal expansion and the brittleness of the photoresist. When increasing the concentration of the developer, the speed of etching increased, as consistent with literature [21]. Even at 100% concentrations and even though microscopic photos gave the appearance of cleared resist, cracking was still apparent and presence of a thin impermeable film was evident by the prevention of LOR.
development after hard baking. It was clear that for this biosensor hard-baking was not a viable optimization method. Interestingly the same phenomenon appeared not just from hard baking but from simply letting the sample dry in ambient air overnight.

12.1.1.1 Troubleshooting Issues of Resist Impermeability

In investigating the causes of film impermeability, it was found even air drying prevented further development. Samples after developing, without hard baking, were left overnight to dry after nitrogen blow dry. The next day, they were re-immersed in developer. It was expected that the solubility would not change and both the photoresist and LOR would continue to etch as normal. Surprisingly this was not the case.

Fig 41. Drying in air produces a protective film above the photoresist, making it impenetrable by photoresist developer. This demonstrates the need for “one-shot” development. Etch rates must be controlled with bake temperatures.
Only LOR around cracked areas etched and did so slowly. This suggests the developer is reaching the LOR not through the top but by seeping through laterally via the small cracked areas. This supports a theory that there is an impermeable barrier preventing the developer from coming in. To rule out developer concentration as a cause, the concentration was changed both by using two developers, TMAH based MF-321 and its more aggressive formula CD-26, at dilution ratios 1:1, 2:1, 3:1. All yielded no etching through the film. This leads to a conclusion that somehow after the first etch, whatever S1811 remains, creates a protective film and becomes insoluble thereafter in spite of being exposed previously to UV.

Fig 42. Cracked area of photoresist. The blue hue around the crack represents the LOR undercut that was etched after 10 min exposure. Even without hard baking, the resist becomes insoluble protecting other areas. This is unlike the LOR which is always soluble in developer regardless previous treatment.

To test whether it was an interaction between the LOR and photoresist which prevented development after drying in air overnight, a primer was placed in between the LOR and
the photoresist layers. HMDS primer is often used with S1811 series photoresists with silicon for adhesion however, deemed unnecessary when using LOR. This primer may be used to separate the film LOR from the S1811 to prevent possible interactions that can block transmission of developer at the layer so, to examine whether there was an interaction between the layers, HMDS was added nonetheless.

Having ruled out many controllable factors, such as hard-bake temperature, developer concentration and interfacial agents, it is clear that it is preferable for any etching of the LOR with S1811 photoresist be done in a one step process. This is an important consideration when choosing to use photolithography over E-Beam and one that is not obvious in literature because of commonplace use of hard-baking using photoresists for other purposes.

Fig 43. Oxygen plasma used to attempt removal of ZEP/LOR bilayer stack. Vein like penetration points appear after 10 min exposure. Marginal lift off appears as most of the region is still coated by the metal evaporated layer, making chemical methods more suitable for lift-off procedure.
1.1.1 Bi-Layer Photolithography

UV lithography for bilayer resist process was done for 10 micrometer lines due to issues with monolayer lift-off stripping. A layer of LOR was spun onto the wafer and baked at 200 degrees Celsius. The higher temperature was desired to reduce the LOR etch rate from the developer. This is especially important in UV photolithography with the resist used because the S1818 photoresist is developed by basic solutions, in this case, tetramethylammonium hydroxide. Increasing the resistance is important as it allows the photoresist layer to be developed without prematurely etching the lower areas. This is in contrast with E-Beam as will be discussed because the E-Beam developer is different.

Postbaking of the photoresist was essential. Initial attempts were made to observe the effects of development time without postbaking. This was done to establish whether the rate of dissolution of the exposed resist and the LOR layer would allow for synchronous dissolution and create the undercut at the same time in a one-step process. This of course assumes that the exposed area will dissolve faster than the LOR which in turn dissolves much faster than the unexposed polymer. After 3 minutes of development with MF-321, the 5um lines were still not clear. Rather a large 100um radius bubble of rapid etching grew, clearing the polymer in that area entirely and removed a significant portion of the electrode. Based on this it can be inferred that once the LOR layer was sufficiently penetrated by the larger contact, the lateral etch rate was fast enough to lift-off the bilayer stack before the inner 5 um lines were able to clear through. This could also be a dose effect so the UV dose will need to be considered, keeping in mind, however, that 90s is still relatively high. Another factor could be that the layer without post-baking began to etch in addition to the LOR. This caused a simultaneous and exponential growth in etching because as the photoresist is removed the gap gets larger and the etch rate increases as a result. It was found previously [78] that the etch rate was proportional to the gap size.
Dose test for larger patterns was required. Developing with MF-321 is the same chemical composition and close to the same concentration of 80% CD-26. A challenge hence is to determine the approximate time of dissolution necessary and dose equivalent to produce an undercut and dissolve the features at the same time. MF-312 is more dilute and may be desirable to slow the process. With E-beam lithography, it was found to show an undercut rate of less than 75 nm per minute. At the same thickness, it is predicted that the dissolution rate of LOR will be the same. Larger features on the pattern did not show any comparable undercut, in the 1 um range, however, this can be due to the thickness of the photoresist over the LOR layer (1um to 0.1nm). Even at 2 min 30 s, it still showed incomplete clearing at the critical features. Instead a double line pattern was seen and can be due to edge diffraction and interference patterns from exposure and reflectance of the 0.1 nm layer of LOR. Resonance may have occurred at 100nm range.

While it is much less costly than E-beam, photolithography does have drawbacks. The smaller the pattern feature size and resolution, the more difficult it becomes to control the feature size and produce IDA lines. This is due to diffraction limits of the UV light and the distance between mask and polymer layer. [79] Concerning lift-off in particular, there are additional constraints. The photoresist thickness needs to be thicker than the metal deposition layer because if the metal layer and the resist layer is too similar, the edges may not disconnect off the other areas easily and remain on the substrate, making the resist removal step difficult. If you increase resist thickness, in order for the UV light to penetrate the polymer down to the substrate (for the pattern to “clear”) the resist thickness needs to be comparable to the width of the features being patterned. This is because the farther down, the more the light can diffract and widen the image unacceptably wide. Widening images can be especially problematic for this biosensor
Fig 44. Pattern developed with MF321 for 40s. At this concentration the etching was aggressive enough to remove both the photoresist and the LOR layer at the same time, in a short time span. Because of overdevelopment, lines which are supposed to be separated blended together as the widths expanded.

A vast number of factors affect the photolithography process in fabricating biosensor electrodes. Factors that affect development include facility architecture, ambient temperature, humidity, air cleanliness, UV light exposure time, developer concentration, developer solution type, development time, resist composition, resist concentration, resist thickness, UV exposure time, UV exposure intensity, ambient humidity, resist bake time and bake temperature. While many of these factors are related to the facility in question, the most controllable ones are UV exposure time, developer concentration and development time. Hence, in order to optimize the process for each facility and each sample it is required to develop calibration parameters and test it under those conditions. To test UV exposure, the UV exposure time is varied with other factors.
remaining the same. This is similar to an E-Beam dose test; it considers how much energy is required to clear the resist to form a clean pattern on the substrate. A clear pattern is one that is neither underexposed, unable to dissolve in the developer solution, nor overexposed to bleed outwards. For both single and bilayer samples, the UV exposure test was performed.