SYNTHESIS, MODIFICATION, AND APPLICATION OF SILOXANE MATERIALS FOR ENVIRONMENTAL SENSING

by

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Abstract

As human populations and resource consumption increase, it is increasingly important to monitor the quality of our environment. While laboratory instruments offer useful information, portable, easy to use sensors would allow environmental analysis to occur on-site, at lower cost, and with minimal operator training. We explore the synthesis, modification, and applications of modified polysiloxane in environmental sensing.

Multiple methods of producing modified siloxanes were investigated. Oligomers were formed by using functionalized monomers, producing siloxane materials containing silicon hydride, methyl, and phenyl side chains. Silicon hydride-functionalized oligomers were further modified by hydrosilylation to incorporate methyl ester and naphthyl side chains. Modifications to the siloxane materials were also carried out using post-curing treatments. Methyl ester-functionalized siloxane was incorporated into the surface of a cured poly(dimethylsiloxane) film by siloxane equilibration. The materials containing methyl esters were hydrolyzed to reveal carboxylic acids, which could later be used for covalent protein immobilization. Finally, the siloxane surfaces were modified to incorporate antibodies by covalent, affinity, and adsorption-based attachment. These modifications were characterized by a variety of methods, including contact angle, attenuated total reflectance Fourier transform infrared spectroscopy, dye labels, and $^1$H nuclear magnetic resonance spectroscopy.

The modified siloxane materials were employed in a variety of sensing schemes. Volatile organic compounds were detected using methyl, phenyl, and naphthyl-functionalized materials on a Fabry-Perot interferometer and a refractometer. The Fabry-Perot interferometer was found to detect the analytes upon siloxane extraction by deformation of the Bragg reflectors. The refractometer was used to determine that naphthyl-functionalized siloxanes had elevated refractive indices, rendering these materials more sensitive to some analytes.

Antibody-modified siloxanes were used to detect biological analytes through a solid phase microextraction-mediated enzyme linked immunosorbent assay (SPME ELISA). The SPME ELISA was
found to have higher analyte sensitivity compared to a conventional ELISA system. The detection scheme was used to detect *Escherichia coli* at 8500 CFU/mL.

These results demonstrate the variety of methods that can be used to modify siloxanes and the wide range of applications of modified siloxanes has been demonstrated through chemical and biological sensing schemes.
Acknowledgements

This thesis, and the projects it describes, have been a continual learning process for me. I cannot thank my supervisor, Dr. Stephen Brown, enough for his advice and generosity throughout my time in his group. Every time we spoke, his enthusiasm for research and his patience with the roadblocks we encountered always shone through and inspired me to keep pursuing my goals. The breadth of his knowledge continues to astound me and I can always count on learning something new in every conversation. My supervisory committee members, Dr. Anne Petitjean and Dr. Richard Oleschuk, have been incredibly supportive in offering advice and feedback on my project and thesis; I feel very fortunate to have had the chance to work with them both.

Thank you also to the many other people who contributed to my studies. The staff members in the Chemistry Office were always there to help and I would like to especially acknowledge the Graduate Assistants, Michelle and Annette, for taking care of all the students over the years. I was also fortunate to be assisted by Dr. Françoise Sauriol in the NMR facility, Dr. Ruiyao Wang in the surface analysis facility, and Dr. Igor Kozin in the undergraduate instrument room. Also, thank you to Colette Steer in the School of Graduate Studies for being a completely unexpected support in my final year of school.

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I am incredibly indebted to my family for their continuous support. Thank you especially to my parents, Janet and Jim Mackey, and brother, Stuart Mackey, who have been there with unconditional love and a listening ear. Your support has kept me going through all these years of school. To my grandparents, Art and Marie Mackey and Cam and Pat Hughes, who believed in the value of education, I wish that you were still here so that I could thank you in person. Finally, thank you to John for your love, understanding, and unlimited generosity.

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Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author, with the following exceptions:

Work on the Fabry Perot interferometer devices (Chapter 2) was carried out in collaboration with members of Dr. Stephen Brown’s research group at Queen’s University (Dr. Jingjing Zhou), Dr. Peter Loock’s research group at Queen’s University (John Saunders, Dr. Jack Barnes), and Dr. Yves-Alain Peter’s research group at École Polytechnique, Montreal, QC (Antoine LeBlanc-Hotte and Dr. Raphael St.-Gelais). The experiments and data described in this thesis are limited to work done by the author.

The high refractive index film project (Chapter 2) was started in collaboration with Jessamyn Little. Undergraduate students Sarah Kawai, Wendy Lee, and Regan Sedgewick performed the experiments described here under my supervision.

Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

Gillian Claire Mackey

May, 2016
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<th>Description</th>
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<tr>
<td>4-MUP</td>
<td>4-methylumbelliferyl phosphate</td>
</tr>
<tr>
<td>Anti IgG-AP</td>
<td>Anti rabbit immunoglobulin G-alkaline phosphatase</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated total reflectance Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>EDAC</td>
<td>N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgG-AP</td>
<td>Immunoglobulin G-alkaline phosphatase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline containing 0.1% Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCBMS-PDMS</td>
<td>Poly((4-carboxethoxy)butylmethyl-co-dimethyl)siloxane</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PDMS-PMS</td>
<td>Poly(dimethyl-co-methylhydrogen)siloxane</td>
</tr>
<tr>
<td>PDMS-PMS-PDPS</td>
<td>Poly(dimethyl-co-methylhydrogen-co-diphenyl)siloxane</td>
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<td>PMHS</td>
<td>Poly(methylhydrosiloxane)</td>
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<tr>
<td>PMOS-PDMS</td>
<td>Poly(methyloctyl-co-dimethyl)siloxane</td>
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<td>PyrOH</td>
<td>1-Hydroxypyrene</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SELEX</td>
<td>Selective evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface-enhanced Raman spectroscopy</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSIMS</td>
<td>Static secondary ion mass spectrometry</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compound</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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</table>
Chapter 1

Introduction

1.1 Environmental Contamination

As human populations grow and resource consumption rises, it is increasingly important to monitor the environment in order to understand the effects of pollution on the earth system: which pollutants are present, where the pollution comes from, how much pollution is present, and how the amounts are changing over time. Pollution, or environmental contamination, is defined as “any change in the physical, chemical, or biological characteristics of the air, water, or soil that can affect the health, survival, or activities of human beings or other forms of life in an undesirable way.” Biological pollutants encompass bacteria, viruses, and protozoa, while chemical pollutants range from pesticides and other organic molecules to gases and metals, and physical pollutants include heat, noise, light, and radiation. Some of the current pollution challenges include climate change due to rising concentrations of carbon dioxide and other greenhouse gases, smog due to combustion, and diseases spread through drinking water, among many others. It is not always possible to prevent the release of pollutants, since some are only recognized as harmful after their release, accidental spills can occur, and the cost of avoiding pollution can be high. Therefore, it is important to have systems capable of detecting pollution when it occurs in the environment, allowing appropriate remediation to take place.

1.2 Water Contamination

Water is a necessary component of all life on earth, while it has also served as a convenient receptor for waste throughout human history. Waste is quickly dispersed when water flows and mixes. Although positive from a sanitation perspective, since accumulations of waste can lead to disease, many
water systems have received too much waste, leading to levels of contamination that impair environmental quality. Many of these chemical, physical, and biological pollutants can cause acute or chronic health problems for people and for their environment.

Biological contaminants that produce disease are categorized as pathogens. Pathogens, which include bacteria, viruses, and other microorganisms, are infectious agents capable of causing disease in a host organism. While many microorganisms exist naturally in the human body and can even be beneficial, a small number cause serious diseases. Disease-causing pathogens have many possible routes of transmission, including air, direct contact or contact with bodily fluids, or by the fecal-oral route. This fecal-oral mechanism of transmission is the primary concern in water quality, since bodies of water are used for both drinking water and sanitation. When fecal waste enters a water source that is also used for drinking water, transmission of pathogens can occur. Waterborne diseases have become an increasing problem as the planet deals with rising human population densities. This has led drinking water sources to be found in close proximity to sewage outflows, septic systems and livestock operations, leading to higher risks of drinking water contamination. Worldwide, 663 million people lack access to improved drinking water and it is estimated that 842 000 deaths could be prevented annually by improved access to drinking water, hygiene and sanitation. Even in developed countries such as the United States, where drinking water is usually treated, millions of gastrointestinal illnesses are attributed to contaminated water each year. In Canada, a 2015 report in the Canadian Medical Association Journal found that 1838 drinking water advisories were in place across the country, with especially high frequency in First Nations communities. The number of these advisories indicates the prevalence of impaired water quality due to pathogenic contamination. Salmonella typhi (typhoid) and Vibrio cholerae (cholera) were the first pathogens to be associated with waterborne transmission. In recent years, many additional pathogens have been identified as waterborne hazards, meaning that there are now a large variety of bacterial, viral,
protozoan, and helminth pathogens of concern in drinking water. Due to this variety of pathogenic risks, government leaders and society at large must be concerned about the maintenance of microbiologically clean water resources for the future. An important aspect of this is the ability to detect and eliminate harmful pathogens from water supplies.

Chemical contaminants can also enter the water system through a number of pathways. For example, modern agricultural practices lead to the release of insecticides, herbicides, and other pesticides, which may have toxic effects on organisms in the ecosystem, as well as fertilizers, which lead to eutrophication. These pollutants end up in the water system due to runoff during rainfall and drainage into aquifers. Both industrial and household wastes add large quantities of chemical contaminants to the water system through sewage outflows and landfills that leak into surrounding bodies of water. Meanwhile, new categories of pollutants are constantly being added and some of the most recent concerns include artificial sweeteners, brominated flame retardants, pharmaceuticals, hormones, perfluorinated compounds, and sunscreens. A wide variety of chemical contaminants can enter the water system from agricultural, household, and industrial sources, leading to health impacts for humans and their surrounding ecosystems.

It is of critical importance to be able to identify contamination problems when they arise, whether due to chronic release or accidental spill, so that clean up or precautions can be undertaken as necessary. This demonstrates the need for a wide variety of environmental analysis techniques to alert regulators to potential issues before major health or ecosystem effects occur.

1.3 Environmental Analysis

Environmental properties are measured for many reasons, including: baseline monitoring of the natural environment and its variability, compliance and enforcement activities by regulators, and effects
monitoring to determine the impact of stressors introduced by humans. For many years, environmental monitoring has required the collection of samples, transportation to a laboratory, and analysis by a trained technician. In this model, only intermittent measurements can be recorded and concerns relating to sample transportation and storage stability have to be addressed. Nevertheless, most environmental analysis is still completed in laboratory settings due to the superior equipment available. High-performance liquid chromatography and gas chromatography, using a variety of detectors, are currently the primary methods of analysis for organic molecules, after purification and concentration of the sample. Meanwhile, metals are often analyzed by inductively coupled plasma attached to either optical emission or mass spectrometry equipment. For biological contamination, culture methods, polymerase chain reaction, and fluorescence assays (for protozoans) are the usual methods of choice. Common among all these analysis methods are the need for large, expensive instruments, and costly expert analysis, leading to a continuing interest in the development of monitoring technologies for on-site (portable, but operated in a lab) and in-field (hand-held, can be used for in situ measurements) use. These technologies may take the form of miniaturized laboratory instruments or specially designed sensors. There are many advantages available through the move to on-site monitoring (Table 1-1) and many researchers have worked to adapt conventional analytical instruments for this purpose. For example, gas chromatographs (with mass spectrometer, flame ionization, and photoionization detectors), ultraviolet-visible and near infrared spectrometers, X-ray fluorescence systems, ion mobility spectrometers, electronic noses, electronic tongues, capillary electrophoresis, and polymerase chain reaction systems have all been utilized on-site. One very exciting area of research is ambient ionization mass spectrometry, which minimizes or eliminates the need for sample preparation and enhances portability. While many applications of adapted laboratory instruments have been demonstrated, the results are characterized by inaccurate quantitation, higher limits of detection, and lower sensitivity in comparison with their conventional counterparts.
Table 1-1: Advantages to be obtained through the development of on-site monitoring technologies

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Description</th>
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<tbody>
<tr>
<td>Constant monitoring</td>
<td>On-site analysis allows continuous monitoring of environmental properties and can provide early warning of contamination events(^{18})</td>
</tr>
<tr>
<td>Cost reductions</td>
<td>Manual sampling and laboratory analysis is more expensive than on-site monitoring,(^ {19}) both in terms of equipment and personnel costs</td>
</tr>
<tr>
<td>Portability</td>
<td>Remote locations would benefit from portable and robust devices to avoid the inconvenience of sample transport</td>
</tr>
<tr>
<td>Sample integrity</td>
<td>Transport to an off-site lab may take days, which may compromise the sample(^ {19})</td>
</tr>
<tr>
<td>Adaptable sampling</td>
<td>Ability to adjust planned sampling locations based on data collected allows more detailed information to be obtained(^ {20})</td>
</tr>
<tr>
<td>Efficiency</td>
<td>On-site analysis techniques require little or no energy to operate and little or no reagents(^ {21})</td>
</tr>
<tr>
<td>Limited sample pretreatment</td>
<td>Techniques developed for on-site analysis must require little or no sample pretreatment steps, simplifying the procedure(^ {21})</td>
</tr>
<tr>
<td>Simplicity</td>
<td>On-site techniques are easy to operate(^ {21})</td>
</tr>
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</table>

For instance, X-ray fluorescence instruments can replace inductively coupled plasma techniques for on-site analysis of metals and require only minutes per sample for analysis, but do not reach an equivalent level of sensitivity.\(^ {26}\) In addition, the use of conventional analytical instruments on-site leaves them susceptible to changing ambient conditions, and sample contamination due to the working environment.\(^ {21}\) There remains a need, therefore, to develop on-site and in-field analysis techniques that address the performance challenges currently observed.\(^ {21}\)

1.3.1 Sensors for Environmental Contamination Analysis

IUPAC defines a chemical sensor as "a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal."\(^ {27}\) Wang and Wolfbeis provided a more descriptive definition: "chemical sensors are miniaturized analytical devices that can deliver real-time and online information on the presence of specific compounds or ions in complex samples."\(^ {28}\) Although a common definition has not been agreed to,
a sensor device is generally held to include a receptor component, which transforms chemical information into a form of energy, and a transducer, which converts this energy into an analytical signal.\(^{27}\) Ideally, sensors exhibit rapid responses, high sensitivity and selectivity to analytes, and are small, lightweight, and convenient to use.\(^{29}\) In reality, many sensors developed in laboratories remain large, complex to use, unreliable, and unstable.\(^{30}\) These devices are challenged by a wide variety of chemical combinations present in their environments, leading to interference, and contamination.\(^{31}\) Nevertheless, chemical sensors are promising components of on-site analysis due to their portability, convenience, and rapid responses. Many types of sensors have been developed already for environmental contamination and these can be categorized based on their method of signal transduction, the most common categories of which are mass-sensitive, electrochemical, and optical.

Mass sensitive resonators monitor the change in mass associated with adsorption or absorption of analytes on the sensor material. This process changes the mass of the device and impacts its resonance properties. Since all analytes have a mass, these sensors can be operated without the need for labeling. However, this also means that the devices function more effectively for larger analytes that lead to greater changes in mass.\(^{32}\) Disk microresonators,\(^{33}\) resonant cantilevers,\(^{34,35}\) magnetoelastic resonators,\(^{36}\) microbalances,\(^{32}\) bulk acoustic resonators,\(^{37}\) and surface acoustic wave devices\(^{38}\) have been used to sense environmental contaminants. Mass, however, is not a selective property, meaning that these sensors must be combined with selective extraction matrices to concentrate particular analytes on the device.\(^{39}\) These sensors are advantageous due to their label-free capacity and ease of use,\(^{40}\) but suffer from the need for washing and drying steps, long incubation times,\(^{41}\) and decreased signal in biological solutions,\(^{42}\) as well as their need for a selective recognition layer.\(^{43}\)

Electrical signals also give rise to a versatile group of sensing techniques. Electrochemical methods are fast, sensitive, inexpensive, robust, and able to be miniaturized.\(^{40,44}\) These sensors can
measure potential across a membrane (potentiometric), current as a function of potential (voltammetric),
current at an electrode of constant potential (amperometric), as well as resistance (conductometric) and
impedance. These sensors are employed in the detection of environmental contaminants ranging
from organic molecules, to gases, to metal ions. Recent examples include pesticide detection in water, nitrous oxide in the atmosphere, and iron ions in water. The characteristics of electrochemical sensing result in some challenges for environmental applications, however, such as fouling of the sensor surface, the limited lifetime of ion selective coatings, signal reversibility, and sensitivity to fluctuations in the ambient environment (e.g. electrical noise). In ion selective membrane-based electrochemical sensing, it is critical to work with a defect-free coating, since any imperfections can dominate over selective electrochemical processes. The monitoring of explosive gases also represents a limitation, since the introduction of electrical components could lead to a safety risk.

Despite the availability of other platforms such as mass and electrical signal-sensitive systems, both the commercial sensor market and research are dominated by optical detection schemes. The reasons for this include their simplicity, rapid response, sensitivity, ease of miniaturization and long distance transmission of information. Compared to electrochemical sensors, optical sensors are not as susceptible to defects in the coating materials, since a small defect in an optical coating will result in only a small effect on the overall signal.

The key parts of optical sensors include light sources, optical components for light manipulation, and detectors for light collection. Nearly all optical sensors require a light source to probe the sample. These can include light emitting diodes, laser diodes, lasers, incandescent bulbs, and gas-discharge lamps. Light can then be manipulated and directed to a sample using classical components such as lenses, prisms, windows, crystals, mirrors, and diffraction gratings, to change the path of the beam. These classical components present challenges for alignment, which are not compatible with the aim of
compact, robust sensors. Instead, many sensors rely on planar and fibre-based waveguides to manipulate light.\textsuperscript{59} Once the light is directed to the sample, the properties of the beam are modified in response to the environmental analyte. Properties that can be measured include absorbance, reflectance, luminescence, fluorescence (including quenching), phosphorescence, refractive index (including surface plasmon resonance), and light scattering. Information can be gathered from the intensity, phase, wavelength, polarization, spectral distribution, or emission kinetics of the light. Sensors detect changes in these properties using photodiodes, photodiode arrays, charge-coupled devices, and photomultiplier tubes. From the wide variety of light sources, properties, and detector types, many different optical chemical sensors have been developed.

Extrinsic (conventional) optical sensors measure the optical properties of analytes using a light beam that passes directly through the sample matrix.\textsuperscript{57} Absorbance, Raman scattering, fluorescence, phosphorescence, and chemiluminescence detection schemes have all been employed in these types of sensors for environmental analysis.

Absorbance analysis may probe the change in transmitted light intensity at a particular wavelength or, more commonly, the change in colour of a sensing material due to the presence of an analyte.\textsuperscript{60-62} Infrared absorbance analysis of environmental properties can be carried out by similar methods.\textsuperscript{63} One area of growing interest for colourimetric detection is the use of noble metal nanoparticles, which experience localized surface plasmon resonance at a wavelength that depends on the aggregation state of the particles.\textsuperscript{62,64,65} Compared to traditional colourimetric signaling molecules, localized surface plasmon resonance-enabled particles are very photostable, an attractive property for a robust sensor system.\textsuperscript{64} Colourimetric methods are particularly convenient relative to other optical detection techniques since a specialized light source is not required and a device as simple as a mobile
phone camera may be used for image capture.\textsuperscript{66} However, this simplicity corresponds to lower sensitivity,\textsuperscript{44} which may be detrimental when attempting to detect trace contaminants in the environment.

Raman scattering phenomena can also be used for environmental sensing. Due to the low intensity of Raman effects, surface-enhanced Raman spectroscopy (SERS) is particularly attractive for sensing and involves the enhancement of the vibrational spectrum of analytes near the surface of a Raman active particle or film.\textsuperscript{67} Raman scattering is a feature of all polarizable molecules, and SERS can thus be used to detect any analyte that is polarizable when interacting with the sensing material.\textsuperscript{68} Although SERS techniques are considered to be somewhat unstable and difficult to adapt to new analytes, researchers have used these methods for the detection of pathogens, as well as chemical contaminants in the environment.\textsuperscript{68-70}

Fluorescence is one of the most sensitive properties used in chemical analysis\textsuperscript{44} and has been used in many sensors as a result. The use of many fluorescent probes that emit at different wavelengths also offers the possibility of multiplexed analysis of multiple environmental contaminants. Metals,\textsuperscript{71} organic molecules,\textsuperscript{72} and protein toxins\textsuperscript{73} have all been detected by fluorescence-based techniques. One challenge of fluorescence measurements is the effect of the stability of the light source on the measurements. This can be avoided by carrying out emission kinetics-based measurements of fluorescent processes, such as fluorescence lifetime analysis.\textsuperscript{74} Despite the high sensitivity, fluorescence sensing is limited by the potential for photobleaching of the probe molecules. The use of quantum dots as photostable fluorescent labels can avoid this challenge, however.\textsuperscript{44} An additional consideration in the use of fluorescent methods is the need for labeling, since the analyte of interest is rarely fluorescent. The process of labeling the analyte or identifying a suitable fluorescent signaling molecule can hinder the use of this sensitive method.
Phosphorescence is a luminescence process that is similar to fluorescence, but exploits forbidden transitions, leading to longer emission lifetimes. This leads to advantages including the option to perform inexpensive lifetime measurements, avoiding interference from fluorescence and scatter by exploiting the longer lifetime, and increased selectivity due to the rarity of the phenomenon. While phosphorescence can be applied to environmental sensing, this process is less frequently used than fluorescence since it is uncommon and requires heavy atoms that facilitate inter-system crossing, a rigid environment, and the absence of oxygen, which quenches phosphorescence. These requirements can be challenging for optical sensors that must be deployed in a range of environments.

Chemiluminescence, in which a chemical reaction produces photons, is another sensitive transduction method. It is useful for small-scale devices because no external light source is required, unlike most optical methods and combines low cost with high sensitivity. Chemiluminescence and its counterpart, electrochemiluminescence, in which an applied voltage adjusts the optical signal, have both been applied in sensors. However, chemiluminescent analysis again requires the use of specialized reagents or labels, which can complicate the sensing process.

Conventional extrinsic optical sensors using the above detection mechanisms can be deployed in a variety of platforms. These set-ups include a light source and detector, as well as any required sensing reagents and sample manipulation architecture. Often, a solid-phase support is used to contain the sensing reagents or immobilize the analyte. Solution-phase detection can be achieved, much like typical benchtop analysis, either in test cartridges or in microfluidic chips. In addition, paper microfluidics and chromatographic strips can be used to take up an analyte solution and add sensing reagents. Finally, optical sensing reagents can be contained in an analyte-permeable solid phase, such as a hydrogel or membrane. Commercial environmental sensors that utilize conventional optical detection are available for pH, dissolved oxygen, turbidity, soil moisture, and oil in water (Table 1-2).
Table 1-2: Commercially available environmental sensors that utilize conventional optical detection methods.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detection Method</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>Fibre-coupled absorbance</td>
<td>Ocean Optics&lt;sup&gt;90&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dissolved O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Fibre-coupled luminescence</td>
<td>Ocean Optics&lt;sup&gt;91&lt;/sup&gt; Hach&lt;sup&gt;92&lt;/sup&gt;</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Light scattering</td>
<td>Hach&lt;sup&gt;93&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soil Moisture</td>
<td>Reflectometry</td>
<td>ESI&lt;sup&gt;94&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oil in Water</td>
<td>Fluorescence</td>
<td>Sea Bird Scientific&lt;sup&gt;95&lt;/sup&gt;</td>
</tr>
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</table>

There are limitations to all these designs, however. Since these systems involve detection of the analyte directly in the sample matrix, the optical properties of the matrix may affect the results. Many of these traditional optical platforms require the use of label or signal molecules, which further complicates the analysis procedure. One way to avoid this complexity is to utilize waveguide-based sensing, which can use light to probe a sample without requiring transmission through the sample. The use of waveguides also offers the possibility to monitor refractive index, a universal optical property, for sensing.

Intrinsic waveguide sensors function when the surrounding matrix modifies the optical properties of the waveguide itself.<sup>57</sup> Some intrinsic sensors measure the changes in properties of the waveguide directly, while other sensors of this type probe the region penetrated by the evanescent wave, allowing analysis of the region just outside the waveguide.

When sensors detect optical properties within the waveguide itself, selectivity can be introduced either by using a waveguide material that preferentially takes up the analyte of interest, or probing a unique optical property of the analyte. In either case, after analytes are extracted from the matrix and into the sensitive waveguide material, the optical properties of the waveguide are altered. These properties may include refractive index, optical path length, and interference spectra.<sup>96-98</sup> In other sensing schemes,
more specific optical effects are probed, such as fluorescence quenching or wavelength shift following the binding or extraction of specific analytes into the waveguide.\textsuperscript{99,100} These methods require the waveguide to extract analytes from the bulk sample, while also maintaining the refractive index and optical transparency properties necessary to function as a waveguide. To avoid these challenges, it is sometimes preferable to sense optical changes immediately outside the waveguide.

Another group of waveguide sensors indirectly probe samples using the evanescent wave phenomenon, which allows analysis of the region just outside the device. When light is propagated through a waveguide, it reflects from the material interface separating the waveguide from the surroundings, leading to a decay of energy just outside this interface. This affects the apparent refractive index of the waveguide itself, allowing changes at the waveguide surface to be detected (Figure 1-1).\textsuperscript{101}

**Figure 1-1:** A schematic of evanescent wave-based optical sensing. Light is guided by a waveguide core of higher refractive index than the cladding layers. The small amount of guided mode energy located outside the core is called the evanescent field. Sensing phenomena, such as analyte binding, that occur in the region of the evanescent wave can be detected through changes to the guided mode.

Since the light is guided primarily within the waveguide core, the optical properties of the analyte matrix do not affect the measured signal. This evanescent wave can be used to monitor the same optical properties as extrinsic optical sensors. Since evanescent wave sensing probes only the sample layer just
above the waveguide surface, it is usually necessary to concentrate the analyte in this sensing region, a feat that can be accomplished using a selective extraction matrix or surface adsorption.

Waveguide-based sensors can take the form of optical fibres and planar waveguide structures. These devices can be used to detect optical properties including refractive index changes, surface plasmon resonance, and properties ordinarily measured in the bulk phase, such as fluorescence and phosphorescence, infrared and ultra violet-visible absorption, and chemiluminescence.

Refractive index-based analysis methods are advantageous in sensors since this property is universal, similar to mass-based detection, but this also leads to complex non-specific signals. Due to the universal nature of refractive index and the capability of waveguides to probe a matrix indirectly, many modern optical sensors utilize this scheme. As was the case for mass-based sensors, however, the universality of refractive index means that sensors must be combined with a selective extraction matrix to concentrate analytes of interest on the device. When the material absorbs an analyte, the refractive index of the waveguide or cladding changes, causing a modification in the transmission properties of the waveguide.

Fibre optic chemical sensors employing refractive index have been used for the detection of gases, moisture, ions, organic molecules, and biomolecules. Another common type of refractive index sensors are planar waveguides, which are formed on flat chips. Planar waveguides operate similarly to optical fibres: light is propagated within the waveguide and the evanescent wave interacts with the surface above the chip, allowing changes in refractive index in this zone to be detected. Chips containing ring resonators and interferometers can be used for evanescent wave-based refractive index sensing with the addition of a selective coating, for example.

Waveguide-based surface plasmon resonance (SPR) is a special category of refractive index analysis involving the excitation of electron plasma on a metal layer at a waveguide surface. The resonance angle changes as analyte binding occurs at the chip surface and this can be probed to detect
many environmental analytes with the addition of a selective adsorption coating.\textsuperscript{3,28,110,111} While SPR is label-free, it is susceptible to changes in the surroundings such as temperature fluctuations\textsuperscript{40} and this must be well-controlled for sensor applications.

A challenge in using a non-specific optical property such as refractive index for environmental sensing is the difficulty of finding materials with selectivity towards particular molecules,\textsuperscript{112} to facilitate specific detection. Researchers using mass-sensitive detectors have been developing selective coatings for years,\textsuperscript{113} and with the increasing sensitivity of optical devices some of the coatings previously used in these sensors could be adapted to optical detection. The selectivity limitation, however, still prevents widespread deployment of these sensors in real-world systems.

Other optical properties can also be measured using the evanescent wave technique and waveguide sensors. Fluorescence in particular is used for waveguide-based sensors and this type of analysis is called total internal reflection fluorescence. Using this method, fluorescent molecules bound at the surface of the waveguide sensor can be preferentially excited and the emission light can be collected, leading to lower background fluorescence signals from the sample matrix.\textsuperscript{101,114} Similar principles have been used for phosphorescence,\textsuperscript{76} absorbance (both UV-Vis and IR),\textsuperscript{115-117} chemiluminescence,\textsuperscript{118} and surface enhanced Raman scattering.\textsuperscript{119}

Despite the research being performed in the area of waveguide-based environmental sensors, few commercial devices based on evanescent wave technology are available since this is still a developing field. There are a number of notable examples, however, of commercialized waveguide sensors, summarized in Table 1-3. Of these, only the Research International systems are designed for on-site use. Additional barriers to commercialization include the need for portable, high quality light sources, more stable light coupling, and the need for better isolation from the environment (mechanical and electrical noise, temperature), as well as the requirements for more reproducible surface chemistry and reduced non-
Table 1-3: Commercially available optical sensors based on intrinsic waveguide property analysis.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detection Method</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxins, pathogens, explosives, environmental contaminants</td>
<td>Evanescent wave-based fluorescence (RAPTOR and BioHawk)</td>
<td>Research International</td>
</tr>
<tr>
<td>Disease diagnosis and toxin quantitation</td>
<td>Planar waveguide fluorescence</td>
<td>MBio Diagnostics</td>
</tr>
<tr>
<td>Drug discovery</td>
<td>Waveguide interferometry</td>
<td>Creoptix</td>
</tr>
<tr>
<td>Bacteria, viruses, yeast, biomarkers</td>
<td>Waveguide interferometry</td>
<td>Ostendium</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Microring resonator</td>
<td>Genalyte</td>
</tr>
<tr>
<td>Drug discovery, antibody screening, bacteria, etc.</td>
<td>Surface plasmon resonance</td>
<td>Biacore</td>
</tr>
</tbody>
</table>

specific adsorption. For environmental applications in particular, new sensors require low detection limits and long-term stability. The ability to produce reversible sensors would also be desirable.

1.3.1.1 Biosensors for Environmental Analysis

A special and growing category of sensors are the biosensors, which utilize a biorecognition element as the receptor. While this definition has been put forward by the International Union of Pure and Applied Chemistry, many researchers expand the field of biosensors to include any sensor that detects a biologically relevant analyte. Compared to other biological analysis techniques, biosensors are unique due to the direct contact between the recognition and signal transduction elements, and the low sample volumes and portability afforded by the small sizes of these sensors. In the ideal case, these devices are capable of distinguishing closely related analytes, detecting analytes at low concentrations in complex matrices, binding analytes strongly enough to survive washing steps, having high signal stability, and exhibiting a broad dynamic range. An incredible diversity of biosensors exists, incorporating a wide array of biological sensing elements and transducer types.

Antibodies, protein components of the animal immune system, are one of the most common recognition elements employed in biosensors. These proteins are composed of two heavy and two light
chains of amino acids. One end of the structure is relatively consistent in sequence, while the other is highly variable and serves as the recognition site for foreign substances (Figure 1-2).\textsuperscript{130}

![Diagram of antibody structure]

**Figure 1-2:** General structure of an antibody.

Antibodies are useful biorecognition elements for many reasons. In principle, antibodies can be produced for any substance (biological or chemical) that is capable of producing an immune response, meaning that they are available for a wide range of analytes.\textsuperscript{131} In addition, their specificity is flexible, meaning that antibodies can be produced to recognize a wide range of related analytes, or to be very specific to one analyte.\textsuperscript{130} For the purposes of immunosensors, in which the antibodies must be immobilized on the sensor, it is also an advantage that many conjugation methods are available in the literature.\textsuperscript{132-136}

Antibodies can also operate in complex matrices without the need for sample purification.\textsuperscript{137} On the other hand, being proteins, antibodies are susceptible to changing environmental conditions and require physiological pH and temperature for operation, limiting their uses in environmental sensing.\textsuperscript{138}

Antibodies are also known to be susceptible to non-specific binding, can lack reproducibility between batches, and can be unstable.\textsuperscript{138} Finally, for pathogen analysis, antibodies will recognize and detect bacteria that may have been killed or otherwise inactivated, which may give overestimates of contamination.\textsuperscript{14}
Some of the difficulties associated with antibodies, including low stability and inconsistent production, have led researchers to investigate other biorecognition elements in greater depth. Aptamers are composed of nucleic acid sequences that have affinity for specific analytes, similar to antibody affinity for antigens, and are therefore known as “synthetic antibodies”.\(^{56}\) Aptamers are produced by an in vitro process called SELEX (Selective Evolution of Ligands by Exponential Enrichment),\(^ {139-141}\) which begins with an enormous library of nucleic acid sequences (\(10^{14} - 10^{15}\)), followed by cycles of adsorption to target and non-target species, recovery of bound sequences, and amplification of these sequences.\(^ {142}\) Although formerly a slow method, this process now takes just hours to complete.\(^ {143}\) Once a useful sequence is identified, it can be produced in large quantities by DNA replication processes.\(^ {144}\) In addition to the advantage of in vitro production, aptamers can be selected to function in non-physiological conditions,\(^ {142}\) and have very high affinity for their targets.\(^ {64}\) Unfortunately, aptamers can be broken down by the presence of nucleases, although protection can be afforded by chemical modifications to the nucleic acid backbone.\(^ {142}\) Currently, aptamers are available for a more limited range of analytes relative to antibodies, but this range may expand significantly in the future.\(^ {143,145}\) Aptamer-based biosensors have been used for algae toxins,\(^ {62}\) mercury ions,\(^ {86}\) and pathogens,\(^ {146-148}\) illustrating their versatility. These recognition molecules represent a growing area of interest due to their advantages relative to antibodies.

Other recognition elements are less commonly used. Carbohydrates, including oligo and polysaccharides can bind pathogenic analytes, since these microorganisms ordinarily bind through carbohydrates located on the surfaces of the cells of an infected host organism.\(^ {64}\) Carbohydrates are more stable to pH and temperature fluctuations than antibodies,\(^ {64}\) and can be packed at a higher density on a sensor surface due to their small size, but tend to have lower affinities than comparable antibodies and a higher tendency for cross-reactivity.\(^ {149}\) Antimicrobial peptides can also serve as recognition elements for biosensors that detect microorganisms. These short sequences are 15 to 45 amino acids in length\(^ {64}\) and
bind to the surfaces of cells to facilitate death.\textsuperscript{150} Although these are promising recognition elements, antimicrobial peptide binding depends partly on ionic interactions between the peptide and cell membrane, which can be altered by changes in the ionic strength of the surrounding environment.\textsuperscript{64}

Finally, bacteriophages, which are viruses that bind to and infect bacteria can also be used for recognition.\textsuperscript{151} Phages are simple to isolate and are stable during storage, attractive features for a biosensing element.\textsuperscript{152} A wide and expanding variety of biorecognition elements are available for use in biosensors. The choice for a specific application will depend on the analyte and the required characteristics (e.g. stability, selectivity, analytical conditions) of the sensor.

Once an environmental analyte has been recognized and bound by the biosensing element, this event must be transduced into a measurable signal. Electrochemical, optical, and mass sensitive devices have all been used to accomplish this goal and these techniques were described above in detail. The most common optical detection methods are fluorescence and surface plasmon resonance,\textsuperscript{64} although a vast array of schemes is available. One particular interest of research today is the move toward label-free biosensing.\textsuperscript{153} The main attraction of label-free methods is the possibility of detecting analytes in situ, either immersed directly in a sample or with minimal sample treatment. Additional motivations include avoiding the expense and time required to label analytes or detection molecules\textsuperscript{103} as well as the potential loss of bioactivity that may result from labeling.\textsuperscript{154} Some of the available label-free biosensing technologies include ring resonators, interferometers, surface plasmon resonance devices, waveguides, optical fibres, and photonic crystals.\textsuperscript{154} While the field of biosensors in general is advancing rapidly, label-free devices are still a developing field and much remains to be understood in terms of their potential for environmental analysis.
1.3.2 Detection of Microbiological Contaminants – Accepted Methods

Testing of water for pathogens is necessary to ensure human safety, with the most important criteria being sensitivity and speed. Since the infectious dose for pathogens can be as low as 1 to 100 organisms or particles, and pathogenic organisms can be present in complex mixtures with harmless agents, detection can be very challenging, with few techniques meeting the required standards. While consistent and effective drinking water treatment can minimize the risk of disease outbreaks, the ability to detect these pathogens is still important, because it is important to confirm the effectiveness of the treatment process and failures do sometimes occur. However, the vast range of potential waterborne pathogens adds challenges to monitoring (Table 1-4). One way of managing the challenge of detecting such a diverse group of organisms is to choose one species as an indicator of contamination. Since most pathogens are introduced to water through mammalian feces, it is feasible to detect an indicator that is reliably present in such sources. The ideal characteristics of an indicator of fecal contamination are: universal presence in human and warm-blooded animal faeces, readily detectable by simple methods, similar treatment survival to pathogens, higher abundance than pathogens, and inability to grow in natural waters. In the late nineteenth century, Escherichia coli was identified as a useful indicator of mammalian fecal contamination, but the limitations of microbiological testing meant that only the broader group, “total coliforms,” could be identified. Thus, total coliforms was the standard drinking water quality test until the 1970s, when it was replaced by the slightly more specific “fecal coliform” test, which was performed at a higher temperature. Even this test was subject to false positives due to other temperature-tolerant microorganisms, however. The development of defined substrate technology, which exploits the β-D-glucuronidase enzyme found specifically in E. coli, allowed the implementation of tests specifically for this indicator organism. Following this improvement, the drinking water quality standard of 1 colony-forming unit (CFU) of E. coli per 100 mL was introduced.
Table 1-4: List of waterborne pathogens, including bacteria, viruses, protozoans, and helminths, characterized by health and water treatment-related properties. World Health Org.155

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Health Significance</th>
<th>Persistence in water supplies</th>
<th>Resistance to chlorine</th>
<th>Relative infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia pseudomallei</td>
<td>High</td>
<td>May multiply</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Campylobacter jejuni, C. coli</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Escherichia coli – Pathogenic</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Escherichia coli – Enterohaemorrhagic</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Legionella spp.</td>
<td>High</td>
<td>May multiply</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Leptospira</td>
<td>High</td>
<td>Long</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Mycobacteria (non-tuberculous)</td>
<td>Low</td>
<td>May multiply</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Other salmonellae</td>
<td>High</td>
<td>May multiply</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>High</td>
<td>Short</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>High</td>
<td>Short to long</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoviruses</td>
<td>Moderate</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Astroviruses</td>
<td>Moderate</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Hepatitis E virus</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Noroviruses</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Rotaviruses</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Sapoviruses</td>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acanthamoeba spp.</td>
<td>High</td>
<td>May multiply</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Cryptosporidium hominis/parvum</td>
<td>High</td>
<td>Long</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Cyclospora cayetanensis</td>
<td>High</td>
<td>Long</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Entamoeba histolytica</td>
<td>High</td>
<td>Moderate</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Giardia intestinalis</td>
<td>High</td>
<td>Moderate</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Naegleria fowleri</td>
<td>High</td>
<td>May multiply</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td><strong>Helminths</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dracunculus medinensis</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Schistosoma spp.</td>
<td>High</td>
<td>Short</td>
<td>Moderate</td>
<td>High</td>
</tr>
</tbody>
</table>

1Incidence and severity of disease, including association with outbreaks. 2Duration over which infective state may be detected in water at 20°C. Short – up to one week. Moderate – one week to one month. Long – greater than one month. 3Duration for 99% inactivation of infective organisms following chlorine disinfection at conventional doses and contact times at 20°C and pH between 7 and 8. Low – less than 1 minute. Moderate – 1 to 30 minutes. High – greater than 30 minutes. Organisms that survive in biofilms (e.g. Legionella and mycobacteria) will be protected from chlorination. 4High – infective dose between 1 and 102 organisms/particles. Moderate – 102 to 104 organisms/particles. Low – greater than 104 organisms/particles. 5May survive for long periods in association with aquatic organisms. 6In warm water.
There are difficulties remaining with this requirement, however. The biological contaminants for which \textit{E. coli} is a poor indicator include \textit{Legionella pneumophila}, \textit{Pseudomonas aeruginosa}, \textit{Giardia lamblia}, \textit{Cryptosporidium parvum}, and enteric viruses\textsuperscript{5}. Since these pathogens are also concerns for human health when found in water supplies, their detection must be carried out separately from \textit{E. coli}. However, the tests for these pathogens are complex\textsuperscript{157} and not practical for routine use. According to the World Health Organization “frequent examination by a simple method is more valuable than less frequent examination by a complex test or series of tests.”\textsuperscript{158} Therefore, until simple methods are available for the analysis of some of the pathogens listed above, routine monitoring of \textit{E. coli} levels in water will continue to be the preferred, if imperfect, choice.

Most pathogen detection methods are based on microbiological or biochemical tests, with culture methods considered the most reliable\textsuperscript{159}. Established methods of bacteria detection in drinking water are described in Standard Methods\textsuperscript{160}. The current methods of \textit{E. coli} and total coliform detection in drinking water are multiple tube fermentation, membrane filtration, and chromogenic-fluorogenic substrate tests\textsuperscript{160}. In the multiple tube fermentation method, a lactose fermentation test is followed by a confirmation step. The test is performed using multiple dilutions of the water sample to provide a “most probable number” of bacteria in the sample\textsuperscript{14}. The fermentation test is useful for coloured or turbid samples and relatively cost-effective to perform, but time consuming and labour-intensive\textsuperscript{14}. In addition, this method can produce an overestimation of the number of bacteria present\textsuperscript{161}. The membrane filtration technique involves filtering the water sample through a 0.45 \textmu m filter to collect bacteria, then placing the filter on a special growth medium and incubating overnight\textsuperscript{160}. After the incubation time, coloured or fluorescent colonies are counted to determine the number of colony forming units in the original sample\textsuperscript{160}. This method demonstrates improved quantitation versus the multiple-tube fermentation method, is relatively simple for a microbiologist to run, and can handle large volumes of water\textsuperscript{14}. However, potential interference from
heterotrophic bacteria and turbid water may make analysis by membrane filtration difficult. Finally, a chromogenic-fluorogenic substrate test, also known as defined substrate technology, exploits enzymes found uniquely in the organisms of interest, such as β-glucuronidase in *E. coli*. The most common example of this technology uses a fluorogenic substrate (4-methylumbelliferyl-β-D-glucuronide) as a food source for the *E. coli*, promoting selective growth of this indicator organism. The metabolism of this substrate can then be visualized by fluorescence detection within 18 to 24 hours. This method can be used as a present/absent test, as well as a quantitative analysis technique.

While the above techniques are the most commonly applied in practice, each has a number of drawbacks. The conventional detection methods meet the sensitivity requirements for drinking water by sacrificing rapidity, since culturing the samples to amplify the signal requires 18 to 48 hours or more. Despite the long test duration, unreliable results may still be produced. In both membrane filtration and multiple tube fermentation, for example, the presence of large numbers of non-indicator bacteria can compromise the test, leading to interference in positive sample identification. As well, these methods rely on the growth of cultures of indicator bacteria for positive sample identification. However, bacteria are sometimes stressed but not killed by water treatment, which can lead to viable but not culturable organisms that are not detected by culture methods. Finally, most of the approved methods can be difficult to interpret and, as a result, require personnel skilled in the performance of such tests. The requirement for complex instrumentation as well as expertise means that most microbiological analysis takes place in centralized laboratories. This centralization means that many smaller communities must send water samples away for testing, a process that can add to the time taken to receive results. Such a delay could allow many people to fall ill in the event of contamination. These issues taken together mean that the development of portable, rapid, sensitive, selective, and easy-to-operate technologies for water
analysis could significantly improve access to safe drinking water, especially for citizens in isolated communities.

In addition to the problems of the indicator tests currently in use, there are a number of concerns with using indicator organisms for water quality analysis in general. For example, protozoan organisms such as *Giardia lamblia* and *Cryptosporidium parvum* are not chlorine sensitive in their oocyst phases, unlike the indicator bacteria, so they may survive chlorination even when indicator bacteria do not. The standard test to detect both of these protozoan pathogens involves the filtration of a large water sample, immunomagnetic capture and separation of the organisms, and immunofluorescence labeling and detection using a microscope. Although this method is the standard, it can result in pathogen recovery rates averaging just 9.3% in some cases. Other microbial contaminants are not always associated with sewage contamination, meaning that the conventional indicators may be absent even when pathogens are present. An example of this problem is *Legionella pneumophila*, which can be found in natural waters and becomes a human health concern when it colonizes potable water supply systems, cooling towers, and whirlpool spas. Unfortunately, the conventional microbiological test for the presence of *L. pneumophila* in water requires both culture and confirmation steps, adding several days before results can be reported. Viruses are also difficult to analyze in water systems. Although culture methods exist for their detection, they are costly and time consuming, and therefore not as convenient as those used for bacteria. The many barriers to the detection of other waterborne pathogens mean that indicator bacteria tests continue to be the standard, although these may not be appropriate surrogates for all types of microbial contamination.

Over the past decades, researchers have developed new indicator tests for waterborne pathogens that are effective in reaching the 1 CFU/100 mL requirement for drinking water. In the process, improved technologies have reduced interferences as well as the possibility for operator error. Commercial methods
based on conventional culture and chromogenic-fluorogenic substrate detection include Colilert and Colisure (Idexx\textsuperscript{168}). These methods differ in the substrate used for coliform detection, but are otherwise quite similar in that both allow quantitation by performing present/absent tests on aliquots of the 100 mL sample, and the number of positive wells can be used to determine the “most probable number” of indicator pathogens in the sample.\textsuperscript{169} These methods also include antibiotics to reduce the growth of interfering bacterial species.\textsuperscript{169} Colilert 18 also has the advantage of higher levels of nutrients, which allow the test to be analyzed after just 18 hours, an improvement over most conventional detection methods, which take 24 to 48 hours to complete. Despite these improvements, Colilert and Colisure both rely on skilled operators to perform and interpret the test, just like the conventional microbiological analyses. In an effort to avoid this, the Colifast automated detection system was developed (Colifast Systems\textsuperscript{170}). The aim of this method was to remove the operator analysis requirement for the fluorogenic substrate test by performing spectrophotometric analysis on the cultured water sample.\textsuperscript{171} The requirement for fluorescence analysis directly in the water sample can be difficult, however, if the water is turbid or unusual in colour. As a result, the Colifast technique has failed to gain regulatory approval.\textsuperscript{172} Recently, a technique was introduced by our group that uses pyrene-\(\beta\)-D-glucuronide as the substrate for \textit{E. coli}.\textsuperscript{173,174} This substrate is cleaved to form 1-hydroxypyrene, which can then be selectively partitioned into a siloxane polymer located in the test cartridge. The fluorescence of the product molecules can then be measured within the optically clear polymer by a waveguide-coupled spectrometer system (Figure 1-3).
Figure 1-3: A schematic design of the extraction-mediated *E. coli* detection method commercialized by Endetec. As the bacteria grow, the cells consume the targeted substrate (pyrene-β-D-glucuronide) and release a fluorescent product (1-hydroxypyrene) that is hydrophobic. This hydrophobic product is extracted into the polymer element, where it is detected by a spectrometer.

This technology has been commercialized as the TECTA™ by Endetec (Veolia175). As with the Colifast system, the TECTA is capable of automated result analysis, reducing the operator requirements of the test. Additionally, the extraction of the fluorescent product of the targeted enzyme reaction is extracted into a polymer phase, decreasing the level of optical interference caused by turbid or coloured water samples. This method has successfully received EPA approval.176 Despite these improvements, limitations still remain to be overcome for pathogen detection, including the long duration of all tests (minimum 18 hours), the requirement to perform the test in specialized laboratories, the presence of viable but non-culturable pathogens, as well as all the challenges of using indicator pathogens to represent all possible pathogenic contamination. At present, however, these microbiological tests are still the preferred choice due to their sensitivity and selectivity.25
1.3.3 Detection of Microbiological Contaminants – Emerging Methods

As a result of the concerns with existing detection methods, researchers continue to search for improvements. New methods need to improve on the existing methods in at least one of three characteristics: sensitivity (detection of small numbers of pathogens), specificity (of one species from another, or viable organisms from dead), or speed, while improvements to portability and ease of use are also desirable. Barriers to improved detection methods include the range of contaminants potentially present in water samples, which may include inorganic and organic molecules, microorganisms, and particulates. The very low numbers of pathogens of interest that may be present in a water sample also present a sensitivity barrier. This means that sample pre-treatment may need to be undertaken to concentrate the pathogens when no superior detection method exists. This may involve filtration, microfluidic filtration, or magnetic separation. While no one technology has emerged to take over from the existing approved techniques, there are many that demonstrate the new directions in the field of microbiological drinking water analysis.

1.3.3.1 Immunological Detection

Pathogenic water analysis can be accomplished using immunological detection methods. These methods take advantage of the capacity of antibodies to recognize foreign substances. For immunoassay detection, enzyme labels can be attached to the antibodies, catalyzing the production of a coloured, fluorescent, or electrochemically active product. Alternatively, antibodies can be directly tagged with a fluorophore or a dye-containing liposome. Immunoassays are commonly used in clinical settings, but the detection limits have not met the standards for drinking water, since they range from $10^3$ to $10^6$ organisms per mL. Improved sensitivity be achieved in many ways, including increasing the surface loading of antibodies using nanocomposites, combining immunoassay with polymerase chain reaction, or including an immunomagnetic separation prior to analysis. Despite these
improvements, immunoassay detection limits are still orders of magnitude greater than desired for drinking water analysis. In addition, while these techniques are quite powerful, they are unable to determine the infectivity of cells (alive/dead) and some methods can be very time-consuming. Immunological methods have the advantage of being adaptable to any pathogen for which an antibody is available, but detection limits must be improved further for widespread application to be possible.

1.3.3.2 Nucleic Acid-Based Detection

Molecular detection methods involve the recognition of nucleic acids characteristic of various pathogens. The pathogens are first concentrated in a reduced volume, the nucleic acids (DNA or RNA) are extracted, the genome segment of interest is amplified and detection takes place. These methods tend to be more rapid than the traditional culture tests used for pathogens and the two main types of tests used are the polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH).

In the most common PCR method, DNA is extracted from organisms, denatured, annealed to a primer sequence, and extended with DNA polymerase. Through repeated cycles, the DNA of interest is amplified and can be detected by electrophoresis. The method is fast relative to culture methods and also is capable of detecting viable but non-culturables. It is also viewed as a sensitive, specific, and accurate technique. However, PCR faces challenges in drinking water analysis due to inaccurate quantitation, labour intensive procedures, false positives due to “naked DNA,” the detection of non-viable organisms, and the potential for inhibition of the polymerase enzyme by humic substances found in natural waters. Researchers are constantly advancing techniques to avoid these drawbacks. For example, selective enrichment of the target organism can counter some of these difficulties by increasing the quantity of target DNA and decreasing inhibitor concentrations. Immunomagnetic separation can also be coupled to PCR to increase the concentration of the target pathogen. One relatively new addition to nucleic acid recognition is reverse transcriptase PCR, which is capable of detecting genes that
indicate viability, while being specific, sensitive, and fast.\textsuperscript{190} Real time PCR on microfluidic chips is also an increasingly attractive option, since it reduces the labour requirements and allows online monitoring for target sequences, eliminating the post-processing procedures.\textsuperscript{24} While PCR has been applied to the detection of many microorganisms in water samples,\textsuperscript{15,191-193} its characteristics still do not allow it to replace culture technology.

In situ hybridization involves the use of labeled nucleic acid probes that hybridize with target ribonucleic acid sequences and this binding can be detected by microscopy or cytometry.\textsuperscript{14,194} The advantages of this technique include the increased speed relative to culture methods and the potential for automated detection.\textsuperscript{184} However, stressed, nutrient starved bacteria in drinking water may not have high levels of RNA to facilitate detection, a nucleic acid sequence must be identified for use as a probe, and, while this technique detects viable but non-culturable cells, it also detects dead cells.\textsuperscript{14} In situ hybridization has been used for some pathogen detection, such as parasites in fish,\textsuperscript{195} and pathogens in water,\textsuperscript{196,197} but is less sensitive than PCR\textsuperscript{198} and therefore requires improvements to meet the needs of drinking water analysis.

1.3.3.3 Biosensors

Researchers are exploring a wide variety of other approaches to pathogen detection. Biosensors were described above in detail and will be discussed here particularly in the context of waterborne pathogen detection. Biosensor-mediated pathogen detection methods include a wide variety of recognition and transduction elements that were described in Section 1.3.1.1. Examples of pathogen biosensors are provided in Table 1-5.

Mass sensing techniques can be applied to waterborne pathogens. \textit{Vibrio cholerae} was bound to a microcantilever functionalized with antibodies, producing a detection limit of $10^3$ CFU/mL,\textsuperscript{203} while \textit{Giardia lamblia} was detected at 10 cysts/mL using a similar device.\textsuperscript{202} Since these techniques are mass
Table 1-5: Examples of the available biosensor devices described in scientific literature for pathogen detection.

<table>
<thead>
<tr>
<th>Signal Transduction</th>
<th>Recognition Element</th>
<th>Organism</th>
<th>Detection Limit and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrochemical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrode potential</td>
<td>Aptamer</td>
<td><em>Salmonella typhi</em></td>
<td>1 CFU/5 mL^199</td>
</tr>
<tr>
<td>Resistance</td>
<td>Antibody</td>
<td><em>E. coli</em> O157:H7</td>
<td>78 CFU/mL^200</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Antibody</td>
<td><em>E. coli</em> O157:H7</td>
<td>15 CFU/mL^179</td>
</tr>
<tr>
<td>Impedance spectroscopy</td>
<td>Aptamer</td>
<td><em>Staphylococcus aureus</em></td>
<td>10 CFU/mL^188</td>
</tr>
<tr>
<td>Impedance</td>
<td>Antibody</td>
<td><em>E. coli</em> O157:H7</td>
<td>10 CFU/mL (buffer), 83 CFU/mL (milk)^201</td>
</tr>
<tr>
<td><strong>Mass Sensitive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cantilever</td>
<td>Antibody</td>
<td><em>Giardia lamblia</em></td>
<td>10 cysts/mL^202</td>
</tr>
<tr>
<td>Cantilever</td>
<td>Antibody</td>
<td><em>Vibrio cholerae O1</em></td>
<td>10^3 CFU/mL^203</td>
</tr>
<tr>
<td>Magnetoelastic</td>
<td>Phage</td>
<td><em>Salmonella typhimurium</em></td>
<td>1.5 x 10^3 CFU/mm^2^36</td>
</tr>
<tr>
<td><strong>Optical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Antibody</td>
<td><em>Giardia lamblia</em></td>
<td>5 x 10^4 cysts/mL^204</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Antibody</td>
<td><em>E. coli</em> O157:H7</td>
<td>10 CFU/mL^205</td>
</tr>
<tr>
<td>SPR</td>
<td>Antibody</td>
<td><em>Legionella pneumophila</em></td>
<td>10^5 cells/mL^167</td>
</tr>
<tr>
<td>Electrochem-luminescence</td>
<td>Antibody</td>
<td><em>E. coli</em> and <em>Salmonella typhimurium</em></td>
<td>1000 to 2000 bacteria/mL^162</td>
</tr>
</tbody>
</table>

Sensitive, some researchers have increased the sensitivity by using antibody-functionalized magnetic nanoparticles\(^{206}\) or gold nanoparticles\(^{207}\) to bind to the analytes, selectively increasing the mass of bound material. None of these techniques have reached the necessary sensitivity for drinking water analysis, meaning that improvements are still necessary.

Electrochemical sensors can also be used for the detection of waterborne pathogens. These techniques are desirable due to their selectivity and the possibility for real-time analysis.\(^{56}\) The change in current, potential difference, resistance, or impedance can be measured by such sensors.\(^{56}\) Semiconductor materials are used in pathogen sensors to detect binding events at the sensor surface, which can affect the device resistance. In one example, *E. coli* O157:H7 was captured using antibodies immobilized on a pad between two electrodes and detected with polyaniline-functionalized antibodies.\(^{200}\) The accumulation of polyaniline, a conductive polymer decreased the resistance and allowed detection of 78 CFU/mL of
pathogen.\textsuperscript{200} Potentiometric biosensors include ion selective electrodes and field effect transistors, the latter of which can be miniaturized for sensing purposes.\textsuperscript{40} For instance, graphene film-based field effect transistors functionalized with antibodies were used to bind \textit{Cryptosporidium parvum} and detection was achieved by monitoring the current at the drain electrode. Detection was demonstrated in buffer at 25 oocysts/mL.\textsuperscript{208} \textit{Salmonella typhi} was detected at 1 bacterium per 5 mL sample nearly instantaneously using single walled carbon nanotubes on a carbon electrode through potentiometric sensing.\textsuperscript{199} In this method, aptamers immobilized on the carbon nanotubes were employed for pathogen recognition and their conformational change upon binding of the pathogen induced a change in the recorded potential.\textsuperscript{199} Amperometric sensors detect the current generated by processes including oxidation or reduction reactions of microbial enzymes or through binding of the pathogens to the surface of an electrode.\textsuperscript{40} This current-based measurement method has been employed in the detection of \textit{E. coli} O157:H7 using antibodies labeled with glucose oxidase for recognition, the reaction of which produces a current, allowing detection of the bacteria at 15 CFU/mL.\textsuperscript{179} These electrochemical methods provide versatile alternatives for pathogen detection, though again none have achieved the necessary qualities for drinking water analysis.

Many pathogen detection systems use optical signals such as fluorescence for signal transduction. The ways in which this is carried out are widely varied. Fluorescent labels are often used for the purpose of detection, especially in the case of immunosensors. For instance, a sandwich immunoassay was carried out inside a capillary waveguide, using a fluorescently labeled antibody for detection. After binding of the fluorescent antibody, excitation light was shone onto the capillary in a perpendicular direction, and the emitted fluorescent light was guided down the capillary where it could be detected at the end of the waveguide.\textsuperscript{209} Using this method, 10 cells of \textit{E. coli} O157:H7 could be detected in a single capillary containing 75 $\mu$L of liquid.\textsuperscript{209} The RAPTOR device, a portable fibre optic biosensor utilizing evanescent
wave sensing, is another recent example of the use of fluorescent sandwich immunoassays for pathogen detection, which has been used to detect *Giardia lamblia*, Cryptosporidium parvum, and *Enterococcus* spp. In a more unusual design, polydiacetylene liposomes were functionalized with antibodies and the binding of *C. parvum* oocysts caused a colour change and a fluorescent signal from the polymer. Fluorescence resonance energy transfer (FRET) can also be used for the biosensing of pathogens. In a recent example, antibodies to rotavirus were immobilized on graphene oxide and the bound viruses were detected using antibodies functionalized with gold nanoparticles, which led the photoluminescence of the graphene oxide to be quenched by the nanoparticles, through FRET. In addition to fluorescence, surface enhanced Raman spectroscopy can also be used for pathogen analysis. The label-free detection potential of surface plasmon resonance (SPR) makes it a commonly used detection method for biosensors, but this advantage is offset by the temperature sensitivity problems exhibited by such devices. In addition, due to the small volumes employed for analysis, environmental water samples with low pathogen concentrations would need to be pre-concentrated before testing. The sensitivity of SPR is relatively low and in a typical example researchers have used an antibody coated SPR system to detect *Legionella pneumophila* at 10⁵ cells/mL. One way of improving the binding efficiency, and therefore the sensitivity, of SPR is by pre-binding a biotin-functionalized antibody to the pathogen of interest, then flowing this solution over an avidin-functionalized SPR chip. This allowed 1 x 10² oocysts/mL of *Cryptosporidium parvum* to be detected, compared to 1 x 10⁶ oocysts/mL for the conventional system. These detection levels are still well above the requirement of 1 CFU/100 mL for drinking water quality, however, and much room for improvement remains.
1.3.3.4 Other Methods

While the methods described above comprise the most common areas of research for new pathogen detection devices, many other techniques have been developed. Although most recognition of pathogens is done using biomolecules, one research group has exploited chemical recognition instead.$^{214}$ Flow cytometry, a cell counting method, can also be employed if the pathogens can be isolated from the water sample. One group found that *Giardia lamblia* could be detected by a combination of filtration, fluorescent labeling, immunomagnetic separation, and flow cytometry analysis at concentrations as low as 10 cysts/L.$^{215}$ However, this may still represent an infective dose. Bacterial analysis has even been completed using matrix assisted laser desorption ionization mass spectrometry, searching for biomarker proteins and detecting as few as 500 CFU/mL gram positive bacteria.$^{177}$ In addition, some researchers are working to improve the existing glucuronidase enzyme test by performing it immediately (without culturing) using a hand-held fluorescence detector. This impaired the detection limit of the test, however, (7 CFU/mL) making it suitable for swimming regulations, but not drinking water.$^{216}$ It appears that no technique has emerged to provide a replacement for conventional analysis in the near future. Therefore, substantial room exists for new ideas and technologies in this field.

1.3.3.5 Outlook for Novel Pathogen Detection Systems

An underlying problem in a number of the techniques discussed above is the detection of all viable pathogens. In some cases, bacteria may be viable but non-culturable. Although these cells will not be detected by culture, toxin genes may be expressed, representing a health hazard,$^{190}$ and the cells may be later revived to a culturable state.$^{217}$ On the other hand, techniques such as immunoassays and biosensors that rely on detection of microbe surface antigens may detect all pathogenic organisms, whether alive or dead.
While many new methods of pathogen detection have been reported, most have not reached the required sensitivity limits for drinking water without the assistance of pre-concentration. These detection schemes may only be useful for highly contaminated water supplies. This problem will be difficult to overcome in many cases because the surface area of the biosensor material may be very low compared to the volume of water that must be analyzed, leading to difficulty capturing a single organism from a 100 mL water sample. Combined with this, obtaining a detectable signal from a single organism will require an instrument with high sensitivity to the target, but very low background interference, or a high degree of signal amplification. Finally, many of the sensors discussed above have not been tested using environmental water samples. Compared to laboratory water, which should be relatively consistent in purity and pH, environmental water samples may contain particulates, additional microorganisms, varying pH values, and chemical contaminants. All these factors may complicate an analysis that was straightforward in laboratory conditions.

Polymerase chain reaction methods, in contrast to many other novel techniques, have met the drinking water detection limit standards, with one group reporting the detection of 3 CFU/L *E. coli* and *Salmonella* spp. in spiked surface water samples. In addition, PCR may be the only available method for many pathogens and can provide detailed species information. However, there remain a number of difficulties with the application of nucleic acid detection methods to water analysis. The analysis methods vary widely between laboratories, which may lead to significant inconsistencies in the results. The role of inhibitors of the enzymes required for the assays that may be present in natural waters is not fully understood and may prevent reliable results being obtained. PCR methods also remain a complex lab technique not amenable to analysis in the field.

Although many groups are working towards the goal of a simple, portable, and easy-to-use sensor for the detection of pathogenic organisms at the standards required for drinking water, no single method
has met all of these requirements. Therefore, the opportunity still remains to improve existing methods or develop new techniques altogether to reach this goal.

### 1.4 Enzyme Linked Immunosorbent Assay and its Applications to Environmental Analysis

Immunoassays are common laboratory analytical techniques that use antibodies to recognize analytes in biological samples. Early in their development, immunoassays were completed using radiolabels.\(^{218}\) The idea of using enzymes as labels instead was raised and brought to fruition in the early 1970s.\(^{218}\) After Avrameas\(^{219}\) successfully coupled enzymes to antibodies using glutaraldehyde, two groups simultaneously developed the technique known as enzyme immunoassay or enzyme-linked immunosorbent assay (ELISA): Engvall and Perlmann,\(^{220}\) using alkaline phosphatase, and van Weemen and Schuurs,\(^{221}\) using horseradish peroxidase. Years later, the same two enzymes were still the labels of choice in ELISA methods, with horseradish peroxidase being slightly preferred over alkaline phosphatase, though others are also available.\(^{222}\)

Antibodies can be produced and used for the detection of environmental contaminants ranging from small molecule toxins to large bacteria and protozoans (Table 1-6).\(^{40}\) Immunoassay-based detection methods for pathogens are advantageous due to their speed relative to culture, sensitivity,\(^{14}\) specificity,\(^{228}\) relatively low cost, and the limited need for sample pretreatment.\(^{131}\) Immunoassays utilize antibodies, which can be raised to detect a variety of contaminant organisms at a range of specificity levels (e.g. family, genus, species, serotype). Pathogens are often detected in the sandwich assay format, in which capture antibodies are attached to a solid surface, then the test sample is added and analytes are bound by the capture antibodies. Following washing, an enzyme-linked antibody for the same analyte is added.
Table 1-6: Examples from the scientific literature of the detection of environmental contaminants using antibody recognition.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Signal Transduction Method</th>
<th>Detection Limit and Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> O157 H7</td>
<td>Coloured immunoliposomes</td>
<td>2500 cells (approx.)</td>
</tr>
<tr>
<td><em>E. coli</em> O157 H7</td>
<td>Colourimetric – Horseradish peroxidase label</td>
<td>1800 CFU/mL</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Electrochemical – Alkaline phosphatase label</td>
<td>3 oocysts/mL</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>Total internal reflection fluorescence</td>
<td>$4.9 \times 10^4$ CFU/mL</td>
</tr>
<tr>
<td>Protein Toxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paralytic Shellfish Toxins</td>
<td>Fluorescent label on secondary antibody</td>
<td>12 pg/mL</td>
</tr>
<tr>
<td>Ricin</td>
<td>Fluorescent nanoparticle</td>
<td>100 pg/mL (buffer), 1 ng/mL (cider and milk)</td>
</tr>
<tr>
<td>pToxin</td>
<td>Fluorescent nanoparticle</td>
<td>10 pg/mL (buffer), 100 pg/mL (cider and milk)</td>
</tr>
<tr>
<td>Small Molecule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>SPR</td>
<td>45 – 64 ng/L</td>
</tr>
<tr>
<td>Atrazine</td>
<td>Chemiluminescence (luminol and peroxidase)</td>
<td>6 ng/L</td>
</tr>
</tbody>
</table>

Excess antibodies are then washed off before the addition of the colourimetric or fluorimetric substrate for the enzyme of interest. The detection of the optical signal is normally completed in solution (Figure 1-4). Improvements in antibody production mean that these biomolecules can be produced rapidly and at low cost. While ELISA is not as sensitive or specific as DNA-based detection methods, it holds the advantage in terms of speed, reliability, and the ability to directly detect toxins associated with bacteria, as well as the organisms themselves. It is also possible to detect the presence of more than one antigen by using multiple antibodies, each labeled with a different enzyme, but interferences between the conditions required for each must be considered. As described above, however, immunoassays have failed to meet the required detection limits for some water analysis applications like drinking water testing. It is desirable, therefore, to improve the sensitivity of immunoassays so that their many advantageous features may be employed in microbiological water analysis. This could involve amplifying the signals produced.
Figure 1-4: A conventional immunoassay for the detection of a waterborne pathogen. A surface-bound antibody binds the pathogenic analyte from solution. After washing to remove weakly bound material, a detection antibody for the analyte is introduced. This antibody is conjugated to an enzyme label (in this case alkaline phosphatase), which later converts a substrate (e.g. 4-methylumbelliferyl phosphate) to a fluorescent product (4-methylumbelliferone).

through the assays, reducing the background signal so that low levels of bacteria can be distinguished, and potentially employing a separation scheme in order to concentrate the analytes in a smaller analysis volume.

1.5 Poly(dimethylsiloxane) and its Applications to Environmental Analysis

Siloxane polymers are defined by their silicon-oxygen backbone and organic or hydrogen side chains. Poly(dimethylsiloxane) (PDMS) is a well-known siloxane with applications in chemical analysis, which analyze extraction, medicine, microfluidic chips, and optics. Among its attractive properties are its low cost, durability, and ease of molding, which are combined with biocompatibility, flexibility, and low liquid permeability as well as hydrophobicity and transparency. These properties
lead to the extensive use of PDMS in sensor design, including pressure sensors,\textsuperscript{239} ion selective field effect transistors,\textsuperscript{49} accelerometers,\textsuperscript{240} and many other applications.

PDMS is one of the most common extraction matrices used in solid phase microextraction (SPME), an analyte extraction technique developed by Janusz Pawliszyn’s group in collaboration with Supelco.\textsuperscript{230,241} In the classic SPME approach, a fibre coated with a polymer extraction matrix, such as PDMS, is inserted into a sample and analytes partition into this matrix until a distribution equilibrium condition is reached.\textsuperscript{242} Other materials, such as mesoporous silicates, polyamides, and immunosorbents, can be used, depending upon the required analyte specificity of the coating.\textsuperscript{243} The extracted analytes can be desorbed and analyzed inside an instrument such as a gas chromatograph.\textsuperscript{242} The distribution equilibrium is described by the partition constant $K_{fs}$, which is the ratio of the analyte concentration in the extraction film ($C_f$) to the analyte concentration in the sample ($C_s$) (Equation 1-1).\textsuperscript{242}

\begin{equation}
K_{fs} = \frac{C_f}{C_s}
\end{equation}

For hydrophobic coatings and an aqueous phase, there is a correlation between $K_{fs}$ and the octanol/water distribution constant, $K_{ow}$.\textsuperscript{242,244} Thus, it can be expected that a hydrophobic analyte in an aqueous solution will preferentially partition into a hydrophobic film, resulting in a $K_{fs}$ value greater than 1. Provided the film volume is small compared to the sample, this results in a concentration of the analyte in a smaller volume. While SPME was originally designed for gas chromatographic analysis, the analyte uptake properties of siloxane materials can be used in combination with a number of types of sensors. SPME with PDMS has been employed in the detection of volatile organic compounds,\textsuperscript{245-247} the determination of food quality,\textsuperscript{248-250} and sampling of analytes in living organisms.\textsuperscript{251,252} Optical sensors that use siloxane extraction have been applied in the detection of volatile organic compounds (VOCs)\textsuperscript{246,253-255} and other gases.\textsuperscript{99}
Many analytical methods and sensor designs take advantage of the concentration factor provided by SPME to improve sensitivity to analytes. The Endetec pathogen detection method for E. coli and total coliforms uses fluorescent substrates combining sugar molecules and pyrene or anthracene labels. These substrates are consumed by the relevant indicator bacteria as they are grown in culture and cleaved to form 1-hydroxypyrene and 1-hydroxyanthracene. These product molecules are comparably more hydrophobic than their corresponding sugar-linked substrates. As a result, the fluorescent products partition into a hydrophobic polymer matrix located in the water sample. The products are detected within this polymer matrix by a waveguided fluorescence system (Figure 1-3).

1.5.1 Solid Phase Microextraction and ELISA

One way of amplifying an optical signal from ELISA is through the concentration of the fluorescent product. This can be accomplished by extraction of the molecules into a second phase of smaller volume. The SPME technique described above can be the basis for such an approach. Since fluorescence measurements are concentration dependent, this should result in an increased sensitivity for optical measurements if the analytes can be concentrated in a hydrophobic film. Since the products of enzyme reactions can function as inhibitors as well, extraction of the enzyme reaction product from the solution could reduce this inhibition effect, increasing the rate of the reaction, and therefore the sensitivity of the assay. SPME may be employed in any situation in which an analyte can be extracted from the environment into a matrix with some selectivity, such as through hydrophobic effects. If the Endetec concept of enzyme reactions that produce hydrophobic products could be replicated for an ELISA procedure, the ELISA product could be concentrated in a polymer matrix for detection. This could be advantageous for improving the detection limit for water analysis.
1.6 Research Objectives

The objective of this research project is to develop new environmental sensing applications for modified siloxane materials. The main part of the work focuses on the development of a pathogen analysis system by combining an enzyme-labeled immunoassay with enzyme activity monitoring through partitioning of a fluorescent, hydrophobic product into a polymer film. The goal is to demonstrate improved performance of this new immunoassay based on four advantages of the proposed system: i) detection of product molecules will be independent of the sample optical properties, reducing the requirements for sample pretreatment; ii) preconcentration of the product molecules by SPME will give a higher concentration in the polymer, increasing sensitivity to the amount of enzyme label; iii) partitioning provides the ability to use substrate/product combinations that cannot be used in conventional systems (i.e. those that do not change from non-fluorescent to fluorescent); iv) partitioning simplifies the automated, instrumental detection of the immunoassay result.

In this study, ELISA has been chosen as the assay of choice. The use of antibodies is advantageous because these elements are both sensitive and selective, are available for a wide range of pathogens, and techniques for their immobilization on solid surfaces are well known. They are also available for pathogens that may not be indicated by *E. coli* contamination and should therefore be detected separately. However, drawbacks including false positives due to dead cells and reactions with the sample matrix must be considered.

The scheme used in the existing Endetec test for *E. coli* will be adapted for this purpose. The method is already capable of detecting enzyme activity by extracting the fluorescent product of an enzyme reaction into a siloxane polymer and the same capability will be required in this case. For this assay, the enzyme will be an alkaline phosphatase label on a detection antibody. Alkaline phosphatase has been chosen over horseradish peroxidase because the latter generally cleaves reactants to form radical
products, and these reactions do not meet our requirement for a hydrophilic substrate to hydrophobic product transformation. Alkaline phosphatase catalyzes the hydrolysis of phosphate monoesters on a variety of substrates. To take advantage of the extraction properties of the siloxane polymer, the substrate will be pyrene phosphate, which alkaline phosphatase can break down to inorganic phosphate and 1-hydroxypyrene. This product, being more hydrophobic than the substrate, should preferentially partition into the siloxane polymer for detection (Figure 1-5).

![Diagram of the proposed ELISA pathogen detection system with solid phase microextraction of the enzyme product. The capture antibodies are immobilized on the siloxane polymer and, after binding of the alkaline phosphatase-labeled detection antibody, pyrene phosphate is cleaved by the enzyme to form 1-hydroxypyrene and inorganic phosphate. 1-hydroxypyrene partitions into the siloxane polymer layer due to its hydrophobicity and is detected using a customized optical system with a 365 nm excitation source.](image)

**Figure 1-5:** The proposed ELISA pathogen detection system with solid phase microextraction of the enzyme product. The capture antibodies are immobilized on the siloxane polymer and, after binding of the alkaline phosphatase-labeled detection antibody, pyrene phosphate is cleaved by the enzyme to form 1-hydroxypyrene and inorganic phosphate. 1-hydroxypyrene partitions into the siloxane polymer layer due to its hydrophobicity and is detected using a customized optical system with a 365 nm excitation source.

This test uses the siloxane polymer in the cartridge for antibody immobilization and absorption of the enzyme reaction product. Multiple methods will be explored for the surface immobilization of the
antibodies, including carboxyl-functionalized siloxanes linked to antibodies through carbodiimide chemistry, physical adsorption of the antibodies, and linking through mediators such as Protein A. These methods will be compared to determine the degree of successful antibody attachment, the level of activity of the surface-immobilized antibodies, and the stability of the antibodies during storage.

Using the newly biofunctionalized siloxane surface, a model immunoassay will be used to investigate the characteristics of the ELISA linked to SPME compared to conventional ELISA. The SPME-ELISA will then be adapted and characterized for waterborne pathogens.

In addition to pathogen detection, the application of modified siloxane materials to volatile organic compound (VOC) detection through solid phase microextraction and optical analysis will be investigated. While siloxane polymers are well-known materials used in the extraction of VOCs from air, there remains a need to produce new functionalized materials that have different analyte specificities and optical properties, and therefore different responses to analyte uptake. New materials will be produced through hydrosilylation to introduce desired functional groups and the application of siloxane films for VOC sensing on two devices: a refractometer and a Fabry-Perot interferometer, will be explored.

1.7 List of References

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Chapter 2
Siloxane Materials for Environmental Sensors for Volatile Organic Compounds

2.1 Introduction

Volatile organic compounds (VOCs) are organic molecules with relatively low boiling points (between 50 and 260°C) and are some of the most important contributors to indoor air quality problems.\(^1\) These contaminants occur in indoor air due to their volatility and their common use in household products such as paints, varnishes, waxes, solvents, detergents, household cleaning products, inks, and toners.\(^1\) Some of the most common VOCs found in indoor air are benzene, toluene, xylenes, styrene, terpenes, acetaldehyde, and formaldehyde.\(^1\) Exposure to airborne contaminants such as these can lead to a range of health problems, from “sick building syndrome” (non-specific irritation of the eyes, nose, throat, and nervous system) to more serious conditions including cancer,\(^1\) adverse birth outcomes,\(^3\) and juvenile asthma.\(^4\) Canadian indoor air quality guidelines currently do not set limits on the concentrations of most VOCs,\(^5\) but many are present at low concentrations in Canadian homes (Table 2-1).\(^6\)

Table 2-1: The concentrations of a sample of VOCs in Canadian homes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detection Frequency in Canadian Homes(^6)</th>
<th>Mean Concentration in Canadian Homes (7 days)(^7) (µg/m(^3))</th>
<th>Exposure Guidelines (Canada)(^9) (mg/m(^3))</th>
<th>Exposure Guidelines (ACGIH TLV) (mg/m(^3))(^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>99.69</td>
<td>1.93</td>
<td>none</td>
<td>2</td>
</tr>
<tr>
<td>toluene</td>
<td>99.95</td>
<td>17.80</td>
<td>2.3 (24 hour)</td>
<td>189</td>
</tr>
<tr>
<td>m,p-xylenes</td>
<td>99.90</td>
<td>14.44</td>
<td>none</td>
<td>434</td>
</tr>
<tr>
<td>o-xylene</td>
<td>99.95</td>
<td>4.33</td>
<td>none</td>
<td>434</td>
</tr>
<tr>
<td>styrene</td>
<td>99.77</td>
<td>1.13</td>
<td>none</td>
<td>85</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>96.89</td>
<td>1.95</td>
<td>none</td>
<td>344</td>
</tr>
</tbody>
</table>

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Recent studies on the concentrations of VOCs in indoor air make use of passive sampling devices. For example, a recent survey of indoor air quality in Canada employed a stainless steel tube packed with carbon particles.\textsuperscript{6} Analysis of the samples, collected over seven days, was carried out by thermal desorption, followed by gas chromatography-mass spectrometry (GC-MS).\textsuperscript{5} Other passive collection methods include empty steel canisters,\textsuperscript{8} personal monitoring badges,\textsuperscript{9} and solid phase microextraction fibres,\textsuperscript{10} all of which can also be combined with GC-MS analysis. Handheld on-site detection can also be carried out with photoionization and infrared detectors,\textsuperscript{11} as well as colourimetric gas sampling tubes.\textsuperscript{12} Common to these techniques, however, is the need for operator involvement: either in placing and retrieving the passive sampling unit, or in handling the on-site detection apparatus. Since VOCs can spread quickly through an indoor environment and sensory detection (scent-based) may happen above the contaminant safe exposure limit,\textsuperscript{13} it would be ideal to develop remote sensors that could operate continuously and alert operators to changing concentrations.

\textbf{2.1.1 Detection Methods for Volatile Organic Compound Sensors}

In order to detect volatile organic compounds using a sensor, the analytes are usually extracted into a sensing material or adsorbed onto a sensing surface. The challenge in either case is to select for particular VOCs or groups of VOCs while excluding other components of the sample matrix and there are a number of strategies that researchers have used to accomplish this. Macromolecule-functionalized siloxanes,\textsuperscript{14} carbon nanotube-polymer composites,\textsuperscript{15} and molecularly imprinted polymers\textsuperscript{16,17} can be used to selectively extract analytes of interest from an air sample. Preferential adsorption of VOC analytes onto a sensing material such as porous silica,\textsuperscript{18} graphene oxide nanosheets,\textsuperscript{19} or metal oxide semiconductors\textsuperscript{20,21} can also be employed. Less selective materials can also be used for VOC sensing by incorporating a signaling functionality into the polymer, such as a solvatochromic dye,\textsuperscript{22,23} a turn-on fluorescent dye,\textsuperscript{24} an organometallic film that reacts with analytes,\textsuperscript{25} or a functional group that reacts with acidic and basic
vapours.26 These materials can be used to impart the desired degree of selectivity for a sensor, whether there is a need to detect a single contaminant or a broad range.

In addition to the choice of extraction or adsorption material, sensors can utilize a wide range of transduction schemes. Many of the types of chemical sensors discussed in Chapter 1 have been used for the detection of VOCs, including electrochemical21,27 and mass sensitive15,17,28 transducers. As with chemical sensors in general, optical detection methods dominate the literature on VOC analysis. Methods that have recently been applied include Rayleigh scattering,29 absorbance,26 colourimetric analysis,22,23,30 localized surface plasmon resonance,18 fluorescence,24,31 deformation-based interferometry,32 and refractive index measurement.19,25,33 Refractive index and deformation-based optical measurements are particularly attractive for VOC detection since these phenomena are universal, and can be used for general screening or more specific applications, provided the extraction medium is selective for the analyte of interest. These methods also do not require labeling procedures that may complicate analysis.

2.1.1.1 Principles of Refractive Index

Refractive index (RI) is a property of substances that describes the speed at which light can travel through the material. Since light travels at constant speed (c) in a vacuum, 2.998 x 10^8 m/s, the refractive index (n) of the material is defined as the ratio of the speed of light in a vacuum (c) to the speed of light travelling through the material (v) (Equation 2-1).34

Equation 2-1

\[ n = \frac{c}{v} \]

The speed of light in a medium varies based on the material’s polarizability and molar volume, as demonstrated by the Lorentz-Lorenz equation (Equation 2-2), where n is the refractive index of the material, \( N_A \) is the Avogadro’s number, \( \alpha \) is the molecular polarizability, and \( V \) is the molar volume.35
Thus, in denser media, the speed of light is slower and the corresponding refractive index is higher. When light travels across the boundary between two media with different refractive indices, the differing light speeds cause the beam to be refracted. The angle of the refracted light depends upon the angle of incidence (the angle of the light beam at the boundary relative to the normal) and the refractive indices of the two materials. The relationship between the light beam angles and refractive indices is described by Snell’s Law (Equation 2-3).

\[
\frac{n_1 \sin \theta_1}{n_2 \sin \theta_2} = \frac{n_1}{n_2}
\]

In Snell’s law, \(n_1\) and \(n_2\) are the refractive indices of the two media, \(\theta_1\) is the incident angle of the light at the media boundary, and \(\theta_2\) is the angle of the refracted light. This can be represented pictorially, as shown in Figure 2-1. When the refractive index of medium 1 is greater than the refractive index of medium 2 (\(n_1 > n_2\)), the angle in the second medium is greater. As the incident angle of the light relative to the normal increases, the refracted angle also increases until it reaches 90°. The incident angle at which refraction is at 90° is called the critical angle, and any incident light that reaches the boundary at an angle greater than the critical value undergoes total internal reflection (Figure 2-2). The critical angle (\(\theta_c\)) can be calculated when the refractive indices of the two materials are known by substituting 90° for \(\theta_2\) in Snell’s law (Equation 2-4).

\[
\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right)
\]
**Figure 2-1:** An illustration of Snell's Law describing a light beam traveling from one medium to another. In (a), as the light beam travels from a less dense to a more dense medium, the angle of the beam with respect to the normal decreases. In (b), as the beam travels from a more dense to a less dense medium, the angle increases.

**Figure 2-2:** An illustration of the effect of the incident angle of a light beam on its path after encountering a boundary at which $n_1 > n_2$. In (a), the incident angle is less than the critical angle and the beam is refracted, in (b), the incident angle equals the critical angle and the beam is propagated at the boundary, and in (c), the incident angle is greater than the critical angle and the beam is reflected.
The principle of total internal reflection is used in many sensor designs based on waveguides. By using a material of higher refractive index than its surroundings and an incident angle of light greater than the critical angle, light can be propagated through the material.

2.1.1.2 Refractive Index Sensing Mechanisms

Refractive index change can be analyzed using a number of platforms. When using a waveguiding material, the process of total internal reflection within the waveguide leads to a small amount of light that propagates outside the waveguide core and into the “cladding” or surroundings. This is called the evanescent wave (Figure 2-3).

![Figure 2-3: A schematic of waveguide-based optical sensing. Light is guided by a waveguide core of higher refractive index than the cladding layers. The guided mode energy located outside the confines of the core is called the evanescent field. Sensing phenomena such as analyte binding that occur in the region of this evanescent field can be detected through changes to the guided mode.](image)

The penetration depth \(d_p\) of the evanescent wave into the cladding material is defined by Equation 2-5, where \(\lambda\) is the wavelength of the incident guided light, \(n_1\) is the refractive index of the core, \(n_2\) is the refractive index of the cladding, and \(\theta\) is the incident angle of the light.\(^{36}\)

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Because the evanescent wave extends a small distance into the surroundings, it is affected by changes in the cladding environment that occur close to the waveguide surface, which in turn influence the waveguide’s effective refractive index. The penetration of the evanescent wave into the immediate surroundings of the waveguide has been used to measure refractive index change by monitoring changes in properties of the propagating light such as the phase,\textsuperscript{37} resonance angle,\textsuperscript{38,39} transmittance,\textsuperscript{40} reflectance,\textsuperscript{14} ringdown time,\textsuperscript{41} ellipsometry (polarization),\textsuperscript{42} and resonance wavelength shift.\textsuperscript{43} Evanescent wave sensing has been used by many researchers in combination with waveguide technologies including tapered fibres,\textsuperscript{43} ring resonators,\textsuperscript{44,45} interferometers,\textsuperscript{37} long period gratings,\textsuperscript{46,47} fibre Bragg gratings,\textsuperscript{48,49} and planar waveguides.\textsuperscript{50,51} The strength of refractive index sensing is its universality, but this can also be a weakness since sensors based on this technique tend to be non-specific. This lack of specificity can be applied to screening-type sensors that respond to a range of contaminants. If analyte identification is required, however, it is useful to consider all available methods that may provide different responses to the analytes of interest. By combining multiple methods to provide a fingerprint response, more specific detection may be possible.

2.1.1.3 Deformation for the Detection of Analyte Extraction into Polymer Films

Another label-free analysis technique is deformation, which can be used for chemical sensing when analytes are absorbed into a suitable sensor material and that material undergoes swelling. When this swelling material is put in contact with a strain-sensitive optical component, such as a Bragg grating or interferometer, chemical sensing can be achieved. In a Bragg grating sensor, a narrow range of wavelengths are reflected from the grating material and, when a source of stress is placed on a fibre Bragg

\begin{equation}
    d_p = \frac{\lambda}{2\pi d \sqrt{n_1^2 \sin^2 \theta - n_2^2}}
\end{equation}

Equation 2-5
grating, such as the swelling or contraction of an extraction coating, the reflected wavelengths change.\textsuperscript{52,53} Planar distributed Bragg reflectors can also be used in chemical sensing since the uptake of analytes into the deformable reflector changes the periodicity of the layers, leading to a change in effective refractive index and therefore the reflected wavelength.\textsuperscript{54,55} Interferometric sensors monitor the interference patterns produced by multiple beams of light, which can change based on factors that influence the beams’ paths or speeds. Planar interferometric sensors, in which the deformation of an extraction material in the presence of analyte causes a spectral shift of the interference pattern, have been used to detect VOCs.\textsuperscript{32,56} Fabry-Perot interferometers consist of two partially reflective materials and multiple reflections to create an interference pattern (Figure 2-4).

\textbf{Figure 2-4}: A schematic of a Fabry-Perot cavity interferometer. Light enters the cavity and is reflected between two mirrors. Depending upon the spacing between these two mirrors, different wavelengths of light constructively and destructively interfere, leading to an interference pattern.

In an in-plane Fabry-Perot system, light is transmitted through one side of the interferometer, and resonances are created through constructive and destructive interference as the light bounces between the two reflective surfaces. The light transmitted through the second reflective surface contains a series of wavelength peaks, which are the resonant wavelengths. By integrating such a sensor with a microfluidic
channel between the two mirrors, sensing can be achieved.\textsuperscript{57} This system can be used as a deformation-based sensor if a swelling-capable polymer is located between the mirrors. Deformation sensors are widely applicable, provided the sensing material swells in the presence of the analyte of interest. While many polymers, including poly(styrene), cellulose acetate, and poly(vinylidene chloride-\textit{co}-acrylonitrile), exhibit this property,\textsuperscript{58} light transparency at the wavelengths of interest is also a requirement of polymers used in these types of optical sensors. Similar to refractive index techniques, deformation is usually non-specific and therefore such devices would need to be employed in a multiplexed detection system\textsuperscript{32} or used as screening sensors.

\textbf{2.1.2 Siloxanes in Volatile Organic Compound Sensors}

Siloxane materials including poly(dimethylsiloxane) (PDMS) are frequently used as components in chemical sensors for VOCs. The reasons for this include the hydrophobicity of PDMS and the material’s strong interactions with volatile organic compounds.\textsuperscript{59} Sensing devices exploit the intrinsic hydrophobicity of PDMS,\textsuperscript{60} which allows the polymer to absorb organic compounds from water and air based upon the $K_{ow}$ or $K_{oe}$ values of the compounds of interest.\textsuperscript{61,62} This extraction process is not specific, however, and many hydrophobic analytes and matrix interferents may be absorbed by the material during sensing. It would therefore be ideal to develop siloxane materials that selectively absorb particular VOC analytes or exhibit different sensing responses to different classes of analytes. These materials could be combined in an array of polymers that exhibit different responses to the same analyte, as in a fingerprint detection scheme.

To produce a fingerprint sensor array, it is necessary to develop a variety of siloxane materials that vary in their extraction specificity or response to different analytes. By modifying the functional groups present in a siloxane material, certain VOCs may be taken up preferentially from the sample matrix. The absorption of different analytes may also lead to unique responses, such as the extent of
refractive index change or swelling of the material. In order to produce a fingerprint sensor, it is therefore necessary to characterize siloxane-VOC analyte interactions for a number of material-analyte combinations and on a variety of platforms. Refractometry, for example, has been used to extract both swelling and refractive index effects during the absorption of VOCs by thin siloxane films\textsuperscript{63} and ellipsometry has also been used to characterize the response of siloxanes to contaminants.\textsuperscript{64} More work of this type is needed to produce new materials with different functional groups, leading to unique specificities, refractive indices, and deformation responses. These new materials must then be characterized for their responses to a large number of VOC analytes.

2.1.2.1 Refractive Index and Deformation-Based Siloxane Sensors for Volatile Contaminants

The use of siloxane materials can improve the sensitivity of refractive index-based optical sensors by 2 to 6 orders of magnitude.\textsuperscript{65} When siloxane materials absorb analytes, two different responses are possible. In the first, the analyte fills free volume in the polymer without swelling, causing an increase in the refractive index of the siloxane regardless of the analyte refractive index. This effect can be explained by the finding that the free volume of a material and its refractive index are inversely related, thus a material with a lower free volume has a higher refractive index.\textsuperscript{66-68} When analyte absorption fills microcavities in a material, the free volume decreases and the refractive index should increase. In the second model of analyte uptake, the siloxane undergoes swelling as it absorbs analyte; this phenomenon is well-known for siloxane interactions with non-polar solvents.\textsuperscript{59} In this conception of uptake, the refractive index of the material is governed by the Lorentz-Lorenz equation for multi-component mixtures, where $n$ is the refractive index of the mixture, $n_i$ is the refractive index of the pure component $i$ and $\phi_i$ is the volume fraction of the component $i$ (Equation 2-6).\textsuperscript{69,70}
This relationship assumes that the volumes of the components are additive. In this model, the refractive index of the polymer-analyte mixture could be higher or lower than that of the pure polymer, depending on the analyte refractive index. This leads to both refractive index and deformation-based effects that can be exploited for VOC sensing. The understanding of these two mechanisms is important in analyzing the results of label-free optical sensing methods.

Many sensors using siloxanes have been built based on the refractive index and deformation properties described above. In some sensor designs, the effects of one of these mechanisms can be isolated, while in others a combination of swelling and refractive index influence the results.

Long period and fibre Bragg gratings coated with siloxanes have been used for VOC sensing, relying on the change in refractive index of the cladding when analytes are extracted. This change in cladding refractive index influences the effective index of the waveguide, leading to altered transmission or reflection properties. Our group has used functionalized siloxane materials coated on long period gratings to analyze VOCs including xylenes, which were detected at 134 ppm, while other VOCs were detected at higher concentrations. Through the use of modified siloxanes, our group has demonstrated two effects on the device sensitivity: i) functional groups that can change the refractive index of the siloxane, leading to greater RI contrast between the polymer and analyte and therefore to greater device sensitivity, and ii) functional groups can change the properties of the material and therefore the specificity to various VOC analytes. Fibre Bragg gratings, which reflect light at specific wavelengths depending on the effective refractive index, have also been used with PDMS to sense VOCs. Lowder et al. were able to detect dichloromethane and acetone using fibre Bragg gratings at detection limits of 4000.
and 6000 ppm respectively.\textsuperscript{71} Other fibre-based sensors have also been produced. For example, light reflected from a fibre end coated with a siloxane material was used to detect VOCs.\textsuperscript{72} Ring resonator devices can also detect changes in the refractive index above the sensor surface, since changes in the effective refractive index affect the whispering gallery mode resonances within the device. These resonators have been used in combination with siloxanes to detect VOCs including \textit{m}-xylene at 0.5 ppm\textsuperscript{73} and ethanol at 7290 ppm.\textsuperscript{74} All these devices described above function based on the change of refractive index of the siloxane extraction matrix in the presence of VOCs. Other sensing methods, however, rely on the swelling deformation that takes place upon analyte uptake.

Deformation-based sensing relies on the change in optical pathlength through which the light travels within the sensor, which affects properties of the transmitted or reflected light. Some light properties that can be used to measure swelling deformation include transmission power, as well as interference and diffraction patterns. Wearable radio frequency identification sensors for VOCs have been developed using the swelling of PDMS, which increases the distance between the antenna and feed loop in the device, allowing detection based on the required transmit power.\textsuperscript{75} Fibre gratings are sensitive to strain and this can be exploited by coating the grating with a siloxane material that swells upon absorption of a VOC, deforming the grating and modifying the reflected wavelengths. This method has the advantage of avoiding the chemical etching procedure usually required to expose grating sensors for evanescent wave sensing and has been used to detect alcohol and acetone vapours.\textsuperscript{76} Planar interferometers coated with siloxanes have also been used for the analysis of volatile organic compounds. These sensors rely on the interference of light beams reflecting from the film-air and film-substrate surfaces; the interference pattern changes through a combination of physical thickness and refractive index. It has been found that most of the effect in these sensors is due to swelling rather than refractive index, meaning that the sensitivity is related to the molecular volume of the analyte.\textsuperscript{56} A number of researchers have demonstrated
the use of these interferometers for gas chromatographic (GC) detectors. For example, spectral interferometry on a siloxane film was used as a detector for VOCs and the authors reported a detection limit of less than 100 ppm. Reddy et al. produced a sensor array of multiple functionalized siloxane polymers for the detection of four VOCs and found linear sensor responses at low analyte concentrations, reporting parts per billion level detection limits for toluene and heptane.

While these label-free refractive index and swelling-based sensors are useful in a demonstration experiment, much refinement would be needed before use in real world sensors would be possible. Such devices would require many well-characterized polymers with different selectivities to detect the full range of VOC contaminants. It is therefore necessary to continue the search for polymers with differing absorption capabilities for VOCs, polymers that exhibit greater responses (RI or swelling) to the absorption of VOCs, and platforms with higher sensitivities with which to detect these responses.

2.1.3 Functionalized Siloxanes – Methods of Production and Limitations

Ready-to-use siloxane polymer preparations usually contain only dimethyl siloxane monomer groups. Alternatively, a variety of functionalized silane monomers are commercially available and, by producing polymers from these starting materials, more extensive modification is possible. One such method of synthesis is called methanolysis and uses glacial acetic acid and methanol to catalyze the polymerization of SiCl₂R₂ monomers to obtain linear polymers with average molecular weights greater than 1000 amu. Alternatively, the slow addition of base can be used to catalyze the polymerization reactions of the same monomers. The choice of method depends upon the characteristics of the monomers being used and the desired properties of the resulting oligomers. The linear oligomers produced under various conditions can then be cross-linked to produce an elastomer. Cross-linking can be carried out in many ways, such as by the addition of trichloro and tetrachloro silanes, which are able to link multiple linear oligomers together to form a continuous network. Another cross-linker is titanium
tetraisopropoxide,™ which can be added at 0.5 to 2 mole percent to cross-link siloxane oligomers and form polymers as thin films.™ While the addition of cross-linker is necessary to cure the films, it is important to consider the other polymer properties that are impacted by this variable, including the loss of elastomeric character,™ and hindered swelling.

The types of polymers that can be produced by the above methods are limited by the commercial availability of the appropriate monomers and the potential reactivity of functional groups in these monomers during the polymerization process. To circumvent these difficulties, vinyl terminated molecules may be attached to a hydrosiloxane by the hydrosilylation reaction. Hydrosilylation is defined as the “addition reaction of organic and inorganic silicon hydrides to multiple bonds, particularly carbon-carbon, carbon-oxygen and carbon-nitrogen.”™ This method was first reported by Sommer in 1947,™ and Speier reported the use of hexachloroplatinic acid as a catalyst in the 1950s.™ Researchers have since developed a wide range of catalysts for this process including platinum catalysts with electron deficient olefins for increased catalyst stability,™ platinum (O)-carbene complexes for tolerance to the presence of different functional groups,™ and ethyl and benzyl sulfido platinum salts for catalysis in the presence of ester and amide-containing compounds.™ While platinum is a popular transition metal for homogeneous catalysis of hydrosilylation, others have been used, including nickel, palladium and rhodium.™ There has been some debate regarding the mechanism of platinum catalysis in hydrosilylation. The original mechanism was put forward by Chalk and Harrod in 1965,™ while a modified version of this mechanism™ was proposed in 1977 and followed by the Lewis mechanism in more recent years.™® No definitive conclusion has been drawn, however, possibly due to the variety of factors influencing the reaction mechanism.™® Nevertheless, hydrosilylation has been used in a variety of siloxane applications, including the curing process for Dow Corning Sylgard® materials™ and the incorporation of functional groups such as fluorinated phenol,™ hexafluoroisopropanol,™ naphthyl,™ aldonamide,™ poly(ethyleneglycol),™
cyclam, among many others. Using this well-known process, along with catalysts expected to be effective in the presence of the functional groups of interest, it should be possible to produce new customized polymer materials for this project.

2.1.3.1 Applications of Functionalized Siloxanes to VOC Sensors

Functionalized siloxanes have been used to sense a variety of gaseous analytes and many researchers have investigated the effects of chemical functionality on analyte selectivity. For example, Ronot et al. used functionalized siloxanes coated on an optical fibre to absorb VOCs from air and analyzed the variation in the power of the transmitted light to determine the concentration of the analytes. The group proposed that the absorption of volatile organics from air into polymers was related to solubility interactions including dispersion, polarizability, dipolarity, basicity, and acidity. The tunable extraction selectivity of functionalized siloxanes has allowed many researchers to direct their VOC sensors for particular analytes. Nitroaromatic vapours are one area of interest for these types of sensors and researchers typically exploit the hydrogen bonding properties of the analytes when producing functionalized siloxane extraction materials. These explosive vapours have been detected using hexafluoroisopropanol, cyclodextrin, nitrotoluene, and aniline modified siloxanes coated on mass-sensitive transducers. These materials have high affinity for the explosives due to their strong hydrogen bonding and dipole-dipole interactions with nitrophenyl groups. Hydrogen bonding interactions were also exploited in the use of a fluorinated phenol-functionalized siloxane to detect an organophosphate nerve agent. Recently, a group of researchers demonstrated the fingerprint sensing potential of functionalized siloxanes by using a group of four polymers with different side chains (methyl, phenyl, fluoro, and cyano substituted) to sense four VOCs, with each material showing different responses to the analytes. Our group has also used a variety of alkyl, fluoro, phenyl, and nitrile-functionalized polymers to generate films of different chemical selectivities.
Siloxane functionality can be exploited to modify the refractive index of the polymer material. This has been employed by our group in three different ways: to produce a polymer coating with an RI in the range of maximum sensitivity for long period grating sensors,\textsuperscript{65} to allow for waveguiding within the polymer itself,\textsuperscript{102} or to improve the refractive index contrast between the analyte and extraction material for maximum sensitivity.\textsuperscript{41} Our group demonstrated the first effect by incorporating phenyl groups into a siloxane coating for a long period grating and observed an increased sensitivity toward VOCs compared to PDMS.\textsuperscript{65} The refractive index of unmodified PDMS is approximately 1.4 (1.4225 for Sylgard 184 at 632.8 nm\textsuperscript{103}), lower than is desired for many waveguiding applications, and therefore the goal is usually to increase the RI to higher values. The two most common ways of accomplishing an increased refractive index are the incorporation of functional groups with high molar refractions, such as aromatics, halogens, and sulfur atoms, and the inclusion of high RI inorganic nanoparticles, such as titanium dioxide.\textsuperscript{104} Our group has used both aromatic phenyl groups and titanium incorporation to increase the refractive index of the siloxane materials used in optical sensors.\textsuperscript{65} Many other researchers have used these two strategies to increase the siloxane refractive index.\textsuperscript{62,105-107}

Due to the number of possible VOC contaminants in air and their chemical similarity, it would be ideal to produce fingerprint sensors, in which multiple sensing materials respond differently to the presence of the same contaminant. An examination of this response could allow detection and identification of specific VOCs in real world situations. In order to allow this, however, a variety of sensing materials are needed, with different extraction profiles due to different chemical functionality, as well as varying refractive indices to provide sensitive responses to a range of contaminants with different RIs.
2.1.4 Research Objectives

The goals of this research are to produce a variety of siloxane-based polymers for sensing applications. These materials will include PDMS, as well as polymers with different functionalities, including phenyl and naphthyl, which will be produced by acid and base-catalyzed polymerization, and hydrosilylation. A newly synthesized naphthyl phenyl methyl-functionalized siloxane will be analyzed to determine the refractive index enhancement provided by combining multiple aromatic functional groups in the material. This polymer is anticipated to have a higher refractive index relative to PDMS and previously produced phenyl-functionalized siloxanes. It is predicted that this increased refractive index will enhance the sensitivity of SPME-based detection of some VOCs, such as cyclohexane, that have RIs close to the RI of PDMS. This prediction will be tested using m-xylene and cyclohexane extraction and detection on a refractometer. This material should have optical properties that contrast well with those of unmodified PDMS, making it an attractive additional component for a fingerprint VOC sensor. The use of deformation-based optical detection of VOCs will also be investigated using dimethyl and diphenyl-functionalized siloxanes to determine the properties of these materials in a deformable Fabry-Perot interferometer sensor.

2.2 Materials and Methods

2.2.1 Acid and Base-Catalyzed Siloxane Polymer Synthesis

2.2.1.1 Poly(dimethyl-co-methylhydrogen)siloxane (PDMS-PMS) Synthesis

A polymer containing 50% dimethyl and 50% methylhydrogen functionality was produced by methanolysis as a precursor to hydrosilylation reactions (Figure 2-5).
This procedure was previously developed by Krista Plett. Acetic acid (0.020 mol) and methanol (0.023 mol) were mixed in a 20 mL scintillation vial. After stirring began, 0.010 mol of dichlorodimethylsilane (Sigma-Aldrich, Oakville, ON) and 0.010 mol of dichloromethylsilane (Sigma-Aldrich) were added dropwise by syringe in two alternating portions of each silane over 8 minutes. The reaction mixture was then stirred for 1 hour to facilitate further reaction. After this time, a nitrogen gas stream was used to evaporate volatiles from the mixture, leaving the PDMS-PMS linear oligomer in the vial. Synthesis of siloxanes by this method previously resulted in oligomers consisting of 8 to 20 repeat units.

2.2.1.2 Poly(diphenyl-co-dimethyl)siloxane (PDPS-PDMS) Synthesis

Linear polymers containing 10% diphenyl siloxane and 90% dimethyl siloxane were synthesized by adapting a previously developed procedure (Figure 2-6). In a 20 mL scintillation vial, 0.039 mol of dichlorodimethylsilane (Sigma-Aldrich) and 4.3 x 10^-3 mol of diethoxydiphenylsilane (Alfa Aesar, Ward Hill, MA) were mixed and cooled on ice. While stirring, 80 drops of 10 M sodium hydroxide solution were added from a Pasteur pipette over one hour. The mixture was then stirred for one and a half hours. After this, 3 mL of dichloromethane were added to dilute the mixture and the organic layer was washed three times with water and six times with dilute sodium hydroxide until a neutral pH was obtained. The solvent was then driven off by bubbling with N2 gas. Some precipitate remained in the polymer liquid, so it was passed through a 0.2 µm syringe filter to remove solid particles.
2.2.1.3 Poly(dimethyl-co-methylhydrogen-co-diphenyl)siloxane (PDMS-PMS-PDPS) Synthesis

A copolymer containing dimethyl, methylhydrogen, and diphenyl siloxanes was also prepared, combining the synthetic procedures for PDPS-PDMS and PDMS-PMS (Figure 2-7).

![Chemical structure](image)

**Figure 2-7:** Synthesis of PDPS-PDMS-PMS from the monomers by the methanolysis reaction.

Diethoxydiphenyl silane, dichlorodimethyl silane, and dichloromethyl silane (0.010 mol each) were added dropwise to a solution of 0.040 mol of methanol and 0.030 mol of acetic acid over 8 minutes. The reaction mixture was stirred for one hour at room temperature, then one hour at 60°C. After this time, a nitrogen gas stream was used to remove volatiles from the mixture, leaving the liquid polymer.
2.2.2 Hydrosilylation-Based Polymer Synthesis

2.2.2.1 Poly(methylethynaphthyl-co-dimethyl)siloxane (PMNS-PDMS) Synthesis

PDMS-PMS (prepared as described in Section 2.2.1.1), which contained SiH functionality, was combined with 2-vinylnaphthylene (Alfa Aesar) to produce a naphthyl-functionalized siloxane (Figure 2-8).

![Figure 2-8: Synthesis of PMNS-PDMS from PDMS-PMS and 2-vinylnaphthylene by hydrosilylation. In this case, m and n = 0.5x.](image)

The PDMS-PMS was used in this procedure on the day it was synthesized to prevent the conversion of the reactive Si-H functionalities to hydroxyl groups in the presence of water, leading to unwanted cross-linking. A PDMS-PMS mass of 1.00 g contained 7.45 mmol of Si-H sites, based on an expected ratio of 1 Si-H site per 3 Si-CH₃ sites (Figure 2-5), although this ratio varied somewhat in each synthesis reaction. The PDMS-PMS was dissolved in 8.3 mL of toluene, and 7.45 mmol of 2-vinylnaphthalene was added to the round bottom flask. 1.86 x 10⁻⁵ mol of Pt(SEt₂)₂Cl₂ (Sigma-Aldrich) were used as a catalyst. A condenser was fitted and the reaction mixture was heated to a 70°C bath temperature while stirring. Once at the required temperature, the reaction was allowed to continue for 4.5 hours. After this, the solution was
cooled to room temperature and moved to a 20 mL scintillation vial. Dichloromethane (5.0 mL) was used to rinse the round bottom flask to avoid losing polymer product and this was added to the vial. The mixture was then placed under a nitrogen gas stream to evaporate the volatile components for at least 4 hours or until the mass did not change. The success of the reaction was monitored by $^1$H NMR.

2.2.2.2 Poly(methylethynaphthyl-co-diphenyl-co-dimethyl)siloxane (PMNS-PDPS-PDMS) Synthesis

The hydrosilylation reaction to produce vinlynaphthyl-grafted PDPS-PDMS-PMS was equivalent to that used for PMNS-PDMS above, except that PDPS-PDMS-PMS pre-polymer was used (Figure 2-9). The success of the reaction was monitored by $^1$H NMR.

**Figure 2-9**: Synthesis of PMNS-PDPS-PDMS from PDPS-PDMS-PMS and 2-vinynaphthalene by hydrosilylation. In this case, $m = n = p = 0.33x$. 
2.2.3 Casting Polymers for Fabry Perot Cavities

2.2.3.1 Sylgard 184 Cross-Linking and Casting

A PDMS material was prepared from the commercial Sylgard® 184 kit (Paisley Products, Toronto, ON), using a 9:1 (by mass) base to curing agent ratio. After this mixture was prepared, 15 – 20 µL were deposited from a 1 mL syringe fitted with a 21-gauge needle into a built-in fluidic reservoir on a Fabry-Perot interferometer (École Polytechnique, Montreal, QC, see Section 2.2.4 below). A microfluidic channel then carried the liquid to the sensing device, where it cured between the interferometer reflectors (Figure 2-10). The resulting material was allowed to cure for two days at room temperature before testing.

Figure 2-10: The on-chip Fabry-Perot interferometer used in this project. a) A side-on view of the interferometer. The input fibre directs light into the cavity, where it reflects between the two Bragg mirrors. The output fibre collects the light and transmits it to the detector. The siloxane polymer is located between the two mirrors. Diagram adapted from St. Gelais et al.\textsuperscript{109} b) An optical microscope image of the chip from above. In this image, a polymer has been deposited in the chip.
2.2.3.2 Titanium Tetraisopropoxide Assisted Cross-Linking and Casting for PDPS-PDMS

A material consisting of 10% PDPS and 90% PDMS was cast using the linear oligomer synthesized in Section 2.2.1.2. To accomplish this, a 50% (v/v) mixture of PDPS-PDMS in hexanes was prepared \( (i.e. \ 500 \mu L \ of \ PDPS-PDMS \ were \ dissolved \ in \ 500 \mu L \ of \ hexanes) \). Next, \( 1.8 \times 10^{-4} \) moles of titanium tetraisopropoxide \( (Ti(O-iPr)_{4}) \) (Sigma-Aldrich) from a 10% (v/v) solution in hexanes were added and mixed to reach a 3 mole percent concentration of titanium tetraisopropoxide relative to siloxane monomer \( (5.8 \times 10^{-3} \) moles\). After quickly mixing the components, the solvent was evaporated using a stream of \( N_2 \) gas before the mixture was cast on the Fabry-Perot device as described for PDMS.

2.2.3.3 Casting Polymers for Refractometer Sensing

Polymer films intended for use on the refractometer needed to be cast on specialized glass slides with a high refractive index. In this experiment, N-SF11-type glass \( (RI=1.7783 \ at \ 635 \ nm, \ Volume \) Precision Glass Inc., Santa Rosa, CA) that was 2.5 cm x 2.5 cm square and 1 mm thick was used. Both the solvent dilution and the titanium content of the films had to be optimized, since the dilution affected the film thickness, and the titanium content affected the degree of cross-linking, the physical properties, and the refractive index of the cured film.

In the optimized procedure, a 1:1 (v:v) mixture of dichloromethane and hexanes was chosen as the casting solvent. This mixture was used to dilute the polymer at 5% (m/v) in solvent \( (i.e. \ 0.05 \ g \ of \ oligomer \ in \ 950 \mu L \ of \ solvent) \). Titanium tetraisopropoxide at 3 mole percent relative to monomer units in the polymer material \( (i.e. \ 1.0 \times 10^{-5} \) moles of Ti(O-iPr)$_4$ and \( 3.5 \times 10^{-4} \) moles of monomer) was then added and the material was mixed quickly. The Ti(O-iPr)$_4$ was pre-dissolved at 10% (v/v) in hexanes solution as described above. The mixture prepared was then added to the slide surface in two aliquots of 600 \( \mu L \). The
second aliquot was added 30 minutes after the first and the resulting films were cured overnight at room temperature before use.

2.2.4 VOC Sensing Using Fabry-Perot Cavities

Fabry-Perot cavity-containing chips were prepared by Dr. Raphael St-Gelais at École Polytechnique, Montreal, as described elsewhere. In summary, the device was fabricated in silicon using photolithography and deep reactive ion etching. The Bragg reflectors were composed of either three layers of silicon and two layers of air (3 wall) or two layers of silicon and one layer of air (2 wall). Channels were etched to allow the placement of the optical fibres against the Bragg reflectors.

Once the polymers were cured in the cavity, the device could be used for VOC sensing. The experimental set-up, developed by John Saunders and Dr. Jack Barnes, consisted of an optical measurement system as well as a gas-exposure apparatus. The input light source was a tunable infrared laser (ANDO AQ4320D, Yokogawa Electric Corp, Kawasaki, Japan). This was coupled through input and output optical fibres (single mode Corning SMF-28, Corning, NY). The fibres were aligned on the chip using a micropositioning stage. The transmission spectrum was collected every 30 seconds using an InGaAs photodiode detector (D400FC, Thorlabs, Newton, NJ) and a lock-in amplifier (Stanford Research Systems SRS844 RFCA, Sunnyvale, CA). The voltage output was read by a 16-bit data acquisition USB device (Measurement Computing PMD 1608 FS, Norton, MA) and interfaced with a computer through a LabView (National Instruments, Austin, TX) software program developed by John Saunders.

This optical measurement set-up was combined with a gas-flow system for VOC sensing. The solvent analytes were \textit{m}-xylene (Sigma-Aldrich) and cyclohexane (Caledon Laboratories, Georgetown, ON). Nitrogen gas was bubbled through the solvent of interest to produce saturated vapour (1.122 x 10^4 ppm for \textit{m}-xylene, 1.213 x 10^5 ppm for cyclohexane). The saturated vapour concentration values were
calculated using the Antoine equation (Equation 2-7), where \( p \) is the vapour pressure of the liquid, \( A \), \( B \), and \( C \) are Antoine parameters\(^{111} \) for the particular liquid, and \( T \) is the temperature of interest (25°C).

\[
\log_{10} p = A - \frac{B}{C+T}
\]

Once the vapour pressure had been calculated, this value was converted to a fraction of the total pressure (atmospheric pressure, assumed to be 101.3 kPa), which is equivalent to the mole fraction for the vapour. By multiplying this value by 1 000 000, the concentration of the vapour was calculated in parts-per-million units. The saturated vapour was passed through a mixing flow meter (Praxair PRXFM-4621(22), Mississauga, ON) where it was combined with nitrogen gas to produce different analyte concentrations. The nitrogen flow meter channel contained a tantalum float while the vapour channel contained a glass float. The mixed gas was then passed through tubing to the chip, where it was directed down towards the surface (Figure 2-11). Transmission spectra were analyzed by a method developed by John Saunders. A peak from the spectrum was chosen and fit to a Gaussian function to find the location of its maximum. This peak was then tracked through the experiment to determine the resonance wavelength shift observed for each gas concentration.

### 2.2.5 VOC Sensing Using Visible-Light Refractometer

Once films were prepared for gas sensing, they were modified to allow gas to flow over their surfaces. The top half of a 3 dram glass vial was scored and separated from the bottom half. This top part was placed on top of the polymer-coated glass slide, and immobilized using Sylgard 184. The Sylgard mixture was prepared by mixing the base and curing agent in a 9:1 (mass) ratio. A syringe and needle were used to deposit the mixture in a ring around the vial. The seal was cured at 60°C for 60 minutes after
overnight curing at room temperature. After curing, a reversible attachment between the planar film and the vial top was formed (Figure 2-12).

**Figure 2-11:** Experimental set-up for exposure of coated Fabry-Perot chips to gases. A flow of nitrogen gas was split, allowing some to travel through a gas bubbler filled with the solvent of interest, producing saturated solvent vapour. This vapour stream was mixed in a flow meter with pure nitrogen gas and the relative flow rates of the two were adjusted to achieve variable solvent concentrations. The mixture was flowed over the chip through a gas exposure cap.

**Figure 2-12:** Gas exposure cap attached to N-SF11 glass slide using Sylgard 184. Left: uncoated slide, right: PMNS-PDPS-PDMS coated slide.
2.2.5.1 Visible Wavelength Refractometer

The visible light Abbé-type refractometer used in these experiments was adapted from a set-up described by Chen et al.\(^6\) The instrument was built by Jason Chen and John Saunders with Jessamyn Little and a schematic is shown in Figure 2-13.

![Schematic of 635 nm refractometer](image)

**Figure 2-13:** Schematic of 635 nm refractometer used for these experiments. P-polarized light was emitted from the laser, passed through beam-expanding optics, and reflected by a mirror into the prism. When the beam reached the substrate-film interface, light above the critical angle was reflected through the prism toward the CCD camera.

The light source was a 5 mW 635 nm linearly polarized laser diode (63-885, Edmund Optics, Barrington, NJ). A series of lenses were used to focus the light beam, containing a range of incident angles, at the top of a prism, which was used to couple light into the film-coated N-SF11 glass. As described by Snell’s law, angles of light greater than the critical angle underwent total internal reflection from the film-glass interface, while angles of light less than the critical angle were refracted. The reflected light passed through the prism again and was then focused onto a CCD camera. The image captured was comprised of a dark region indicating refraction and a light region indicating reflected light. The interface between these two regions represented the critical angle (Figure 2-14). The pixel position of the interface was determined using the plot profile function in ImageJ.\(^{112}\) Using a series of solutions of known refractive
Figure 2-14: Left: A typical refractometer image from a calibration using $n = 1.5466$ (at 635 nm) Cargille liquid. The light region represents reflected light, while the dark region represents light that was refracted. The transition between these two is called the interface and the pixel position of this transition can be calibrated to refractive index. Right: Profile of the image performed using ImageJ. Each vertical pixel column was binned to produce a grey value.

index (Cargille Certified Refractive Index Liquids, Cargille Labs, Cedar Grove, NJ), the position of this interface can be calibrated to represent a range of refractive indices. An example of such a calibration is shown in Figure 2-15. This calibration was then used to determine the refractive index of polymer films.

Figure 2-15: Refractive index calibration using Cargille Liquids, correlating the known refractive index for each solution at 635 nm with the interface pixel position on the image. Replicates: $n=1$. 

\[

grey\%\text{Value} = -6919.9x + 11025 \\
R^2 = 0.99716
\]
2.2.5.2 Refractive Index Measurement

Before placing the film-coated glass on the prism, it was necessary to use a refractive index matching liquid \( (n = 1.7250 \text{ at } 589 \text{ nm, Cargille Labs}) \) to avoid a glass-air-glass transition for the light beam travelling from the prism to the slide. For each film, refractive index measurements were taken at three positions to characterize the variability within each polymer material.

2.2.5.3 VOC Sensing on High Refractive Index Thin Films

The gas exposure set-up used was the same as that used for Fabry Perot experiments (above) (Figure 2-11). The mixed gas was flowed over the film by attaching the rubber tubing from the flow meter to a bent needle, which was inserted into a plastic vial cap. Another needle was used to vent vapours from the cap. As the analyte was taken in by the film, the film refractive index changed due to the different refractive index of the analyte relative to the film, as well as due to film swelling. This change was monitored by collecting images from the refractometer using a LabView program developed by John Saunders. These images were profiled by binning each vertical pixel column in the LabView software and these data, containing total intensities for each pixel column, were exported as an Excel-compatible spreadsheet. Many successive images produced a 2-dimensional matrix of data. By calculating average slopes of intensity vs. pixel across sections of each image to approximate a first derivative calculation, the interface position could be identified from the point with maximum negative slope.

Before beginning a gas exposure experiment, the film was equilibrated with \( \text{N}_2 \) gas flow for at least 20 minutes to purge contaminants such as water. In early experiments, it was found that there might be a conditioning effect in terms of polymer swelling, whereby the first exposure to a solvent produced a small response and further exposures produced more dramatic responses. As a result, during later experiments, this conditioning was included in the experimental design: the film was repeatedly exposed
to saturated solvent vapour (1.122 x 10^4 ppm for m-xylene, 1.213 x 10^5 ppm for cyclohexane), followed by pure N₂ gas, until a repeatable response was seen to each vapour exposure. Following conditioning, the film was exposed to a range of concentrations of solvent vapour in nitrogen gas, allowing 30 minutes for equilibration at a particular concentration. To characterize the responses of the films, a wide range of concentrations, ranging from 0 to 20% of saturated vapour concentration (0 to 2244 ppm for m-xylene, 0 to 2.426 x 10^4 ppm for cyclohexane) was used for both cyclohexane and m-xylene. Once the sensitivity of the system had undergone preliminary characterization, a further exposure experiment was conducted, using more concentrations below 10% saturated vapour, to determine the detection limit.

2.3 Results and Discussion

2.3.1 PMNS-PDMS and PMNS-PDPS-PDMS Synthesis

The synthesis of vinylnaphthyl-grafted siloxane was accomplished successfully and ¹H NMR spectra of the reactants and products were used to monitor the process. It was found that the Si-H peak disappeared after hydrosilylation and peaks consistent with the additional aromatic group (naphthalene) appeared. The integration of these peaks approximately aligned with the expected number of protons in the system, indicating a successful reaction for both PMNS-PDMS (Figure 2-16 and Figure 2-17) and PMNS-PDPS-PDMS (data not shown). The most obvious indicator was the disappearance of the Si-H peak located at 4.72 ppm from the product spectrum. In the PMNS-PDMS spectrum, the integration of the Si-CH₃ peak matches the expected number of methyl protons in each repeat unit (9), but the rest of the NMR spectrum shows more protons than expected. This was not investigated, but could be explained by the lack of purification procedures after the hydrosilylation, which would leave unreacted components, especially 2-vinylnaphthylene and the products of side reactions, in the product.
Figure 2-16: $^1$H NMR spectrum of PDMS-PMS in CDCl$_3$ at 400 MHz. Analysis: $\delta$ 0.12 ppm (9H, m, Si-\text{CH}_3), \delta$ 4.72 ppm (1H, s, Si-H). The rectangular box highlights the Si-H peak, the disappearance of which was used to indicate a successful hydrosilylation reaction.

Figure 2-17: $^1$H NMR spectrum of PMNS-PDMS in CDCl$_3$ at 400 MHz. Analysis: $\delta$ 0.01 ppm (9H, m, Si-\text{CH}_3), \delta$ 0.50 – 3.00 ppm (4H, Si-\text{CH}_3-\text{CH}_2-\text{naphthyl} and Si-\text{CH}_2-\text{CH}_2-\text{naphthyl}), $\delta$ 7.00 – 8.00 ppm (7H, m, naphthyl). The rectangular box highlights the absence of an Si-H peak, indicating a successful hydrosilylation reaction.
2.3.2 PMNS-PDPS-PDMS Film Casting and Optimization

Initial investigations suggested that the addition of naphthyl functionality would increase the refractive index of siloxane films, as expected from previous work, which found that the RI of vinyl naphthyl grafted siloxanes was between 1.4 and 1.6, depending upon the monomer composition. Measuring the first PMNS-PDMS films on the refractometer gave a maximum RI of 1.5664 ± 0.0001 at 635 nm when ten mole percent titanium was added. In order to further increase the refractive index of these materials, a copolymer containing dimethyl, diphenyl, and methylnaphthaline functionalities was prepared. This should further increase the refractive index, since aromatic functionalities have high molar refraction values. While it may have been possible to make a polymer composed solely of methylnaphthaline siloxane, this was expected to be problematic because pure poly(methylhydrogensiloxane) has a tendency to polymerize quickly, making it difficult to control chain length in the pre-polymer. Thus, combining the three polymers allowed us to limit the quantity of dichloromethylsilane in the methanolysis reaction while still obtaining a high refractive index that was tuneable based upon monomer composition.

Once the PMNS-PDPS-PDMS was prepared, films were cast using this material under a variety of conditions. The aim was to obtain a polymer with a high refractive index, and good optical properties to allow refractometer measurements. Specifically, the goal was to obtain clear refractometer film images, indicating homogeneous optical properties, as well as materials that were free from aggregation and with the material spread evenly across the glass. After testing 1, 3, 5, and 7 mole percent titanium crosslinker, it was found that 3% produced the most consistent film quality, with even spreading across the glass surface. A solvent dilution factor of 5% (m/v) polymer in solvent was found to allow the polymer to spread across the glass without causing the film to be too thin. An additional factor is the choice of casting solvent; it was necessary that this solvent effectively dissolve the polymer of interest, be miscible with the
Ti(O-iPr)$_4$ liquid, and, finally, it must allow the polymer to spread evenly over the glass surface. Previously, it was found that hexanes provided better casting conditions than dichloromethane, however, the PMNS-PDPS-PDMS was best dissolved in dichloromethane. Thus, a solvent comprised of 1:1 hexanes to dichloromethane was chosen to combine the properties of both. The refractive index of this polymer, (3% Ti) was 1.585 (at 635 nm), which was 0.019 units higher than the maximum RI obtained for the PMNS-PDMS films and 0.027 RI units higher than the PMNS-PDMS film cast under equivalent conditions. Thus, including diphenylsiloxane in the copolymer was successful in further increasing the film refractive index.

2.3.3 VOC Sensing with Fabry-Perot Cavities

The results described here have been published in Sensors and Acuators, B: Chemical.$^{114}$ Transmission spectra from the Fabry-Perot cavities were collected using the instrumentation described above. Nitrogen gas was passed over the chip until a stable spectrum was obtained, then the flow meter was adjusted to allow VOC-contaminated nitrogen to flow at the desired concentration. As this occurred, the peaks in the transmission spectrum migrated as shown in Figure 2-18. This migration was followed by fitting a Gaussian curve to the peak in each spectrum. Once all this data was collected and analyzed, a graph of resonance wavelength over time, representing the uptake kinetics for a particular polymer-analyte combination, was produced. In both materials, PDMS and PDPS-PDMS, $m$-xylene appears to diffuse into the polymer and reach equilibrium at a slower rate than cyclohexane. $m$-Xylene diffusion did not reach equilibrium over 15 minutes in either film, while cyclohexane diffusion took less than 10 minutes in PDMS (Figure 2-19).
Figure 2-18: The shift of a resonance peak for a 2 walled Fabry-Perot cavity coated with PDMS in the presence of 1300 ppm cyclohexane. The resonance wavelength moves from its initial value at 1557 nm to 1563 nm, as indicated by the arrow, where it reaches equilibrium.

In SPME, when agitation of the uptake mixture is not perfect, a boundary layer of depleted concentration develops above the siloxane. In these cases, the equilibration time ($t_e$) is proportional to the thickness of the boundary layer ($\delta$), the film-air partition coefficient ($K_{fa}$), and the film thickness ($b - a$), and inversely proportional to the diffusion coefficient in the solution ($D_s$) (Equation 2-8).

$$ t_e = t_{95\%} = 3 \left( \frac{\partial K_{fa}}{D_s} \right) (b - a) $$

Thus, both the partition coefficient of the analytes with respect to the polymers, and the analytes’ diffusion coefficients in the gas phase should impact the equilibration time in these experiments. The gas phase diffusion coefficients for $m$-xylene and cyclohexane are quite similar. As a result, it is most likely the difference in air-PDMS partition coefficients between cyclohexane and $m$-xylene that determines the equilibration time. Other researchers have determined the log $K_{fa}$ values to be 3.32 for $m$-xylene and 2.7 for cyclohexane. Since the partition coefficient is much higher for $m$-xylene, it would be expected that a longer equilibration time would be observed for this analyte. Diphenyl functionalized siloxanes are
Figure 2-19: Uptake of \textit{m}-xylene and cyclohexane by PDMS and PDPS-PDMS in a 2-walled Fabry Perot cavity monitored by the change in resonance wavelength of the sensor. a) 720 ppm \textit{m}-xylene in PDMS, b) 580 ppm cyclohexane in PDMS, c) 720 ppm \textit{m}-xylene in PDPS-PDMS, d) 580 ppm cyclohexane in PDPS-PDMS.

calculated to have similar partition properties to PDMS, with log $K_{fa}$ values for xylenes at 3.70 and cyclohexane at 2.90. This correlates well with the degree of resonance shift observed for the two analytes in the two-walled cavity, with 720 ppm \textit{m}-xylene causing a shift of 12 ± 5 nm (average and standard deviation for two trials) for PDMS and 15.2 ± 0.8 nm for PDPS-PDMS (average and standard deviation for three trials), while 580 ppm cyclohexane caused a shift of 2 nm for the PDMS device and 4 nm for the PDPS-PDMS device. This difference in sensitivity for the two analyte gases correlates well with the literature partition coefficients for the two analytes.
The response of the Fabry-Perot device used here can be attributed to a swelling response of the polymer, rather than its refractive index change. Since the refractive indices of the polymers (Sylgard 184: 1.3997 at 1554 nm\textsuperscript{103} and 10\% PDPS, 3\% Ti = 1.4235 at 1550 nm) are intermediate between those of the two analytes (m-xylene = 1.4769, cyclohexane = 1.4147 at 1550 nm\textsuperscript{119}), the wavelength shift would be expected to proceed in opposite directions for the two analytes if refractive index was the dominant sensing principle. This is not observed, however, since both m-xylene and cyclohexane caused positive resonance wavelength shifts for both materials. Modeling of the sensor\textsuperscript{114} predicted the dominance of swelling over the refractive index response for this system, which is consistent with the results observed in this work.

Once data for a range of analyte concentrations was collected, a more complete picture of uptake was available. In the example shown in Figure 2-20, cyclohexane vapour at concentrations ranging from 200 ppm to 6100 ppm was passed over a chip coated with PDMS. It can be seen that the response was concentration sensitive. When the flow was reverted to N\textsubscript{2} gas, the resonance wavelength returned to its baseline value, showing good repeatability.

From the collected kinetic data, resonance wavelength shifts for each concentration in a polymer-analyte combination could be calculated. This was accomplished by averaging the last five resonance wavelength data points calculated for each concentration of VOC. For m-xylene, all three devices tested: PDMS in a two-walled device, PDMS in a three-walled device, and PDPS-PDMS in a three-walled device, produced similar results (Figure 2-21). The sensitivities were 0.0152 ± 0.0007 nm/ppm (PDMS, 3-wall), 0.0172 ± 0.0008 nm/ppm (PDMS, 2-wall), and 0.018 ± 0.001 nm/ppm (PDPS-PDMS, 3-wall). This suggested that the m-xylene uptake properties of PDMS and PDPS-PDMS formulations used in this experiment were comparable.
Figure 2-20: Exposure of 2-walled device coated with PDMS to various cyclohexane concentrations. The missing data points represent the spectrum changing too rapidly to track or peaks being obscured by the sudden change.

Figure 2-21: Resonance wavelength shift of siloxane-coated Fabry-Perot devices after exposure to various concentrations of m-xylene. 2W indicates a 2-walled device, while 3W indicates a 3-walled device. Both PDMS and PDPS-PDMS coatings are compared. The y-error bars represent standard deviations of replicate measurements. Replicates: n=2 (2W-PDMS, 3W-PDMS), n=3 (3W-PDPS-PDMS).
For cyclohexane uptake, PDPS-PDMS appeared to be the more sensitive material (Figure 2-22), with a sensitivity of $0.0055 \pm 0.0007$ nm/ppm, compared to $0.0038 \pm 0.0002$ nm/ppm for PDMS. However, only one set of measurements was completed for each material, meaning that the standard deviation for these shift values was not determined. In addition to the functionality of the films, the cross-linking processes for the two polymers were different and these factors could combine to influence uptake properties. In contrasting the two analytes, the resonance shift versus concentration curve confirmed the information obtained from the kinetic measurements described above, that $m$-xylene appeared to have a 4 to 5-fold higher partition coefficient in these siloxanes from air, relative to cyclohexane. Given the large uncertainty on this comparison, this relationship correlated well with the literature partition coefficients for these analytes in siloxanes and octanol.\textsuperscript{78}

![Figure 2-22: Resonance wavelength shift of siloxane-coated Fabry-Perot devices after exposure to various concentrations of cyclohexane. 2W indicates a 2-walled device, while 3W indicates a 3-walled device. Replicates: n=1.](image)

The missing data for 6100 ppm cyclohexane in Figure 2-20 can be attributed to the data collection method, which was unable to track rapid movement of the resonance peak. This result indicated that the dynamic range of the system lay below 6100 ppm, since this concentration could not be properly

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characterized by the sensor. An additional limitation of the sensor was observed in the variable baseline resonance wavelength recorded throughout some experiments (data not shown). This can be attributed to multiple factors, including insufficient re-equilibration time after the return to N₂ gas flow, as well as inconsistencies in fibre alignment. In these cases, the resonance wavelength shift was calculated using the initial baseline peak position from the start of the experiment. Additional instability in the measured peak position during uptake was likely the result of data fitting errors. The use of an average of multiple points to determine the peak shift was expected to mitigate this issue.

Detection limits for these sensors were calculated using the calibration curve method described by Loock and Wentzell. The values obtained are summarized in Table 2-2.

**Table 2-2:** Summarized detection limits for VOC analysis using Fabry Perot cavity devices coated with siloxanes. Value in brackets represents the detection limit for cyclohexane in a 2-wall device when an apparent outlier point (3100 ppm) was removed from the calibration based on a Grubbs test of the fit residuals.

<table>
<thead>
<tr>
<th>Absorbent Material</th>
<th>Analyte</th>
<th>Device Type</th>
<th>Detection Limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS</td>
<td>cyclohexane</td>
<td>2-wall</td>
<td>2100 (840)</td>
</tr>
<tr>
<td></td>
<td>m-xylene</td>
<td>2-wall</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-wall</td>
<td>590</td>
</tr>
<tr>
<td>PDPS-PDMS</td>
<td>cyclohexane</td>
<td>3-wall</td>
<td>2100</td>
</tr>
<tr>
<td></td>
<td>m-xylene</td>
<td>3-wall</td>
<td>540</td>
</tr>
</tbody>
</table>

As expected, the detection limits for m-xylene were lower than those for cyclohexane, which correlated to a greater partition coefficient for the former relative to the latter. The use of a 2-walled versus a 3-walled device appeared to decrease the detection limit for the polymer-analyte combination tested (PDMS with m-xylene), although more data would be required to confirm this finding. For cyclohexane, both PDPS-PDMS and PDMS produced similar detection limits and further experiments would be needed to determine any effect of polymer functionality in this case.
By comparison, other Fabry Perot cavity-based sensors report lower detection limits for VOC detection. For example, Reddy et al. quoted a 25 ppb detection limit for heptane using an out of plane Fabry Perot interferometer coated with a silicone-type material. However, this may be an idealized value since the lowest amount of analyte actually measured was approximately three orders of magnitude higher than the stated detection limit (e.g. 0.1 ng measured vs. quoted detection limit of 0.64 pg). In another report, a PDMS-based interferometer provided results similar to those reported here, with a detection limit near 1500 ppm for ethanol. Thus, while the sensor here requires some improvements, its detection limit was comparable to some other systems in the literature. Lower detection limits could be obtained by improving the stability of the system, since fibre alignment was easily compromised. In addition, the 10 to 20% error on the gas flow meter limited the accuracy of the analyte concentrations, further affecting the limit of detection.

2.3.4 VOC Sensing using Visible Light Refractometer

In initial VOC detection experiments, the PMNS-PDPS-PDMS film was exposed to m-xylene vapour at a variety of concentrations. During three replicate experiments, the response to the analyte was greater in the second and third exposures relative to the first (Figure 2-23). This could be due to a “conditioning effect,” since the response was greater after the first time the film was exposed to each vapour concentration. The mechanism for this effect is not clear, but may be the result of repeated swelling causing the film to reorganize into a more open orientation after multiple vapour exposures. As a result, further experiments were completed only after films were exposed repeatedly to saturated vapour until a consistent response was observed.
Figure 2-23: Three successive sets of exposures of the PMNS-PDPS-PDMS film to different concentrations of m-xylene reveal a response that is not reproducible. Trials 2 and 3 show a much stronger response than Trial 1, suggesting that “conditioning” the film with the analyte may be necessary for optimal response. Replicates: n=1.

After conditioning a new PMNS-PDPS-PDMS film, both m-xylene and cyclohexane vapour exposures were completed at a range of concentrations from 0 to 20% of saturated vapour in air. The goal of these experiments was to produce a wide-ranging characterization of film response in order to focus further experiments on the analyte concentration region of interest. The results of these experiments can be seen in Figure 2-24. During these experiments, m-xylene exposures led to an approximately linear refractive index response, causing the refractive index to decrease in all cases. This decrease was expected based on the Lorentz-Lorenz equation, since the refractive index of m-xylene is 1.4944 at 589 nm, which was less than the refractive index of the film (1.5846). Cyclohexane exposure led to a non-linear response pattern, with low concentrations (2046 ppm and below) causing no observable change in film refractive index, and higher concentrations producing a magnified effect on refractive index. Again, the refractive index of the film decreased as cyclohexane was added, agreeing with the Lorentz-Lorenz model, since the cyclohexane refractive index is 1.4235 at 589 nm. These initial experiments also
showed that 20 minutes was insufficient for equilibration of the film with solvent vapour, so 30 minutes was allowed in further experiments.

Once it was clear that the sensor responded to concentrations below 10% saturated vapour for both analytes, the vapour exposure experiment was repeated with more concentrations in the lower range. For \( m \)-xylene, an increase in refractive index took place at 160 ppm, followed by a decrease when the analyte concentration was increased above this level (Figure 2-25a). This could be explained by considering two possible models of analyte uptake into a polymer. In the lowest concentration samples, analyte may have filled free volume in the material without causing swelling, leading to a higher refractive index, even though the \( m \)-xylene has a lower refractive index than this polymer.\(^{67}\) Free volume has been described as microcavities, or holes, in a polymer material and is used to explain molecular transport within a polymer.\(^{122,123}\) At higher analyte concentrations, swelling would occur after the free volume is occupied. This would lead to a refractive index decrease related to the higher film and lower analyte refractive indices and the volume fractions of the two components, as described by the Lorentz-Lorenz model.\(^{69,70}\) If this is the reason for the reversing trend in refractive index at low analyte
Figure 2-25: Response of PMNS-PDPS-PDMS film to a) m-xylene vapours at concentrations of 160 to 820 ppm and b) cyclohexane vapours at cyclohexane concentrations of 2000 to 10000 ppm. Replicates: n=2.

Concentrations, increasing the refractive index contrast of the film relative to the analyte will not lead to higher sensitivity devices. Instead, it may be necessary to change the polymer casting procedure by reducing the concentration of crosslinker, or to increase the repetitions of the conditioning experiment. Since this set of data was not linear, it was not possible to use it to calculate a limit of detection. Using the data for 0 to 20% m-xylene instead, which showed a greater response and a more linear trend due to the lack of low concentration measurements, the detection limit calculated by Loock and Wentzell’s equation was determined to be 4.0 x 10^2 ppm. For cyclohexane, a decreasing RI trend was observed (Figure 2-25b). Using Loock and Wentzell’s spreadsheet, the detection limit in this case was estimated to be 4.0 x 10^3 ppm. These are estimates of the detection limits of these systems, since more data would ideally be required, such as repeated measurements of a concentration near the detection limit, or triplicate measurements of the calibration curve.

It is clear that these approximate detection limits would not be useable for ambient air quality measurements. Other applications in which a sensor of this sensitivity would be useful might include the detection of solvent spills, since the explosive limits for many vapours fall within the useful range of our
sensor. For ambient air quality sensing, it would be necessary to either refine the sensitivity of the refractometer or produce a polymer with a much higher sensitivity to the analyte of interest to elicit a greater response to a given concentration. It is important to note that the set-up of this instrument inherently limited the sensitivity of the experiment. The precision of the refractometer itself was approximately $2 \times 10^{-4}$ RI units, which corresponded to over 200 ppm $m$-xylene based on our calibration, constraining the minimum attainable detection limit. However, this precision value is typical of commercial refractometers ($3 \times 10^{-4}$ RI units), so it may be necessary to use a different RI analysis platform to improve the resolution of refractive index change below $2 \times 10^{-4}$ RI units. The silicon-on-insulator ring resonators produced by the National Research Council of Canada, which have a refractive index resolution of $2 \times 10^{-6}$ RI units, would be an example of this and could improve the limit of detection for these films by approximately two orders of magnitude, provided there are no other limitations on the sensor.

The use of the PMNS-PDPS-PDMS polymer demonstrated that films with high refractive indices showed decreasing RI values as lower RI analytes were added, consistent with a swelling model of uptake. This response pattern was different from other siloxane polymers exposed to the same VOCs. For example, a previous group member, Krista Plett, found that a PDPS-PDMS film with 8.5% diphenyl functionality (RI=1.425) showed an increasing RI trend when exposed to xylenes and decreasing RI when exposed to cyclohexane. This was because the RI of the film was intermediate between the two analyte RI’s. The use of films with varying RIs could allow the development of a fingerprint sensor, using the RI response of a range of different films to a given analyte to determine its identity. In addition, the sensitivities to cyclohexane previously reported by our group for functionalized siloxanes ranged between $1$ and $7 \times 10^{-8}$ RI units/ppm cyclohexane vapour, whereas the values reported here are between 2 and 3 x
10⁻⁷ RI units/ppm cyclohexane vapour. An increased refractive index contrast between the film and analyte likely accounted for this difference.

2.4 Conclusions

A number of siloxane materials have been developed here for use in sensing applications. The functionalization of siloxanes was shown to change both the optical and chemical properties of these films. In addition, sensors involving these siloxanes have been shown to work on the principles of both refractive index and deformation.

Dimethyl and diphenyl-functionalized siloxanes were coated in Fabry-Perot cavities and used for VOC sensing. These devices were shown to operate on a swelling principle, as demonstrated by the positive resonance wavelength shifts that were observed regardless of the relationship between the refractive index of the film and the analyte. Swelling measurements were shown to produce similar responses for both films tested, with sensitivities of 0.0152 ± 0.0007 nm/ppm (PDMS, 3-wall), 0.0172 ± 0.0008 nm/ppm (PDMS, 2-wall) and 0.018 ± 0.001 nm/ppm (PDPS-PDMS, 3-wall) for m-xylene, and 0.0055 ± 0.0007 nm/ppm, (PDPS-PDMS, 3-wall) and 0.0038 ± 0.0002 nm/ppm (PDMS, 2-wall) for cyclohexane. These results suggested that, at least in the case of these two materials, the chemical functionality of the siloxane was not an important factor in sensing. Detection limits in the hundreds of parts per million for m-xylene (190 to 590 ppm) and hundreds to thousands of parts per million for cyclohexane (840 to 2100 ppm) were obtained. These are not practical detection limits for sensors of indoor air quality, given that American guidelines for threshold limit values for cyclohexane and m-xylene are 100 ppm. Nevertheless, the characterization of a new sensor platform using siloxanes advanced the study of possible VOC sensor designs.
A high refractive index siloxane containing naphthyl, phenyl, and methyl functionalities was synthesized and cast as a film for VOC sensing. The inclusion of both naphthyl and phenyl functional groups allowed the tuning of the refractive index of the polymer to 1.5846, compared to the refractive index of Sylgard 184 PDMS, which has a refractive index of 1.4225 at 632.8 nm. Thus, the uptake of both cyclohexane and \( m \)-xylene produced a decrease in the refractive index of the polymer. This expanded the range of siloxane polymers available for fingerprint VOC sensing because most siloxanes have lower RI values and having a material that responds differently could be an asset in such a sensor. However, the limits of detection for VOC sensing using this material were not sufficient for air quality regulations (4.0 \( \times 10^2 \) ppm for \( m \)-xylene and 4.0 \( \times 10^3 \) ppm for cyclohexane). On the other hand, the PMNS-PDPS-PDMS film was more sensitive to cyclohexane by a factor of approximately 10, compared to previous materials produced in our group. This can be attributed to the higher RI contrast between the film and vapour analyte in this case. An interesting effect was observed in which low concentrations of VOCs seemed to produce no response or a slight increase in RI of the film, while higher concentrations caused a decrease. This may be related to the two mechanisms of uptake, in which low levels of analyte uptake do not cause the material to swell and cause the RI to increase, while higher levels of analyte cause the material to swell and the RI to change based on the analyte refractive index. Regardless of the reason, this observation means that the material is not sensitive to low levels of solvent vapour, and this non-linear response would make improvements in the detection limit difficult.

Refractive index and deformation sensors using siloxanes were used for VOC sensing. However, it has been shown that these techniques, while versatile in the analytes that can be detected, do not easily reach useful detection limits. It is also not straightforward to identify analytes in a complex mixture based on these label free techniques. Thus, in further chapters, labeled antibodies and fluorescence will be explored as a method of detecting bacteria since this method allows both more sensitivity and specificity.
2.5 Acknowledgements

Many people contributed to the success of this project. Dr. Krista Plett performed much of the initial siloxane modification work that was built upon here. Her guidance in my early days as a researcher was also invaluable. Members of Dr. Yves-Alain Peter’s group at École Polytechnique, Montreal, QC were very helpful in the Fabry-Perot cavity experiments. Dr. Raphael St.-Gelais produced the chips used in these experiments and both he and Antoine Leblanc-Hotte were of great assistance in understanding their principles. John Saunders developed the data collection and analysis programs used here. Jingjing Zhou was also involved in the data collection and analysis.

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2.6 List of References


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Chapter 3

Chemical Surface Modification of Siloxane Polymers

3.1 Introduction

3.1.1 Polymer Surface Modification

It is often necessary to functionalize siloxane-based polymers to increase their utility in a variety of applications. Materials required for microfluidic channels often require modification after they are molded. The surfaces may be treated to allow better sealing between components, to increase hydrophilicity for improved flow, and to allow electrokinetic processes within polymer channels. In biological sciences, siloxanes can be modified to improve cell adhesion and encourage tissue growth using the polymer as a scaffold. Meanwhile, other groups seek to passivate siloxane devices to avoid biomolecule and non-polar molecule adsorption, as well as biofilm formation. Alternatively, surface modification may be used to promote the attachment of biomolecules. This last application is particularly useful in the development of biosensors. The surfaces of many commercial polymers such as poly(dimethylsiloxane) (PDMS) do not possess the chemical functionality necessary to immobilize biomolecules for biosensor applications. Consequently, surface modification by liquid reagents, ionized gas methods, ultraviolet light, or other techniques is often the first step in biofunctionalization.

3.1.2 Polymer Surface Modification Techniques

Treatment of polymers such as PDMS by a variety of liquid reagents is possible. These so-called “wet chemical” methods are advantageous in that they do not require specialized equipment and can be used to treat three-dimensional structures, however non-specific reactions, modification instability, and the generation of hazardous waste can be problematic. Silanes represent a large category of wet
chemical surface modification reagents. These molecules are often used in combination with an initial treatment, such as plasma,\textsuperscript{14,15} corona,\textsuperscript{9} or oxidizing wet chemistry (H\textsubscript{2}O\textsubscript{2}),\textsuperscript{7} which renders the polymer material more reactive. Using a trifunctional silane reagent, such as 2-aminopropyltriethoxysilane, can provide three anchor points to the surface, while leaving an amine group available for biomolecule linkage.\textsuperscript{16,17} Similar modifications can also be accomplished with amine\textsuperscript{15} and thiol-containing silanes\textsuperscript{17} possessing a single anchor functional group. While post-curing modification is commonly used, silanes can also be incorporated into siloxane polymers during the curing process, which can render PDMS more hydrophilic.\textsuperscript{5} A siloxane-specific surface modification technique is siloxane equilibration, which is catalyzed by the presence of acid or base and allows these elastomers to self-heal after damage or stress.\textsuperscript{18,19} The primary mechanism of self-healing is believed to be chain exchange, wherein the backbone of the siloxane chain is broken and reformed, as shown in Figure 3-1.\textsuperscript{18,19}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{The siloxane chain exchange mechanism believed to be responsible for the self-healing phenomenon observed by Zheng and McCarthy\textsuperscript{19} and Osthoff \textit{et al.}\textsuperscript{18} The numbers next to the silicon atoms are intended to identify individual atoms in the process. Wavy bonds indicate continuing polymer chains. Figure adapted from Zheng and McCarthy.\textsuperscript{19}}
\end{figure}

This phenomenon was shown to occur in siloxane materials that contained acid or base catalysts, but not in materials that did not contain these catalysts.\textsuperscript{18} While the method was originally demonstrated to heal the material, Sheardown’s group has recently found that the method can also be used to introduce functionalized siloxane oligomers to a previously cured PDMS material in the presence of acid.\textsuperscript{20-22} This method can be particularly versatile if silicon hydride functionality is introduced, since a variety of
molecules with terminal alkene groups could later be introduced via a hydrosilylation reaction.\textsuperscript{20} Other less common techniques have been used to modify PDMS by wet chemical means. Boiling water, for example, has been used to impart hydroxyl functionalities through unreacted silicon hydride sites on cured PDMS.\textsuperscript{4} Non-covalent modification, such as charged surfactants, electrolyte multilayers, phospholipid bilayers, and proteins can also be used to adjust the properties of PDMS surfaces.\textsuperscript{9} Overall, these techniques are convenient when the functional group required can be introduced in a specific manner and the scale is small enough that the reagent waste is not a major drawback.

Plasma is a very common method of modifying the surfaces of a wide variety of polymers. The goals of plasma modification include sealing PDMS channels for microfluidic applications,\textsuperscript{1} introducing reactive sites for further graft functionalization,\textsuperscript{10,17} producing hydrophilic microfluidic channels for effective aqueous solution flow and reduced biofouling,\textsuperscript{3} and improving cell adhesion.\textsuperscript{5} This technique uses a high vacuum chamber to produce a mixture of electrons and ions at high energy\textsuperscript{9} and is believed to either introduce hydrophilic functional groups to the surface or form oxidation products.\textsuperscript{23} It preferentially affects the top nanometer of a material and is preferred when solvent waste generation and the degradation of materials are of concern.\textsuperscript{12} Various gas plasmas, including argon,\textsuperscript{17} oxygen,\textsuperscript{24} acrylic acid,\textsuperscript{10} allyl amine,\textsuperscript{6} and air\textsuperscript{15,25} have been used to modify PDMS and other polymeric materials, with the choice depending on the type of modification required. Despite its widespread use, traditional plasma generators are difficult to use due to the requirement of vacuum conditions.\textsuperscript{12} In addition, many variables affect the outcome of the modification, making repeatability poor.\textsuperscript{12} Plasma is not a preferable technique when specific functional groups are required, except in combination with further modification steps.

Other techniques for surface modification are less common. UV treatment of polymers can produce reactive sites that can later be functionalized.\textsuperscript{12} UV graft polymerization has also been used to produce hydrophilic surfaces on PDMS.\textsuperscript{26} Chemical vapour deposition involves the transformation of gas
molecules to a solid in the form of a thin film or powder.\(^9\) Fluorosilanes, for example, have been incorporated into the surface of PDMS by vapour phase deposition to reduce the adsorption of biomolecules.\(^8\) These examples illustrate the variety of modification methods available. The choice depends upon the facilities available in the laboratory, the type of modification required, the polymer in question, and cost considerations.

A major challenge with surface modification of PDMS is its tendency to reorient after treatment, a process known as hydrophobic recovery.\(^27\) This is driven by the disparate surface free energies of the polymer surface and the surrounding atmosphere\(^28\) and this has been particularly well-studied for plasma-treated PDMS.\(^23,24,27,28\) The proposed mechanisms of this process include silanol reorientation into the bulk material, condensation of surface silanol groups, loss of volatile species, and changes in surface roughness, but the main cause is thought to be migration of unmodified siloxane chains to the surface from the bulk.\(^9\) At low degrees of oxidation, it is proposed that diffusion of polar groups to the bulk dominates.\(^29\) The hydrophobic recovery effect may be more pronounced in plasma treatment than other surface modification methods. For example, Seguin et al.\(^{17}\) monitored the stability of silanized PDMS over multiple weeks and found that, although the material recovered some hydrophobicity, its original surface characteristics were never regained. Similarly, Mikhail et al.\(^{20}\) found that surfaces modified by siloxane equilibration were stable for months. Nevertheless, the time interval between surface modification and characterization must always be considered.

### 3.1.3 Polymer Surface Modification for Biomolecule Attachment

Biomolecules can be attached to polymer surfaces by a variety of techniques, including physical adsorption, affinity, and covalent bonding.\(^12\) While adsorption is a convenient and straightforward process, covalent immobilization provides a more stable bond to the surface and may increase the bioactive lifetime of the material.\(^12\) This is particularly important if the material is subjected to flow or
extended periods in solution, which could cause leaching of adsorbed proteins. As a result, it is useful to explore more robust attachment methods. However, to carry out covalent immobilization, reactive functional groups on the polymer surface are required and these are not readily present in polymers including PDMS. Consequently, it is necessary to incorporate functionality that can lead to covalent bonding onto the PDMS through the methods presented above. It is important to consider the available functional groups on the biomolecule of interest when identifying the coupling chemistry to use. Given that biomolecules are usually linked through their amine, thiol, and carboxylic acid groups, the surface functional groups that can be used for biomolecule attachment include many complementary groups, such as carboxylic acid, succinimide (activated ester), epoxy, aldehyde, maleimide, pyridyl disulfide, vinyl sulfone, and amine. There are many reagents that can mediate these linkage processes by activating one of the two functional groups involved. Thus, chemical surface modification techniques allow access to the variety of covalent biomolecule attachment methods available.

### 3.1.4 Analysis of Polymer Surface Modification

Once the modification of a given polymer has been completed, it is necessary to verify that the expected change in surface properties has taken place. Many methods are available for such analysis, depending upon the type and quality of information required. Before analyzing a surface, it is necessary to define what constitutes the surface region of a material. However, every surface characterization technique has a different sampling depth, and so the region designated as the surface changes depending on the measurement employed. These depths range from the micrometer range, in attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), to the nanometer range in X-ray photoelectron spectroscopy (XPS) and static secondary ion mass spectrometry (SSIMS). All the available techniques have varying strengths and weaknesses. For example, quantitative analysis is most attainable using XPS. On the other hand, contact angle measurements and SSIMS provide the most
surface sensitive analysis of chemical change.\textsuperscript{32} Ideally, a range of techniques used in combination can provide the most complete picture of a treated surface.

3.1.4.1 Dye-Based Surface Analysis Methods

Chromogenic and fluorogenic dyes that complex with specific functional groups may be used to analyze a modified polymer surface.\textsuperscript{12} Quantitation can be carried out in one of three ways: i) by measuring the decrease in fluorescence or absorbance of the dye solution after exposure to the polymer, ii) by removing a complexed dye by ion exchange and measuring the spectrum of the resulting solution or iii) by measuring the optical label signal directly on the treated surface.\textsuperscript{33} Various dyes have been used to detect amines\textsuperscript{17} and carboxylic acids.\textsuperscript{25,33,34} For example, a UV light absorbing compound called $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide (EDAC) bonds to carboxylic acid groups. As a result, the production of these functional sites on a polymer surface can be followed by monitoring the decrease in absorbance of an EDAC.HCl solution upon exposure to a modified polymer.\textsuperscript{25} A major difficulty with these methods is that some molecules may be non-specifically adsorbed on the polymer surface, giving falsely high measurements of functional group concentrations. Provided this difficulty can be averted, dyes may be useful in the analysis of the formation of specific functional groups.

3.1.4.2 Contact Angle Analysis

Water contact angle measurements, which illustrate the degree of hydrophilicity on a surface, are advantageous due to their rapidity and are attractive as a result, but are unable to distinguish between different types of polar functional groups.\textsuperscript{12} Nevertheless, they have been used to observe the changes in a polymer’s hydrophilicity after a wide range of surface treatments.\textsuperscript{7,8,23,25,26} This technique determines the wetting characteristics of a solid material and is related to the three interfacial tensions involved in a droplet’s shape on a solid surface.\textsuperscript{35} Interfacial tensions are the reversible work required to create a unit of
interfacial area and thus represent the excess surface free energy.\textsuperscript{36} In the ideal case, these interfacial tensions are related to the contact angle by Young’s equation (Equation 3-1).

\begin{equation}
\cos\theta = \frac{\gamma_{sv} - \gamma_{sl}}{\gamma_{lv}}
\end{equation}

$\gamma_{sv}$, $\gamma_{sl}$, and $\gamma_{lv}$ represent the interfacial tensions at the solid-vapour, solid-liquid, and liquid-vapour interfaces respectively, and $\theta$ is Young’s angle.\textsuperscript{35,37} This angle is the intersection between the liquid-solid and liquid-vapour interfaces (Figure 3-2).

\textbf{Figure 3-2:} A water droplet on a PDMS surface. The blue line represents the liquid-solid interface while the red line represents the liquid-vapour interface.

A measurable angle is produced when the liquid surface tension is greater than the solid surface tension, while total wetting of the surface occurs in the opposite case.\textsuperscript{32,35} Thus, contact angle is an indirect measurement of the solid surface tension,\textsuperscript{35} which can be related to chemical changes at the polymer surface. PDMS exhibits a low surface tension compared to other polymers, at 19.9 dyn/cm,\textsuperscript{32} meaning that relatively large contact angles are obtained when water is used as the test liquid, since the surface tension of water $7.199 \times 10^3$ dyn/cm at 25°C.\textsuperscript{38} When surface modifications increase the hydrophilicity of PDMS, the contact angle between water and the polymer should decrease.

One common method of determining a contact angle is the sessile drop method, in which a liquid droplet is placed on a surface and the contact angle is measured at equilibrium.\textsuperscript{39} This is frequently
accomplished using a goniometer, which consists of a stage to hold the sample, a pipette to deposit liquid, a light source, and a camera or telescope for visualization.\textsuperscript{35} Although Young’s angle is the equilibrium contact angle, most real surfaces allow for a number of metastable states, meaning that Young’s angle is not obtained in practice by the sessile drop method.\textsuperscript{35} Dynamic measurements of droplets can provide more information about the surface properties. When the drop volume is slowly increased, its contact angle will increase as well until a maximum value is reached. The droplet will then expand its diameter to accommodate the increased volume, while maintaining a constant contact angle, and the angle measured during this process is the advancing contact angle.\textsuperscript{39} Conversely, removing liquid from a droplet decreases the contact angle until a minimum is reached, then the droplet contracts and the receding contact angle can be measured.\textsuperscript{39} The difference between these two values is the hysteresis and is a measure of the surface heterogeneity. Specifically, the advancing angle is more closely related to hydrophobic, low surface energy, regions, while the receding angle is determined by hydrophilic, high surface energy, regions.\textsuperscript{36,39}

Although contact angle measurements are highly surface sensitive, there are many sources of error. The homogeneity and profile of a surface (smooth vs. rough) can affect the consistency of measured values, for example.\textsuperscript{40} In addition, impurities in the test liquid can affect the interfacial tension, which can in turn affect the measured contact angles.\textsuperscript{40} Any processes involving swelling of the surface by the test liquid can also affect the observed angle.\textsuperscript{40} There is also a dependence of the angle on drop size, meaning that the volume of the drop used must be kept constant throughout the experiment.\textsuperscript{41,42} All of these factors mean that there is an inherent error of $\pm 2^\circ$ in the measurements.\textsuperscript{35} However, the speed and convenience of this analysis method, combined with its surface sensitivity, still make it an attractive technique.
3.1.4.3 Spectroscopic Techniques for Surface Analysis

X-ray photoelectron spectroscopy (XPS) is often used to observe changes in the atomic composition of the top few layers of a surface by directing X-ray photons at a material, causing the ejection of photoelectrons. The atom’s identity and oxidation state are determined from the binding energy of the emitted photoelectrons. Many researchers have taken advantage of this method to detect surface changes in polymers. While it is possible to use curve-fitting algorithms to identify functional groups such as carboxylic acids from the carbon 1s electron peak, this is not always reliable because all of the oxidation states for a single atom overlap into a complex peak. To avoid this, XPS information can be enhanced by using tags for specific functional groups. However, the use of tags complicates the analysis by introducing a step that depends on a consistent degree of reaction between the functional group and the tag. Other X-ray techniques have also been used in surface analysis, including near edge X-ray absorption fine structure and specular X-ray reflectivity. While X-ray techniques can provide detailed information, they are limited by low sample throughput and high cost.

Fourier transform infrared spectroscopy allows the interrogation of substances for functional groups whose chemical bonds absorb infrared radiation at certain wavenumbers. Attenuated total reflectance FTIR passes the infrared radiation through a crystal before it is sent to the sample, producing an evanescent wave, which increases surface sensitivity. This method has been used to characterize PDMS, polyethylene terephthalate, and polypropylene surfaces. The penetration depth ($d_p$) of ATR-FTIR beam is dependent upon the wavelength of light ($\lambda$), the refractive index of the waveguide ($n_1$) and surroundings ($n_2$), and the angle of incidence of the light beam ($\theta$) (Equation 3-2).

\[
\text{Equation 3-2} \quad d_p = \frac{\lambda}{2\pi \sqrt{(n_1^2 \sin^2 \theta - n_2^2)}}
\]
Given that typical ATR-FTIR penetration depths are around 1 µm,\textsuperscript{32} this method is not as surface sensitive as other techniques, but it has the advantage of not requiring an evacuated chamber.\textsuperscript{12} These measurements can complement more surface sensitive techniques by providing detailed analysis of a range of functional groups simultaneously, a feature that is not available with dye-based methods or XPS tags.

3.1.4.4 Other Surface Analysis Methods

Other methods of surface analysis are available, though used less frequently. Using electroosmotic force measurements, Zeta potential may be used to characterize modified polymer surfaces destined for microcapillary electrophoresis.\textsuperscript{3} Mass spectrometry, especially time of flight secondary ion mass spectrometry is capable of analyzing ionizable groups present in the top nanometre of a surface, but matrix effects on ionization prevent quantitative analysis of surface functional groups.\textsuperscript{12,13} Atomic force microscopy can also be employed, primarily to study the changes in surface morphology that may occur during modification procedures.\textsuperscript{8,12,45,47} Scanning electron microscopy can also be used for elemental analysis of a modified surface.\textsuperscript{12} Ellipsometry can help evaluate the thickness of a layer deposited on a substrate material.\textsuperscript{8} Adhesion measurements can more directly quantify the thermodynamic and physical properties of a material, including surface free energy and elastic modulus.\textsuperscript{29} The variety of methods available means that multiple techniques can be combined to produce more detailed information about a surface.

3.1.5 Research Objectives

In order to facilitate the covalent attachment of proteins and antibodies, the surfaces of siloxane polymers require modification. A common scheme for the immobilization involves the incorporation of surface carboxyl groups, which can be combined with primary amines on the protein (e.g. lysine side
chains) to form amide linkages. These linkages are stable over a wide range of pH values and utilize the lysine side chains, which are often found on the exterior of proteins. In order to achieve this, carboxyl functionality must be incorporated into the siloxane surface. Two methods of modifying siloxanes to incorporate carboxylic acids were investigated for this project. In the first, a polymer containing ester groups was synthesized and cured, then the surface esters were hydrolyzed, leaving carboxylic acids on the material (Figure 3-3).

![Diagram](image)

**Figure 3-3:** Process for curing an ester functionalized polymer, then removing the ester group by hydrolysis.

In the second, PDMS was cured into the required shape, a thin siloxane polymer layer containing ester functionality was incorporated into the siloxane by chain exchange, and then the surface ester groups were hydrolyzed to leave carboxylic acids (Figure 3-4 and Figure 3-5). These methods were compared in terms of ease of fabrication, ability to perform solid phase microextraction (SPME), resilience to acid treatment, and optical properties. SPME and optical properties were examined by observing the uptake of 1-hydroxypyrene from solution into films made by each method. Resilience to acid hydrolysis was assessed visually by loss of uniformity and pitting in the surface. Since carboxylic acid functional groups were the ultimate goal, the hydrolysis method for the methyl ester-functionalized surface was also evaluated. This was done by methods including labels and contact angles. The ultimate goal was to produce a siloxane polymer with surface carboxyl functionality, to be used for covalent protein immobilization. This polymer
must retain the SPME properties of unmodified Sylgard and show good uptake of 1-hydroxypyrene in order to be of use.

**Figure 3-4:** The process of incorporating an ester-functionalized siloxane oligomer into cured Sylgard PDMS by chain exchange. The wavy bonds indicate that polymer chains continue beyond the scope of the diagram. For simplicity, only one siloxane chain is shown.

**Figure 3-5:** Acid-catalyzed hydrolysis using heat causes the methyl esters to be removed, leaving a carboxyl-functionalized polymer near the siloxane surface. The wavy bonds indicate that polymer chains continue beyond the scope of the diagram.
3.2 Materials and Methods

3.2.1 Poly((4-carbomethoxy)butylmethyl-co-dimethyl)siloxane (PCBMS-PDMS) Synthesis

3.2.1.1 Synthesis of methyl-4-pentenoate

The production of the ester-functionalized siloxane first required the synthesis of methyl-4-pentenoate (Figure 3-6).

\[
\text{CH}_3\text{I} \quad \text{K}_2\text{CO}_3 \quad \text{acetone} \quad \text{reflux} \quad \text{OH} \quad \text{O} \quad \text{CH}_3
\]

**Figure 3-6:** Synthesis of methyl-4-pentenoate from 4-pentenoic acid was accomplished by esterification in the presence of methyl iodide and potassium carbonate.

This procedure was adapted from Esteban et al. First, 42.06 g of potassium carbonate (Sigma-Aldrich, Oakville, ON) were suspended in 400 mL of reagent grade acetone in a 1 L round bottom flask. 25.0 g (0.250 mol) of 4-pentenoic acid (Sigma-Aldrich) were added, a condenser was placed on the flask, and the mixture was stirred for 20 minutes at room temperature. After this time had elapsed, the reaction mixture was cooled to 0°C by immersion in an ice bath for 20 minutes. Once cooled, 15.2 mL (0.244 mol) of iodomethane (Sigma-Aldrich) were added to the mixture. The reaction was then heated in an oil bath to reflux at 78°C and was reacted at this temperature for 24 hours. After cooling to room temperature for 2 hours, the mixture was vacuum filtered through a 1 cm path of Celite 545 on a glass frit. Following filtration, the crude mixture was concentrated on a rotary evaporator. Further purification was performed by distillation.
3.2.1.2 Poly(dimethyl-co-methylhydrogen)siloxane (PDMS-PMS) Synthesis

A polymer containing 50% dimethyl and 50% methylhydrogen functionality was produced by methanolysis (see Chapter 2), as a hydrosilylation precursor. Acetic acid (0.020 mol) and methanol (0.023 mol) were mixed in a 20 mL scintillation vial. After stirring began, 0.010 mol of dichlorodimethylsilane (Sigma-Aldrich) and 0.010 mol of dichloromethylsilane (Sigma-Aldrich) were added dropwise by syringe in two alternating portions of each silane over approximately 8 minutes. The reaction mixture was stirred for 60 minutes to facilitate further reaction. After this time, a nitrogen gas stream was used to evaporate volatiles from the mixture, leaving the PDMS-PMS oligomer in the vial.

3.2.1.3 Synthesis of PCBMS-PDMS by Hydrosilylation

PDMS-PMS was combined with methyl-4-pentenoate to produce an ester-functionalized siloxane (Figure 3-7).

Figure 3-7: Synthesis of PCBMS-PDMS from PDMS-PMS and methyl-4-pentenoate by hydrosilylation. In this case, m and n = 0.5x.

The PDMS-PMS used in this procedure was produced on the same day to prevent unwanted cross-linking due to the reactive Si-H functionalities, which can be converted to hydroxyl groups in the presence of water, allowing cross-linking. A mass of 1.00 g of PDMS-PMS, containing 7.45 mmol of Si-H sites (1
Si-H group for every 3 Si-CH$_3$ groups), was mixed with 8.33 mL of toluene, 0.933 mL of methyl-4-pentenoate (7.54 mmol), and 8.3 mg of Pt(SEt)$_2$Cl$_2$ (1.9 x 10$^{-5}$ mol) (Sigma-Aldrich) as a catalyst, in a round bottom flask. A condenser was fitted and the reaction mixture was heated to a 75°C bath temperature while stirring. Once at the required temperature, the reaction was allowed to continue for 4.5 hours. After this, the solution was cooled to room temperature and moved to a 20 mL scintillation vial. A 5 mL volume of dichloromethane was used to rinse the round bottom flask to avoid losing polymer product and this was added to the vial. The mixture was then placed under a nitrogen gas stream to evaporate the volatile components for approximately 4 hours or until the mass did not appear to change.

### 3.2.2 PCBMS-PDMS Film Casting

#### 3.2.2.1 Planar Film Casting

PCBMS-PDMS bulk films were prepared by first dissolving 100 mg (equivalent to 8.05 x 10$^{-4}$ moles of monomer, based on an average monomer molar mass of 124.215 g/mol) of the prepared oligomer liquid in 4 mL of solvent, consisting of a 1:1 (v:v) hexanes/CH$_2$Cl$_2$ mixture. Next, a solution of titanium tetraisopropoxide (Sigma-Aldrich) was prepared by dissolving the liquid Ti(O-iPr)$_4$ at 10% (v/v) in hexanes. This cross-linking catalyst was added to the polymer mixture to a final concentration of 2 mole percent. This percentage was based on the moles of Ti(O-iPr)$_4$ relative to the estimated moles of PCBMS-PDMS monomer in the mixture (i.e. 1.6 x 10$^{-5}$ moles Ti(O-iPr)$_4$ for 8.05 x 10$^{-4}$ moles monomer). This mixture was then solvent cast onto 1 cm x 1 cm squares cut from glass microscope slides. The volume deposited on each slide was 200 µL. The polymers were then cured by placing them in an oven at 115°C for 3 minutes.
3.2.2.2 Cuvette Film Casting

PCBMS-PDMS did not appear to be optically clear when cast on glass slides. Due to the cost and complication of scaling up the production of this polymer, while performing purification to improve its optical clarity, it was more practical to cast the film as a layer on top of previously cured Sylgard 186 PDMS for fluorescent measurements in the polymer. First, Sylgard 186 (Dow Corning, purchased from Paisley Products, Toronto, ON) was made at a 9:1 ratio of base to curing agent and diluted in hexanes at a 1:1 (m:v) ratio. Then, 60 µL of this solution were dispensed into the bottom of a PMMA Semi-Micro Cuvette (BrandTech, Essex, CT) using a 1 mL syringe with a 21G needle attached. Once this polymer had cured at room temperature (36 hours), a film of PCBMS-PDMS was cast on top of the PDMS layer in layers. The sequential addition of reagents to the cuvette prevented polymer curing prior to film deposition. Two solutions were prepared for the film casting. The first (A) was the oligomer solution and consisted of 0.2 g PCBMS-PDMS in 0.8 mL of dichloromethane (20% m/v). The second (B) was the cross-linker solution and contained 10 µL of Ti(O-iPr)$_4$ dissolved in 290 µL of solvent (20% (v/v) dichloromethane in hexanes). Once the solutions were made, the polymer film was cast in layers. In each layer, the following materials were added: 20 µL of the polymer solution (A), followed by 4.74 µL of the crosslinker solution (B). Thus, each layer contained 4 mg of oligomer (equivalent to 3.2 x 10$^{-5}$ moles of monomer) and 5.3 x 10$^{-7}$ moles of Ti(O-iPr)$_4$. Two such layers were deposited in each cuvette, providing a total of 8 mg of PCBMS-PDMS to each cuvette with 1.6 mole percent Ti(O-iPr)$_4$ added. The modified surfaces were cured overnight at room temperature. Partial coverage of PCBMS-PDMS on Sylgard was also attempted to see if this would improve the uptake properties of the material. This was accomplished by tilting the cuvettes at a slight angle (30° or 60°) during casting and curing of PCBMS-PDMS. An example of the angle-cured cuvettes can be seen in Figure 3-8.
3.2.3 Acid Hydrolysis of PCBMS-PDMS and Analysis by EDAC Absorbance

Each PCBMS-PDMS film (cast on glass slides, see Section 3.2.2.1) was placed in a 20 mL scintillation vial and immersed in a 2.5 mL solution containing the appropriate acid type and concentration. Sulfuric acid was used at concentrations ranging from 1 to 5 M. The vials were placed in a constant temperature water bath to maintain the required temperature, between room temperature and 60°C. Control films that were not subjected to acid hydrolysis were immersed in a 2.5 mL solution of distilled water in a 20 mL vial and exposed to the same temperature conditions as the acid-treated films. After this treatment was completed, all of the films were rinsed by swirling in distilled water for 10 seconds and then gently dried by blotting with a tissue.

EDAC.HCl was used to monitor the production of carboxylic acid groups on a polymer surface by the decrease in absorbance of an EDAC.HCl solution upon exposure to a modified polymer. This method was used to optimize the acid hydrolysis step to determine the optimal conditions for the production of surface carboxyl groups. Each of the film-coated slides was then immersed in 2.5 mL of a 10 mM solution of EDAC.HCl (Sigma-Aldrich) in distilled water and allowed to soak for two hours. After this time, each slide was removed with tweezers and 2.5 mL of distilled water were pipetted over
the surface to remove non-specifically bound EDAC.HCl. This rinse water was allowed to drain into the 2.5 mL EDAC.HCl solution, giving a final volume of 5 mL. Each of these solutions was then diluted 50-fold in distilled water in order to give solutions with absorbance levels in the linear range of the spectrometer.

The UV absorbance spectra for all of these solutions were measured using a Perkin Elmer XLS+ UV/Vis (Woodbridge, ON) spectrophotometer. Spectra were recorded from 200 to 500 nm in quartz cuvettes and the absorbance readings at 211 nm were related to the EDAC.HCl solution concentration. These concentrations were calculated using values produced from a calibration curve of standard EDAC.HCl concentrations in distilled water.

3.2.4 Uptake of 1-Hydroxypyrene in Modified Polymers

3.2.4.1 Single Chamber Custom Spectrometer

Since the ultimate goal of this project involved detection of fluorescent probes within a polymer at the bottom of a cuvette, it was necessary to design a system capable of performing these measurements. Cuvettes were held in a custom-built sample chamber, (Eric Marcotte, Endetec, Kingston, ON) which was attached to a probe that held the excitation source and emission collection fibre. The emission collection fibre was placed along the normal to the cuvette while the excitation source was placed at 67.5° to the normal (Figure 3-9). A plastic spacer was used to raise the cuvette to align the polymer film with the optical elements. The detector was a USB4000 computer-controlled spectrometer manufactured by Ocean Optics (Dunedin, FL) containing a Toshiba TCD1304AP linear CCD array detector. The light source was a 365 nm LED (Nichia, Tokushima, JP). The system was controlled by Ocean Optics OOIBase32 software on a laptop through a USB connection (Figure 3-10).
**Figure 3-9:** Top-down view of polymer-containing cuvette in custom-built cuvette chamber. The 365 nm excitation LED enters the chamber at a 67.5° angle to the normal and excites fluorophores within the polymer. The light produced is focused by a lens and transmitted to the spectrometer by a collection fibre, which is placed along the normal to the cuvette.

**Figure 3-10:** Optimal set-up for measurements of fluorescence within the polymer. The cuvette, with polymer at the bottom, is raised by a “spacer” in order to align the light source and collection fibre with the polymer. The spacer beneath the cuvette could be removed for solution measurements.
3.2.4.2 1-Hydroxypyrene Uptake

In order to assess the ability of modified polymers to perform SPME, the uptake of 1-hydroxypyrene into modified films was evaluated. To do this, the film of interest was cured at the bottom of a plastic disposable semi-micro cuvette. The cuvette was placed in the custom spectrometer set-up (Figure 3-10), aligned so that the polymer layer was probed by the fluorescence measurement. A 4 mM solution of 1-hydroxypyrene (PyrOH) (prepared in our lab by Dr. Ray Bowers) in ethanol was prepared first. Before each uptake experiment, this solution was diluted with water to produce a 40 µM aqueous solution, 1 mL of which was added to the test cuvette. The uptake was monitored based on the appearance of fluorescence signal at 389 nm over time using an integration time of 1500 msec.

3.2.5 Sylgard 186 Film Preparation for Chain Exchange Experiments

3.2.5.1 Sylgard Films on Planar Substrates

Glass microscope slides were cut into 1 cm x 1 cm sections using a tungsten carbide glass cutting tool (Sigma-Aldrich). Each piece of glass was cleaned using isopropanol and allowed to dry. Sylgard 186 PDMS was prepared at a 9:1 base to curing agent mass ratio and diluted with hexanes at a 1:4 (mass of polymer to volume of solvent) ratio. Using this mixture, 200 µL of polymer solution was deposited on each glass square and allowed to cure overnight in air at room temperature.

3.2.5.2 Sylgard Films in Semi-Micro Cuvettes

Semi-micro cuvettes made of PMMA and UV type materials were purchased from BrandTech. Sylgard 186 PDMS was prepared at a 9:1 base to curing agent mass ratio and diluted with hexanes at a 2:1 (mass of polymer to volume of solvent) ratio. The mixture was taken up into a 1 mL plastic syringe, the end was cleaned and a 21G needle was attached. 60 µL of polymer solution was deposited into the bottom of each semi-micro cuvette and the cuvette was tapped to distribute the material across the bottom surface,
ensuring contact with both optical measurement windows. The films were cured for at least 36 hours at room temperature prior to use.

3.2.6 Chain Exchange Modification of Sylgard 186

3.2.6.1 PDMS-PMS Synthesis

PDMS-PMS was synthesized by modifying the method described in Section 3.2.1.2. First, 0.040 mol of acetic acid and 0.046 mol of methanol were mixed in a 20 mL scintillation vial. After stirring began, 0.020 mol of dichlorodimethylsilane and 0.020 mol of dichloromethylsilane were added dropwise by syringe in two alternating portions of each silane over approximately 12 minutes. The reaction mixture was then stirred for 8 minutes to facilitate further reaction. This shorter reaction time was employed to produce a lower molecular weight oligomer that would be more soluble in isopropanol. After this time, a nitrogen gas stream was used to evaporate volatiles from the mixture, leaving the PDMS-PMS oligomer in the vial.

3.2.6.2 PCBMS-PDMS Synthesis

This oligomer was prepared as described in Section 3.2.1.3. After evaporation of the hydrosilylation solvent, the oligomer liquid was weighed and immediately dissolved at 37.5% (m/v) in isopropanol (*i.e.* 375 mg PCBMS-PDMS in 625 µL isopropanol).

3.2.6.3 Poly(methyloctyl-co-dimethyl)siloxane (PMOS-PDMS) Synthesis

A 1.00 g mass of PDMS-PMS oligomer (7.45 mmol of Si-H sites) was dissolved in 8.33 mL of toluene in a 50 mL round bottom flask. Next, 1-octene (7.39 mmol) (Sigma-Aldrich) and 8.3 mg (1.9 x 10⁻⁵ mol) of Pt(SE₂)₂Cl₂ were added. The mixture was heated in the round bottom flask, equipped with a stir bar and an attached condenser, to a temperature of 75°C. The reaction was allowed to proceed at this
temperature for 4.5 hours. After this time, the reaction mixture was cooled and diluted approximately 1:1 with dichloromethane. Then, the volatile solvents were evaporated using a stream of nitrogen gas, leaving behind a viscous, dark brown liquid oligomer, PMOS-PDMS. After evaporation, the liquid oligomer was weighed and immediately dissolved at 37.5% (m/v) in solvent solution. The solvent in this case was 1:9 dichloromethane to isopropanol by volume since PMOS-PDMS was not readily soluble in isopropanol alone.

3.2.6.4 Chain Exchange Modification of Prepared Siloxanes on Cured Sylgard 186

The chain exchange method\textsuperscript{18,19} was adapted from the work of Chen \textit{et al.}\textsuperscript{21,22} This procedure was modified in order to incorporate ester-functionalized siloxane oligomers into a cured PDMS material. The oligomers were synthesized as described above and the same conditions were used to treat both planar films and cuvette films. The oligomers used included PDMS-PMS (see Section 3.2.6.1), PCBMS-PDMS (see Section 3.2.6.2), PMOS-PDMS (see Section 3.2.6.3), and poly(methylhydrosiloxane) (PMHS) (Sigma-Aldrich). The prepared polymer solutions (1 mL, containing 37.5% polymer, 62.5% solvent) were each mixed with 40 µL of concentrated hydrochloric acid. A 75 µL aliquot of the appropriate polymer-acid solution was then added to the PDMS film (Section 3.2.5) to be modified. This solution was removed and replaced once per hour for a total of 3 hours of treatment. This method minimized material use while allowing greater exposure of the reactants to the surface. After the treatments were completed, the films were rinsed with dichloromethane followed by isopropanol (or isopropanol alone in the case of the plastic cuvettes) and allowed to dry for 15 minutes in air.

3.2.6.5 Surface Hydrosilylation for Sylgard 186 Modified with PDMS-PMS

For surface hydrosilylation, Sylgard films previously modified by chain exchange with PMHS or PDMS-PMS were used. A 100 to 400 µL solution containing 10 to 40% (v/v) methyl-4-pentenoate and
1% Karstedt’s catalyst (platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex solution, Sigma-Aldrich) solution by volume in isopropanol was added to each film. This mixture was allowed to react on the film surface overnight at room temperature.

3.2.7 Contact Angle Analysis of Chain Exchange and Acid-Catalyzed Hydrolysis

The surface modification of the films by chain exchange and acid-catalyzed hydrolysis was analyzed using water contact angle measurements, which indicate the hydrophilicity of the surfaces. As ester functional groups are incorporated into the PDMS surface and then converted to carboxylic acid groups (Figure 3-4 and Figure 3-5), the material should become more hydrophilic and the water contact angle should decrease. These measurements were carried out using a Krüss DSA 100 Drop Shape Analyzer (Krüss USA, Matthews, NC). The camera tilt angle was 2° and the Tangent 1 analysis method (profile of whole drop shape modeled based on a conic section equation) was used to determine the contact angles within the supplied software. Multiple droplet deposition methods were attempted, as described below. After analysis of each drop, the film was shaken to remove larger water droplets and smaller drops were carefully removed using a lens cleaning tissue (Thorlabs, Newton, NJ).

3.2.7.1 Optimal Contact Angle Analysis Method

In preliminary experiments, three methods of obtaining contact angle measurements were compared: 1) a 5 µL drop allowed to equilibrate for 5 minutes before measurement, 2) a 5 µL drop allowed to equilibrate for 30 seconds before measurement, and 3) an advancing and receding drop measurement. Group 3 was completed by depositing a 2 µL water droplet on the surface, leaving the needle in the drop. Then, the droplet was increased in volume at a rate of 15.19 µL/min until a 40 µL drop was obtained. Then, the process was reversed and volume was removed from the drop at 15.19 µL/min until the drop detached from the needle or a contact angle could not be calculated by the software. During
this process, the software was set to measure contact angles using the Tangent 1 method every 0.5 seconds using a pre-determined drop baseline. Five Sylgard 186 slide-cured films were used for the measurements and, in the case of static droplets, three measurements were taken on each film. For advancing angles, one continuous set of measurements was completed on each film, with twenty data points (last 10 seconds of the advancing or receding process) averaged for each measurement. After obtaining an average contact angle for each film, the average and standard deviation for the five films analyzed by the same method was calculated and the methods were compared.

During optimization, it was noted that static water droplets on Sylgard 186 failed to reach an equilibrium contact angle value. To identify the source of this effect, Sylgard 186 and an UltraEverDry surface (Hazmasters, Ottawa, ON) prepared according to package directions, were compared. By placing the same size drop of water (5 µL) on both surfaces and measuring the change in contact angle over time, the effects of evaporation, which should be similar in both cases, could be separated from interactions between the water and the surface.

3.2.7.2 Optimization of Chain Exchange Modification by Contact Angle Measurements

To determine the optimal duration of the chain exchange step, Sylgard films on glass slides were treated for varying lengths of time to incorporate the ester functionalized oligomer (PCBMS-PDMS) and then hydrolyzed in acid to reveal the carboxyl functional groups. Before any treatment, all cured Sylgard 186 films were characterized by advancing contact angle (Method 3 above). Sylgard films were modified with PCBMS-PDMS to introduce ester functionality by chain exchange under different conditions: 1) 1-30 minute treatment with 100 µL solution, 2) 1-1 hour treatment with 100 µL solution, or 3) 3-1 hour treatments, each with 75 µL solution. The composition of the solution used was the same as in previous experiments, i.e. a 37.5% (m/v) oligomer solution in solvent was prepared and mixed at a 100:4 (v:v) ratio with concentrated hydrochloric acid. To control for other reactions that might create hydrophilicity on the
Sylgard surface, PDMS films were also modified with PMOS-PDMS to introduce alkyl functionality and treatment schemes 1 and 3 were used to modify these films as well. Following the chain exchange step, the films were rinsed with isopropanol and dichloromethane and dried at room temperature for 10 minutes. Then, all films were hydrolyzed in 2.5 mL of 5 M HCl at 35°C for 24 hours. The films were then rinsed in distilled water and dried for at least 30 minutes in air, using lens tissue to remove any large water droplets. Advancing contact angle measurements were completed again following this acid hydrolysis step.

For the advancing contact angle measurement, a robotic syringe was used to deposit a drop of 20 µL volume onto each sample to be analyzed and the needle was kept within the drop. After defining the baseline of the drop, the syringe was set to add water to the droplet at a rate of 15 µL/min for 60 seconds. The software was set to collect a contact angle measurement for the left and right sides of the drop image every 0.5 seconds. From this, an advancing contact angle was obtained by averaging the measurements during the last five seconds of this process, when the angle approached equilibrium.

3.2.7.3 Optimization of Acid-Catalyzed Hydrolysis by Contact Angle Measurements

Planar Sylgard films modified by the chain exchange procedure so that they contained either PMOS or PCBMS functionality were subjected to acid-catalyzed hydrolysis in a range of conditions. In all cases, 2.5 mL of aqueous acid solution were used. Treatments took place in hydrochloric acid at a range of concentrations and for a duration of 6 or 24 hours. All reactions took place in a constant temperature bath set to 37.5°C, which provided an internal bath temperature of 35°C. Advancing contact angle measurements were completed as described above (see Section 3.2.7.2) for all films before any treatment, and after acid hydrolysis was completed.
3.2.8 ATR-FTIR Analysis of Modified Sylgard Surfaces

Modified planar Sylgard films were analyzed using ATR-FTIR to determine the surface functional group content. The instruments used were a Bruker (Milton, ON) ALPHA Platinum ATR-FTIR containing a single reflection diamond and a ThermoFisher (Ottawa, ON) Nicolet 6700 equipped with SmartOrbit ATR accessory.

3.3 Results and Discussion

3.3.1 PCBMS-PDMS Synthesis

3.3.1.1 Methyl-4-pentenoate Synthesis

The esterification reaction was followed by TLC plate, with visualization using potassium permanganate. While the literature procedure suggested a reaction time of 5 hours, the TLC results suggested that more than 24 hours were required to complete the process. Purified yields of above 50% were obtained for methyl-4-pentenoate once this increase in reaction time was implemented. During the distillation, the fore-run was collected between 55°C and 120°C, while the product was collected between 120°C and 130°C, since the boiling point of methyl-4-pentenoate is 125-127°C. The purified material was a transparent oil with a slight yellow tint and a strong odour. $^1$H NMR was completed on the product to determine its content and purity (Figure 3-11).
Figure 3-11: $^1$H NMR of methyl-4-pentenoate following purification in CDCl$_3$ at 400 MHz. Analysis: δ 2.38 ppm (4H, m, CH$_2$=CH-CH$_2$-CH$_2$-), δ 3.66 ppm (3H, s, -O-CH$_3$), δ 5.00 ppm (1H, dd, $^3$J = 10 Hz, CH$_2$=CH-), δ 5.02 ppm (1H, dd, $^3$J = 16 Hz, CH$_2$=CH-), δ 5.75 ppm (1H, ddt, $^3$J = 18, 12, 4 Hz, CH$_3$=CH).

3.3.1.2 Hydrosilylation to Produce PCBMS-PDMS

During the hydrosilylation, a colour change of the reaction mixture from light yellow to dark brown was observed 30 minutes after heating began. In the literature, this is frequently attributed to the platinum catalyst and indicates that the reaction is proceeding.$^{49,50}$ The success of the hydrosilylation to produce PCBMS-PDMS was monitored by NMR. Since the Si-H functionality has an obvious $^1$H NMR peak at 4.7 ppm, the disappearance of this peak, along with the appearance of the expected methyl-4-pentanoate peaks can be associated with a successful linkage reaction. As seen in the NMR spectra, PDMS-PMS clearly contains the Si-H peak (Figure 3-12), while PCBMS-PDMS only displays a small signal in this region (Figure 3-13).
Figure 3-12: $^1$H NMR of PDMS-PMS before hydrosilylation in CDCl$_3$ at 400 MHz. Analysis: $\delta$ 0.12 ppm (9H, m, Si-CH$_3$), $\delta$ 4.72 ppm (1H, s, Si-H).

Figure 3-13: $^1$H NMR of PCBMS-PDMS following hydrosilylation in CDCl$_3$ at 400 MHz. Analysis: $\delta$ 0.08 ppm (9H, s, Si-CH$_3$), $\delta$ 0.52 ppm (2H, t, Si-CH$_3$), $\delta$ 1.38 ppm (2H, quint, Si-CH$_2$-CH$_2$), $\delta$ 1.65 ppm (2H, quint, Si-CH$_2$-CH$_2$-CH$_2$), $\delta$ 2.32 ppm (2H, t, Si-CH$_2$-CH$_2$-CH$_2$-CH$_2$), $\delta$ 3.66 ppm (3H, s, -O-CH$_3$).
In addition, the new peaks observed in the spectrum of PCBMS-PDMS can be assigned to the newly added side-chain. The relative integration of the PDMS-PMS spectrum showed that the actual ratio of Si-H sites to Si-CH₃ was not exactly 9:1, as would be expected from the mole ratio added in the PDMS-PMS reaction. This could be attributed to higher volatility of dichloromethylsilane or its instability in the presence of water. The integration values for the side chains make sense in this context. Although a 9:3 ratio for Si-CH₃ to O-CH₃ would be predicted, the lower incorporation of Si-H into the starting polymer would explain the 11:3 ratio seen here.

3.3.2 Acid Hydrolysis of PCBMS-PDMS and Analysis by EDAC Absorbance

The hydrolysis of the methyl ester functional groups on PCBMS-PDMS films was assessed by exposing the hydrolyzed films to a 10 mM EDAC solution according to the method of Brown et al. EDAC absorbs ultraviolet light around 211 nm and should bond to carboxylic acids, decreasing the absorbance of the EDAC solution following exposure to hydrolyzed polymers. This absorbance decrease could be correlated to the degree of carboxylic acid group formation. Initial experiments showed that the use of the hydrochloride salt, EDAC.HCl, provided lower background signals than the non-ionized EDAC, so EDAC.HCl was used in all experiments reported here.

Various hydrolysis conditions were used on the films in order to determine the best combination of parameters. The temperature, acid type, concentration, and duration were all investigated. By immersing the hydrolyzed films, as well as untreated PCBMS-PDMS films, in EDAC.HCl, removing and washing the films, and measuring the difference in dye concentration between untreated control and hydrolyzed films, the level of carboxylic acid formation could be estimated. Treatment of films in 2.5 mL of 5 M H₂SO₄ for 4 to 6 hours at 45°C was the most effective (Figure 3-14).
Figure 3-14: The change in EDAC.HCl solution concentration after exposure to PCBMS-PDMS films that had been hydrolyzed in a) 3 M H\textsubscript{2}SO\textsubscript{4} at 35°C for 2 to 7 hours, b) 5 M H\textsubscript{2}SO\textsubscript{4} at 35°C for 2 to 7 hours, c) 5 M H\textsubscript{2}SO\textsubscript{4} at 45°C for 2 to 6 hours. The change was calculated from the difference in absorbance for control and acid hydrolyzed films. The error bars represent the standard deviation for replicate films (n=3).

Room temperature treatments did not lead to successful hydrolysis, while treatments at 55°C caused significant damage to the PCBMS-PDMS films and so these temperatures were not investigated further. A drawback with this optimization method was that each film seemed to behave somewhat differently, leading to highly variable EDAC.HCl concentration changes (± 10% of the total EDAC.HCl concentration in many cases) even within film sets treated under the same conditions. Nonetheless, it was found that hydrolysis in 5 M sulfuric acid for 4 - 6 hours at 45°C led to changes in EDAC.HCl concentration of 3.5 ± 1.3 mM to 4.9 ± 1.0 mM.
While hydrolysis treatment of PCBMS-PDMS films in basic conditions was also attempted, initial results did not show any change in EDAC.HCl concentration after exposure to the modified films. After some initial experiments, this method was abandoned.

3.3.3 Uptake of 1-Hydroxypyrene in Bulk PCBMS-PDMS Materials

Sylgard 186 cast in a cuvette readily takes up 1-hydroxypyrene (PyrOH) from aqueous solution (Figure 3-15). PCBMS-PDMS was cast as a layer on top of previously cured Sylgard 186, due to the impracticality of producing and purifying larger quantities of the functionalized siloxane. The idea was that PyrOH would diffuse through the PCBMS-PDMS into the Sylgard and this analyte would be detected within the optically clear Sylgard material, while PCBMS-PDMS would facilitate biofunctionalization. However, it appeared that creating a flat layer of PCBMS-PDMS on top of the Sylgard impeded uptake of PyrOH substantially (Figure 3-15). Attempts to solve this problem by changing the casting procedure had a limited effect, so it was decided that the best option would be to produce a heterogeneous surface. In this way, the Sylgard surface was partially exposed to the solution to allow for uptake, while the part of the surface that was coated with PCBMS-PDMS was available for covalent immobilization of biomolecules. As shown in (Figure 3-15), this method was quite successful, with films that had approximately half of the Sylgard surface left exposed showing good levels of PyrOH uptake. This necessitated curing the polymer in the cuvette at an angle of 60°, which was difficult to execute reproducibly.
Figure 3-15: Uptake of 40 μM PyrOH into Sylgard 186 and PCBMS-coated Sylgard 186, cast at the bottom of a plastic cuvette, from aqueous solution monitored in custom fluorescence spectrometer set-up. “Flat” indicates that PCBMS-PDMS was cast in a flat layer over the entire Sylgard 186 surface. The angles listed for the other PCBMS-PDMS films indicate that the cuvettes were placed on an angle, causing the PCBMS-PDMS to form a partial layer over the Sylgard while leaving some PDMS exposed.

3.3.4 Adaptation of Siloxane Chain Exchange Method to Incorporate Ester Functional Groups

The siloxane chain exchange mechanism has been reported to heal PDMS and Sheardown’s group\textsuperscript{20-22} has reported success in using the same method to incorporate functionalized siloxanes into cured PDMS. It was, however, necessary to adapt the process used by the Sheardown group to accomplish the chemical modifications required for this project. Among the considerations were: the choice of hydrosilylation catalyst, the effect of any chemistry on the plastic cuvettes containing the siloxane films, and the solubility of the reaction components in alcohols.

In trying to replicate the literature procedure, it was found that lab-synthesized PDMS-PMS liquids, which were allowed to polymerize for 1 hour after the components were mixed (see Section 3.2.1.2), were unable to dissolve in alcoholic solvents. A shorter 12 minute reaction time was chosen to create lower viscosity siloxanes and these oligomers were more soluble in alcoholic solvents. While this
method was successful in making the polymer soluble in alcohol, the large amount of polymer required to make a bulk treatment solution (consisting of 37.5% PDMS-PMS (m/v) in solvent) was not considered sustainable. This required multiple batches of PDMS-PMS, since the siloxane-forming reaction could not be easily scaled up to such a high degree.

To reduce the amount of reagent required, the Sylgard films were instead treated by placing a 75 µL droplet of the PDMS-PMS in methanol mixture on the surface, which was enough volume to cover the 1 cm² surface without overflow. Using this method, PDMS-PMS was successfully attached to Sylgard, as measured by ATR-FTIR measurements, which showed a peak for Si-H in the modified samples (Figure 3-16).

![ATR-FTIR spectra of Sylgard films after chain exchange treatment. The Si-H stretching vibration can be observed at 2166 cm⁻¹.](image)

**Figure 3-16**: ATR-FTIR spectra of Sylgard films after chain exchange treatment. The Si-H stretching vibration can be observed at 2166 cm⁻¹.
These results suggested that PMHS was adhering to the surface of the film through chain exchange, although the presence of HCl did not appear to greatly affect the intensity of the Si-H peak. However, it would be difficult to make a quantitative determination of the amount of PMHS attached based on peak intensity alone, since many other factors may affect this signal. These include the degree of contact between the polymer and the ATR element, and inhomogeneity of the film treatment.

3.3.4.1 Surface Hydrosilylation of PDMS-PMS with methyl-4-pentenoate on Sylgard 186

Following the incorporation of PDMS-PMS into the Sylgard 186 surface by chain exchange, a hydrosilylation reaction was carried out to attach methyl-4-pentenoate at the silicon hydride locations. When these experiments were undertaken, some unexpected observations were made, particularly the appearance of bubbles during the hydrosilylation reaction. In some cases, this was accompanied by loud popping noises. To investigate the source of this reaction, the components of the process: PMHS, methyl-4-pentenoate, isopropanol, and Karstedt’s catalyst, were mixed in different combinations. It was found that PMHS, isopropanol, and catalyst were required for the gas production to occur. Based on literature reports, bubbles may be the result of H₂ gas produced either during the catalyst induction period or as the product of a side reaction with alcohols. This second explanation seemed more likely in this case, since isopropanol was present in the reaction as the solvent. The reason for the use of isopropanol solvent, which was used instead of the usual hydrosilylation solvent, toluene, was compatibility with the plastic cuvettes. Since this reaction would need to be carried out on PDMS cast in disposable plastic cuvettes, a plastic-compatible solvent that was also capable of dissolving the hydrosilylation components was required. Isopropanol was chosen as the best solvent since it fulfilled these criteria and had been used previously in a similar procedure. We investigated the role of isopropanol in the hydrosilylation reaction by collecting ¹H NMR spectra of the reaction mixtures, each lacking one reaction component, described above. It was observed that the peak for the isopropanol hydroxyl group disappeared from all NMR
spectra, except when the hydrosilylation catalyst (Karstedt’s catalyst) was omitted, suggesting that a reaction between the solvent and catalyst had occurred. It may be possible to use an alternative solvent for this reaction that does not dissolve the cuvette or react with the components, such as hexane. However, an alternative approach was available by completing the hydrosilylation on the linear oligomers prior to chain exchange surface functionalization, negating the need to pursue the surface hydrosilylation further.

3.3.4.2 Siloxane Chain Exchange of PCBMS-PDMS on Sylgard 186

An alternative to hydrosilylation on the surface is hydrosilylation of linear PDMS-PMS oligomers in solution. The hydrosilylation to form PCBMS-PDMS was carried out as described in Section 3.2.6.2, in toluene solution in a round bottom flask, with the \( \text{Pt(SEt}_2\text{)}_2\text{Cl}_2 \) catalyst. As expected based on earlier work, this reaction was successful in incorporating the ester functionality and allowed the use of the preferred solvent and catalyst. Then, the newly synthesized PCBMS-PDMS could be incorporated into the PDMS surface by chain exchange.

Some attempts were made to use the commercial PMHS material (Sigma-Aldrich) in the hydrosilylation reaction, but procedures carried out with this oligomer repeatedly led to solid polymer masses, which formed while the reaction mixture was heated at 75°C. As a result, we returned to the use of lab-made PDMS-PMS, which was a mixture of methylhydro- and dimethyl-functionalized siloxane, and was observed to have higher stability during heating.

After the hydrosilylation with methyl-4-pentenoate was carried out to convert PDMS-PMS to PCBMS-PDMS, the siloxane chain exchange procedure was used to incorporate the PCBMS-PDMS oligomers. After completing the chain exchange, a small carbonyl stretch peak was detected in the ATR-FTIR spectrum of the siloxane surface, indicative of the incorporation of PCBMS-PDMS to the polymer (Figure 3-17).


**Figure 3-17:** ATR-FTIR spectra of unmodified Sylgard, Sylgard modified with PMOS-PDMS, and Sylgard modified with PCBMS-PDMS. The inset shows the carbonyl stretch region.

This result suggested that successful ester-functionalization of Sylgard had occurred. These modified materials were carried on to further optimization.

### 3.3.5 Optimal Contact Angle Analysis Method

It was necessary to find a consistent and reliable contact angle analysis method to use when optimizing the surface treatment procedures. In comparing static measurements after 30 seconds and 5 minutes, as well as advancing measurements, the average contact angles and standard deviations obtained were substantially different. For static droplet measurements, a 30 second equilibration time led to an average angle of $118.6 \pm 0.3^\circ$, while a 5 minute equilibration time led to an average angle of $96 \pm 1^\circ$. This
difference can be attributed to interactions between water and Sylgard, producing a decreasing angle that does not reach equilibrium. This was confirmed by performing measurements using both Sylgard and an impermeable material with a high contact angle, UltraEverDry, which is superhydrophobic. In kinetic measurements, during which a 5 µL drop was placed on the surface and contact angles were measured over the next five minutes, it could be seen that the contact angle on Sylgard decreased by 25° while the UltraEverDry contact angle stayed nearly constant. While both droplets experienced evaporation, it appeared that an interaction between water and Sylgard occurred, preventing the establishment of equilibrium (Figure 3-18).

![Figure 3-18: Contact angle values measured over 5 minutes for a 5 µL water drop on the surface of Sylgard and UltraEverDry.](image)

This is a commonly encountered problem when using water as the contact angle test liquid due to its low molar volume,\(^{52}\) which allows it to permeate into the material, creating a heterogeneous surface.\(^ {32}\)

Thus, the decision was made to use dynamic contact angles, which are frequently used in the literature\(^ {1,4,53,54}\) when static angles do not provide reliable equilibrium values due to liquid evaporation and permeation of the liquid into the surface.\(^ {35,55}\) A droplet that is slowly advanced over the surface better
approximates the equilibrium angle, since water permeation does not have as great an effect on the surface.\textsuperscript{55} These measurements tend to be highly repeatable\textsuperscript{56} and have the added advantage of characterizing surface heterogeneity.\textsuperscript{35} An example of the data obtained by dynamic measurements is shown in Figure 3-19.

![Figure 3-19: Advancing and receding contact angle data for two trials on the same Sylgard film surface. In both cases, a 2 \( \mu \)L drop was deposited before the start of monitoring and the drop was then expanded to 40 \( \mu \)L at a rate of 15.19 \( \mu \)L/min and then contracted at the same rate. The sudden change in slope (offset slightly for the two trials) indicates the reversal of water flow.](image)

In two trials on the same Sylgard film, after an initial adjustment, the droplets seem to follow the same trend, with contact angles stabilizing around 114° when the drop reached approximately 40 \( \mu \)L in size. When the flow of water was reversed, a sudden decrease in contact angle was observed. This decreasing trend continued until the droplet was almost completely removed. However, the drop shape was strongly distorted (Figure 3-20), possibly due to the surface changes caused by permeation of water into the siloxane and this drop shape distortion prevented the measurement of reliable receding contact angle data. Therefore, only advancing angles were used since water permeation did not play a role in these measurements. The average advancing angle achieved in these experiments was 113 ± 1°. Although this
dynamic method showed reduced precision relative to the static measurement after 30 seconds of equilibration, it should avoid the complications of droplet evaporation and permeation into the surface. Thus, it was chosen for further experiments.

3.3.6 Optimization of Chain Exchange-Based Siloxane Modification by Contact Angle

Literature examples of chain exchange modification suggested that treatment times greater than 30 minutes did not increase the level of incorporation of the functionalized material, although surface roughness increased.\textsuperscript{20} Thus, the effect of chain exchange treatment times was investigated to determine if shorter times produced similar surface properties.

For the treatment conditions in which only a single aliquot of the chain exchange treatment solution was added to each slide, a volume of 100 µL was used to allow coverage of the entire 1 cm\textsuperscript{2} slide surface. When three consecutive treatments were performed, a lower volume (75 µL) was necessary because successive additions led to a build up of residual solution on the surface that could not be removed by pipette. As a result, the second and third treatments were prone to overflow if volumes greater than 75 µL were used.

\textbf{Figure 3-20}: a) Image of advancing water droplet on Sylgard. b) Image of a receding water droplet on Sylgard.
Based on the change in contact angles after hydrolysis of the ester groups (Figure 3-21), a shorter equilibration treatment of 30 minutes led to similar film hydrophilicity, suggesting the presence of equivalent (within error) levels of carboxyl functionality. Since this treatment regime reduced the time required for experiments and the amount of reagent consumed per film, it was used for further experiments.

![Figure 3-21: The change in advancing water contact angle from initial value to post-hydrolysis on modified Sylgard films. PMOS (octyl) and PCBMS (ester) solutions were added to Sylgard 186 films and subjected to chain exchange for varying treatment times: 1-30 minute treatment, 1-1 hour treatment, 3-1 hour treatments. The error bars represent the standard deviation of a total of nine measurements: 3 on each of 3 films.]

3.3.7 Optimization of Acid Catalyzed Hydrolysis by Contact Angle Measurements

The advancing contact angle method was used to optimize the acid hydrolysis of the methyl esters on PCBMS-modified Sylgard. The change in contact angle from the initial value (before any treatment) to after hydrolysis was determined for all films and averaged for each equivalent set. The change in advancing contact angle for multiple PMOS-modified control films under acid hydrolysis conditions of 5
M HCl for 6 hours and 24 hours at 35°C were -17 ± 5° and -15 ± 5°, respectively. In comparison, the
effect of 5 M HCl treatment at 35°C on PCBMS-PDMS-modified films was a change in contact angle of
-31 ± 4° after 6 hours and -32 ± 3° after 24 hours. The compiled results for all tested conditions are shown
in Figure 3-22.

![Figure 3-22: The change in advancing contact angle from the initial values for PMOS and PCBMS
treated films that were hydrolyzed in HCl at 35°C at a range of concentrations for 6 hours or 24 hours.
The error bars represent the standard deviation of 9 measurements: 3 on each of 3 replicate films.]

The contact angles for the PCBMS-modified films decreased by a significantly greater amount for the
samples treated for with 5 M HCl for 6 hours (t_{calc}=6.22, 95% confidence, 16 degrees of freedom) and 24
hours (t_{calc}=8.41, 95% confidence, 16 degrees of freedom), compared to samples modified with PMOS
and hydrolyzed under the same conditions.

Overall, the concentration of HCl used for hydrolysis appeared to be the main contributing factor,
with the time allowed for the reaction playing a lesser role. Three hour hydrolysis treatments had been
tested previously and showed poor results, indicating that the reaction time may be a factor until some
threshold level of treatment is reached. The 24 hour treatment in 5 M HCl at 35°C was chosen as the
optimal treatment condition as it provided one of the largest decreases in contact angle, which correlates
to a maximum increase in hydrophilicity. This hydrophilicity increase was assumed to be due to the formation of carboxylic acid groups at the surface from the esters of PCBMS-PDMS.

### 3.3.8 Uptake of 1-Hydroxypyrene in Chain Exchange-Modified Sylgard

In order to determine the SPME characteristics of the chain-exchange modified Sylgard cuvettes, a set of cuvettes was prepared and subjected to the chain exchange and acid hydrolysis steps described above to incorporate carboxylic acid functionality. This experiment was completed prior to the optimization of chain exchange treatment time, so the 3 x 1 hour scheme was used here. However, since changing the chain exchange treatment duration did not have a significant effect on the surface chemistry of the material, this variable was not expected to impact the uptake experiment. Three cuvettes contained unmodified Sylgard 186, while three others were treated with the PCBMS-PDMS equilibration solution and acid hydrolyzed at 35°C for 24 hours in 400 µL of 5 M HCl. When a 40 µM PyrOH solution in buffer (10 mM Tris, pH 8, 5 mM MgCl$_2$, 0.1 mM ZnCl$_2$) was added to a cuvette containing the surface-modified Sylgard 186 material, the rate of extraction was comparable to the rate in unmodified Sylgard (Figure 3-23). This suggested that the chain-exchange modification of Sylgard did not significantly decrease the ability of the PDMS material to take up PyrOH from solution, which was important for the success of further experiments.
Figure 3-23: Uptake of PyrOH into unmodified Sylgard 186 and Sylgard modified with PCBMS-PDMS by acid equilibration.

3.4 Conclusions

In the course of this project, multiple methods of modifying PDMS to prepare for biomolecule attachment have been attempted. The effect of these modifications on the solid phase microextraction properties of the siloxane have also been investigated.

The bulk modification of linear PDMS-PMS to incorporate a methyl ester was successfully carried out, as shown by $^1$H NMR studies. Once these materials were cast and cured, hydrolysis was performed to remove the methyl ester, leaving behind a carboxylic acid functional group. Different acid and base conditions, including solution composition, temperature, and treatment time, were used for the hydrolysis step and the level of carboxylic acid formation was monitored by the removal of EDAC.HCl from the solution. Treatment of the films in 5 M H$_2$SO$_4$ at 35°C for 6 hours produced a significant change in the absorbance of an EDAC.HCl solution that was exposed to the modified surface. The modifications
to this material, however, prevent its successful use in the solid phase microextraction of 1-hydroxypyrene from aqueous solution, which was required for the eventual SPME-ELISA system. To avoid this problem, a heterogeneous surface was produced, with a Sylgard film cured on the bottom of a cuvette, followed by a PCBMS-PDMS film that covered only part of the Sylgard material. While this casting process solved the 1-hydroxypyrene uptake challenge, it was not ideal since these heterogeneous surfaces were difficult to produce consistently.

An alternative surface modification procedure was attempted through the chain exchange mechanism. Silicon hydride-functionalized oligomers were incorporated into cured PDMS materials through chain exchange in the presence of acid, and this process was monitored by ATR-FTIR. While this modification was successful, subsequent attempts to perform hydrosilylation on the silicon hydride-functionalized surface to incorporate methyl-4-pentenoate failed due to incompatibility between the plastic cuvettes, solvent, and catalyst being used. By first performing the hydrosilylation of PDMS-PMS and methyl-4-pentenoate in solution, the optimal reaction conditions (including temperature, catalyst, and solvent) could be employed and PCBMS-PDMS oligomers were produced. These oligomers were subsequently incorporated into the cured PDMS films by chain exchange and this modification was again observed by ATR-FTIR analysis.

Following optimization of an advancing contact angle analysis method, surface modifications could be monitored by the degree of hydrophobicity of the surface, approximated by the water contact angle. This method was used to optimize the chain exchange and subsequent acid hydrolysis steps on the PDMS material. It was found that the chain exchange could be carried out for between 30 minutes and 3 hours, with little change in the resulting surface properties. The effect of the acid hydrolysis step was clearer and showed that treatment of the ester-functionalized films in the presence of 5 M HCl at 35°C for
either 6 or 24 hours resulted in significant increases in the hydrophilic character of the surfaces. This was attributed to cleavage of the methyl ester to form carboxylic acid groups on the material surface.

The assessment of solid phase microextraction of 1-hydroxypyrene by the chain exchange modified siloxane showed that this material maintained similar uptake properties to unmodified Sylgard 186. As a result, this modification method was deemed to be preferable to the bulk modified ester-containing material produced earlier. The chain exchange-functionalized films were therefore carried forward for the investigation of covalent biomolecule immobilization.

Further research in this area could focus on more readily cleavable groups to form carboxylic acids on the polymer surface. This could include t-butyl esters, which are hydrolyzed under mild acidic conditions relative to methyl esters. In addition, more detailed surface chemistry analysis would make characterization of the modification more detailed. XPS, particularly with labeled groups, should be attempted on unmodified and modified films.

3.5 Acknowledgements

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3.6 List of References


Chapter 4

Attachment of Antibodies to Siloxane Surfaces for ELISA

4.1 Introduction

4.1.1 Biofunctionalized Sensors

Biological recognition elements are important components of environmental biosensors. These elements are responsible for interacting with the analyte of interest, and communicating this interaction to a signal transducer, which produces a signal to be recorded. Recognition can be performed by a variety of biological components, such as cells, enzymes, antibodies, phages, biomimics, or nucleic acids.\(^1\) Two main types of processes can be explored: bioconversion (\(i.e.\) enzyme conversion of substrate to product), and affinity (\(i.e.\) binding of analyte and recognition element to form a complex).\(^2\) Many biorecognition systems have been used to produce biosensors for clinical and environmental applications, using the available recognition (antibodies, aptamers, carbohydrates, antimicrobial peptides, bacteriophages) and transduction (electrical, mass-sensitive, optical) elements.\(^3-11\)

Antibodies interact with antigens based on an affinity binding known as “lock and key,” which relies on a three-dimensional match between the analyte and recognition element. This three-dimensional shape recognition makes antibodies and the biosensors that use them highly versatile analysis tools, able to detect everything from small molecules, to much larger microorganisms.\(^1\) Antibody specificity can be very useful in detecting analytes in a complex mixture without purification.\(^12\) In order to detect antibody-analyte interactions, labeled and label-free techniques can be employed. Labeled methods use antibodies attached to enzymes, radiolabels and fluorescent tags, while label-free analysis can be accomplished through methods such as surface plasmon resonance, evanescent wave sensing, electrochemistry, acoustic
waves, or microcantilevers, where the binding between recognition element and analyte is detected directly. The use of enzyme-labeled antibodies confers a number of advantages including signal amplification by the enzyme, and the possibility for visual recognition through colour-change reactions. The most common laboratory assay that uses enzyme-labeled antibodies is the enzyme-linked immunosorbent assay (ELISA).\textsuperscript{13} However, while ELISA techniques are commonly employed in fields such as clinical diagnosis, with detection limits in the pg/mL range, there are still key applications for which this sensitivity is insufficient. Improvements to the sensitivity and, especially, the detection limit of ELISA methods could greatly extend the range of applications for this versatile analytical technique.

4.1.2 Methods of Attaching Antibodies to Surfaces

In most biosensors, it is necessary to immobilize the biorecognition element. This immobilization can facilitate binding event recognition on a sensor surface, allow washing or liquid flow over the sensor surface, and potentially facilitate reuse of the sensor. Many methods of protein immobilization on solid materials are possible, including adsorption, entrapment, covalent, and affinity binding.\textsuperscript{14-16} It is important to optimize this process for maximum efficiency of immobilization to avoid waste of expensive biorecognition elements, while preventing non-specific binding, which may obscure the signal.\textsuperscript{17} Some of the major considerations with any binding procedure include: efficient attachment to avoid reagent waste, binding the biomolecule in the correct orientation, avoiding denaturation, long-term stability of the surface, and having an optimal surface concentration of biomolecule for the application of interest. Since there are a wide variety of biomolecules available for immobilization, many sensor surfaces to use as platforms, and a range of applications, it is necessary to optimize the biomolecule attachment method for the particular sensor being developed.
4.1.2.1 Adsorption of Proteins on Surfaces

Adsorption techniques, based on the electrostatic, hydrogen bonding, dispersion, and van der Waals interactions between surfaces and proteins,\(^{18,19}\) tend to be straightforward, useful on a range of materials, and involve mild conditions.\(^15\) For proteins, adsorption tends to be more effective on hydrophobic materials (low surface energy), while post-immobilization activity tends to be higher on hydrophilic materials (high surface energy).\(^20\) Adsorption can decrease protein function in a number of ways: the protein may adsorb such that its native conformation is disturbed, the active site may be blocked by the adsorption site, or a high concentration of adsorbed protein could hinder activity due to steric effects.\(^{20,21}\) Other concerns with adsorption methods include reversibility,\(^15\) as adsorbed proteins may be removed in the presence of certain buffers or detergents,\(^22\) and the lower surface concentrations achieved compared to covalent immobilization.\(^23\) Nevertheless, proteins can be effectively immobilized by adsorption under certain conditions and on certain surfaces. For example, proteins of a variety of sizes were successfully adsorbed onto polystyrene materials and, when polystyrene-poly(methylmethacrylate) block copolymers were used, the proteins showed preference for the styrene functionalized material.\(^24\) Antibodies can therefore be immobilized by adsorption, but the simplicity of the process must be balanced against the potential denaturation, lower stability, and lower surface loadings that may result.

4.1.2.2 Covalent Immobilization of Proteins on Surfaces

Covalent immobilization of proteins usually involves harsher conditions than adsorption,\(^15\) but also generally leads to higher surface loading,\(^23,25\) and more resilient binding.\(^23,26\) These advantages are counterbalanced by activity loss caused by changes to the conformation of the protein during binding.\(^22\) For example, one study found that immobilized enzyme activity is just 20% to 30% of the unbound activity.\(^27\)
There are many possible protocols available for covalent binding and all require a surface that contains functional groups that can be exploited for protein attachment. The most common covalent binding chemistries involve carboxylic acids binding to primary amines, thiols binding to thiols, and aldehydes bonding to amines. A more recently developed immobilization technique involves boronic acid-functionalized surfaces that react with carbohydrates in the constant region of the antibody, leading to oriented immobilization. Self-assembled monolayers can be used as intermediate surfaces for a number of covalent immobilization schemes, increasing the possible density of biomolecules and the biocompatibility of the surface.

Many covalent attachment schemes require activating agents to promote the reaction between surface and biomolecule, and numerous reagents are available for this purpose: a recent summary has been provided in Goddard and Hotchkiss. Carbodiimides and succinimides are frequently used to link carboxylic acids and primary amines, forming amide bonds. A widely applied procedure involves N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDAC), which can be combined with N-hydroxysuccinimide (NHS) to achieve coupling efficiencies of up to 90 percent. The advantage of combining the two reagents is that the NHS-ester is more stable against hydrolysis than the O-acylisourea produced by the carbodiimide reaction (Figure 4-1). When immobilizing proteins by covalent methods, adsorption may still occur, but adsorbed biomolecules may desorb during experiments or storage, which is undesirable when long-term stability is required. In a study by Williams et al., it was found that the immobilization of proteins in the presence of a detergent, Tween 20, was the most effective approach to reducing non-specific adsorption. Another method of reducing non-specific binding is the use of tether groups, such as self-assembled monolayers and poly(ethylene glycol). If hydrophilic tether groups are used, this method may also increase the activity of the immobilized proteins. Depending on the
Figure 4-1: a) The process of activating surface carboxylic acid functional groups with EDAC and NHS to produce amine-reactive NHS esters. The carboxylic acid first reacts with EDAC to produce an O-acylisourea functional group. NHS can then react with this functionality to produce an NHS ester, which is capable of reacting with amines on proteins to form amide bonds. b) The amine-reactive NHS ester reacts with primary lysine residues on the protein, forming a covalent amide bond between the surface and the protein. Scheme adapted from Thermo Fisher Scientific.38

application and expected storage time for a sensor, it may be important to ensure that only covalently bound antibody is present on the surface and one of the above techniques can be used in this case.

Overall, provided the partial loss of biological activity is not a concern, there are many well-developed methods available for the covalent attachment of a range of biomolecules to surfaces. This
attachment is strong and usually irreversible, leading to sensors that can be stored after functionalization and potentially reused multiple times.

4.1.2.3 Affinity Immobilization of Proteins on Surfaces

Two concerns in the covalent attachment of proteins to surfaces are the lack of control of biomolecule orientation and the potential for inactivation or denaturation due to the covalent bond. Recent research has focused on affinity-based immobilization approaches, which can facilitate direction-specific attachment of biomolecules to surfaces. This feature is particularly important when a specific recognition site must be accessible to solution-phase analytes and this is the case for antibodies on biosensors.

Proteins A and G are produced by bacteria and bind the constant (F<sub>c</sub>) region of certain classes of immunoglobulins. Using this affinity for the region of the antibody not responsible for antigen binding, oriented immobilization can be achieved. In this case, Protein A or G must first be immobilized by a conventional protein binding scheme and immunoglobulins can then be introduced and allowed to bind to the functionalized surface. The antibody and protein complex may also be covalently linked following affinity binding to provide a more stable attachment. The use of Protein A or G as an intermediate linker, rather than direct covalent antibody attachment, does not necessarily increase the signal in ELISA methods. However, this assembly can allow sensor surface regeneration since the bound antibodies can be removed by washing and replaced with a new solution of immunoglobulins. Researchers have also reported somewhat higher levels of antigen binding when Protein A-bound antibodies are employed, relative to covalently bound antibodies. Protein A or G can therefore be useful intermediate layers in protein binding schemes, especially when a specific antibody orientation or the ability to regenerate the surface is a priority.
Avidin-biotin affinity can also be exploited for protein immobilization. Due to the high strength of this affinity (K_D = 10^{-15} M), and the simplicity of biotin labeling, this pairing has been widely used in sensors for the immobilization of biorecognition elements. By biotinylating both a protein of interest and a surface, avidin or streptavidin can be used to bind the protein to the sensor material. Avidin or streptavidin can also be covalently bound directly to the surface and then used to link biotinylated recognition elements. Site-directed attachment can be achieved by using a biotinylation method that targets the glycosylated constant region of an antibody. While the use of this method does add multiple labeling steps to the protein immobilization procedure, the high affinity of the interaction and the ability to achieve oriented attachment may be desirable.

Other affinity-based attachment methods are available as well, although less commonly used. These include the use of innate histidine-rich regions in antibodies for binding to supports containing metals, immobilizing via the oligosaccharide functionality in the constant region of the antibody, and employing antibody fragments containing only the antigen binding region. These methods, though more complex, are useful in case specific directional orientation is desired.

4.1.2.4 Solid Phase Entrapment of Biomolecules

Solid phase entrapment is a mild technique in which the protein is not bound by chemical bonds to the solid support. One common entrapment method uses inorganic silicates formed by low-temperature sol-gel processes to encapsulate biomolecules. The advantages of these techniques include the ability to introduce additives to preserve protein function, and the control over pore size means that small molecules may diffuse in and out of the material while larger biomolecules remain trapped inside. Depending on the choice of material, optical transparency may allow sensing within the entrapment matrix. However, the fact that the biomolecules are entrapped within a material means that analytes (antigens or substrates) must be small enough to move through the material readily.
entrapment is a useful method provided the biomolecules could be encapsulated without inactivation and the signal transduction method is compatible with the material.

4.1.3 Protein Immobilization on Siloxanes

Many of the procedures described above have been used for the immobilization of proteins on or in siloxane materials. The inherent hydrophobicity of poly(dimethylsiloxane) facilitates protein adsorption,\textsuperscript{56} but may lead to denaturation. The hydrophobicity of the material can be reduced by the adsorption of small proteins called hydrophobins, which produce a somewhat hydrophilic surface to which proteins can be adsorbed with better activity preservation.\textsuperscript{57} Where a covalent link needs to be established with the protein, a functionalized siloxane surface must be used. This can be accomplished by synthesizing a siloxane material from functional oligomers,\textsuperscript{40} but is more frequently achieved by surface modification of a cured PDMS material. Post-curing modification of siloxanes was reviewed in Chapter 3. Chemical modification methods that have been used as precursors to covalent immobilization of proteins include silanization,\textsuperscript{58} and siloxane chain exchange.\textsuperscript{40} When covalent immobilization is not necessary, encapsulating the proteins within a siloxane may be a useful alternative.\textsuperscript{59} There are various methods for attaching proteins to siloxanes, with the choice depending largely on the desired application. For example: antibodies need access to antigens, so encapsulation methods may not be ideal in immunoassay-type sensors, except for small molecule detection. Some bulk chemical modifications to siloxanes to allow biomolecule attachment may also alter the material properties impairing their desirable characteristics (solid phase microextraction, elastomeric properties, optical clarity). To avoid these challenges, chemical modification of the cured siloxane, which localizes treatment at the surface, can be a useful technique for protein immobilization.
4.1.4 Analysis of Protein Immobilization

Once biomolecules are attached to the solid surface, it is necessary to determine the success of the attachment procedure. An important decision is whether to measure protein immobilization based on the surface concentration or the decrease in concentration in the protein solution. Measurements on the surface are preferable because these directly determine the immobilized amount of protein and the decrease in solution concentration may fall below the detection limit of the quantitation assay.\textsuperscript{16}

A wide range of optical analysis methods are available for protein quantitation. Fluorescent dyes are often used for detecting proteins immobilized on a surface. Fluorescently labeled proteins and antibodies have been analyzed using plate reader technology\textsuperscript{44} and microscopy.\textsuperscript{60,61} For example, the immobilization of IgG, labeled with tetramethyl rhodamine isothiocyanate, was monitored by fluorescence microscopy.\textsuperscript{42} A number of colourimetric assays for proteins can be applied to surface immobilization experiments.\textsuperscript{16} These include the Biuret reaction,\textsuperscript{62} the Lowry method,\textsuperscript{62} the Bradford assay,\textsuperscript{63} and the bicinchoinic acid protein assay.\textsuperscript{62} The development of a colourimetric signal may depend not only on the protein concentration but also upon its conformation and amino acid composition.\textsuperscript{16}

Bioactivity can also be a useful method of analyzing immobilized protein. In particular, if an enzyme has been immobilized, the kinetics of its reaction with a substrate can be measured, as in the case of alkaline phosphatase and \textit{p}-nitrophenyl phosphate.\textsuperscript{15} The concentration of an enzyme should be proportional to its initial reaction rate, when the substrate concentration is not limiting, leading to pseudo-first order kinetics.\textsuperscript{64} The advantage of such a method is that enzyme activity is simultaneously probed,\textsuperscript{15} but this measurement does not determine the total quantity of immobilized biomolecule, preventing a specific activity determination.\textsuperscript{29} Combining activity analysis with a protein quantitation method can provide more information in this regard.\textsuperscript{65}
Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) can also be applied to the analysis of proteins. Characteristic infrared absorption bands exist for protein backbones, namely the amide I (1650 cm\(^{-1}\), C=O stretch) and amide II (1550 cm\(^{-1}\), C-N stretch, N-H in-plane bend) absorptions, and these can facilitate protein identification and structural analysis.\(^{66}\) Although the infrared peaks are typically small, they can be used to identify not only the presence of proteins, but also probe their secondary structure.\(^{20,67,68}\)

Many other methods of analysis are available, depending on the application, surface, and the available instrumentation. Atomic force microscopy\(^{22,59}\) and scanning tunneling microscopy\(^{22}\) may be used to assess surface coverage by proteins and the uniformity of this coating.\(^{24}\) If laboratory conditions permit, radioactive labels on the proteins, such as I\(^{125}\), can be employed for quantitative analysis.\(^{29,69}\) Contact angle measurements may also be used as a simple method to observe the changes in surface energy after protein immobilization.\(^{70}\) Overall, the choice of protein analysis method depends on the type of information required: structure, activity, surface coverage, or quantity. While colourimetric and radiolabeled assays provide quantitative information, activity analysis may be better suited when the amount of active protein is of greatest concern. Meanwhile, ATR-FTIR is useful when structural analysis is required.

**4.1.5 Research Objectives**

This project aims to produce an antibody-functionalized siloxane surface suitable for use in ELISA in combination with solid phase microextraction. Multiple antibody immobilization methods will be attempted, including physical adsorption of the protein on the surface, direct covalent immobilization of the antibody, and affinity binding of the antibody to covalently immobilized Protein A. Spectroscopic, contact angle, and activity-based measurements will be investigated to determine useful methods of
protein analysis for this system. The binding methods will be optimized and compared in terms of quantity of active protein attached, and stability of the functionalized surface.

4.2 Materials and Methods

4.2.1 Examination of Protein Quantitation Methods

4.2.1.1 Protein Immobilization and Analysis by ATR-FTIR

To characterize ATR-FTIR as a method of analyzing protein attachment, BSA was adsorbed to Sylgard 186 films and surface reflectance spectra were collected. Planar Sylgard films were prepared as described in Chapter 3. To summarize, glass microscope slides were cut into 1 cm by 1 cm sections using a tungsten carbide glass cutting tool (Sigma-Aldrich, Oakville, ON). Each piece of glass was cleaned using isopropanol and allowed to dry. Sylgard 186 PDMS (Paisley Products, Toronto, ON) was prepared at a 9:1 base to curing agent mass ratio and diluted with hexanes at a 1:4 (mass of polymer to volume of solvent) ratio. Using this mixture, 200 µL of polymer solution were deposited on each glass square and allowed to cure overnight in air at room temperature.

Bovine serum albumin (BSA) (Sigma-Aldrich) was dissolved in phosphate buffered saline (PBS) (137 mM NaCl, 2.70 mM KCl, 10.0 mM Na₂HPO₄, 2.00 mM KH₂PO₄, in distilled water, pH 7.4) at various concentrations. The cured siloxane films were each immersed in separate 20 mL scintillation vials containing 2.5 mL of protein solution and left overnight at room temperature to allow adsorption. After this time, the films were removed from the solution, rinsed with PBS, and analyzed by a Bruker (Milton, ON) ALPHA Platinum ATR-FTIR containing a single reflection diamond.
4.2.1.2 Contact Angle Measurements of Protein Immobilization on Siloxane Surfaces

Analysis of protein-modified films by contact angle was used to characterize the quantity of protein, rather than active protein, attached to the surface. For this experiment, planar Sylgard films were prepared as above (see Section 4.2.1.1), with the only change being that Sylgard 184 (Paisley Products) was used instead of Sylgard 186. Fifteen cured Sylgard films were exposed to 2.5 mL solutions of BSA in phosphate buffer (10.0 mM Na$_2$HPO$_4$, 2.00 mM KH$_2$PO$_4$) at pH 8 and room temperature overnight. The BSA concentration ranged from 0 to 20 mg/mL. After the overnight adsorption procedure, the films were removed from the protein solution and washed three times with PBS (pH 7.4, prepared as described above) containing 0.1% Tween 20 (Fisher Scientific, Ottawa, ON). Each wash was 10 minutes in length and used 2.5 mL of wash buffer. Following the detergent washes, the films were rinsed with distilled water and dried carefully with lens cleaning paper.

Advancing contact angle measurements were carried out by the method described in Chapter 3, using a Krüss DSA 100 Drop Shape Analyzer (Krüss USA, Matthews, NC). In summary, the camera tilt angle was 2° and the Tangent 1 analysis method was used to determine the contact angles within the supplied software. For the advancing angle measurement, a robotic syringe was used to deposit a drop of 20 µL distilled water onto each sample to be analyzed and the needle was kept within the drop. After defining the baseline of the drop on the surface within the software, the syringe was set to add volume to the droplet at a rate of 15 µL/min for 60 seconds. The software was set to collect a contact angle measurement for the left and right sides of the drop every 0.5 seconds for 60 seconds. From this, an advancing contact angle was obtained by averaging the measurements during the last five seconds of this process, when the angle approached equilibrium.
4.2.1.3 Enzyme Activity for Quantitation of Immobilized Protein

Although enzyme activity is not a direct measure of immobilized protein density, it is capable of giving results that can be compared with solution activity data. This information can then be converted to an “effective activity” measurement. For immobilization experiments, anti rabbit immunoglobulin G-alkaline phosphatase conjugate (raised in goat) (Anti IgG-AP) (Sigma-Aldrich) was used as a model biomolecule. This Anti IgG-AP was a useful test case for antibody immobilization because it is an enzyme-labeled immunoglobulin, and this enzyme can be used for activity analysis. Before use in immobilization experiments, it was necessary to characterize the solution phase activity of Anti IgG-AP.

4.2.1.3.1 Role of Buffer pH in Alkaline Phosphatase Enzyme Activity

Alkaline phosphatase, the enzyme label on Anti IgG-AP, has higher activity in basic pH solutions. As a result, the solution-phase activity of this enzyme was characterized in a number of buffer systems to determine the optimal aqueous conditions for activity measurements. The activity of 5.0 ng/mL alkaline phosphatase enzyme (AP) (bovine intestinal mucosa, ≥6500 DEA units/mg, Sigma-Aldrich) was measured in 10.0 mM Tris buffer (Trizma-HCl, Sigma-Aldrich) containing 5.00 mM MgCl₂ and 0.100 mM ZnCl₂ at pH values between 8.0 and 9.0 as well as carbonate buffer (100 mM sodium bicarbonate, 5.00 mM MgCl₂, 0.100 mM ZnCl₂) at pH values between 9.5 and 10.5. The enzyme was combined in the buffer with 40 µM 4-methylumbelliferyl phosphate (4-MUP) (Sigma-Aldrich). The conversion of this substrate, which produces the fluorescent product 4-methylumbelliferone upon phosphate cleavage, was monitored at 445 nm in the multi-chamber spectrometer system (Figure 4-2). The detector was an Ocean Optics USB2000+ custom CCD array spectrometer (Ocean Optics, Dunedin, FL) and it was capable of handling bundled collection fibres from 16 identical cuvette chambers. Only four of the sixteen chambers were used for these experiments. Each chamber included two 365 nm LEDs, entering the chamber at angles of 67.5° from the normal (Figure 4-3).
Figure 4-2: Multi chamber spectrometer system. a) An overall view of the instrument, showing the computer-controlled spectrometer and sample chambers. b) A sample chamber is shown with its collection fibre in place. Wires to the left and right of this fibre indicate the LEDs. c) A sample chamber from the opposite direction is shown with a cuvette in place for in-polymer measurements. d) A close-up view of the OEM2000Plus Ocean Optics spectrometer. Top left is the collection fibre bundle and top right is the excitation LED assembly.
Figure 4-3: Top view of a cuvette in the chamber of the custom-built multi-chamber fluorescence spectrometer system. Two LEDs emit light at 365 nm and these enter the solution at an angle of 67.5° to the normal. Excitation of the fluorophores leads to emission that is collected by a lens and fibre located at the centre of the cuvette.

The focal length of the optical components was 2 mm from the lens. A computer running proprietary software from Endetec (Kingston, ON) controlled the LEDs for each chamber. This software controlled the sequential illumination of the LEDs in each chamber and recorded time-resolved spectra in a data file. To stabilize the cuvettes during solution measurements, the top of each cuvette was wrapped on three sides with electrical tape to provide a tighter fit in the spectrometer chamber (Figure 4-4).

Figure 4-4: Semi-micro cuvettes used for solution-phase fluorescence measurements in the 4-chamber spectrometer were wrapped on three sides with black electrical tape to provide a tighter fit in the cuvette chambers.
4.2.1.3.2 Solution-Phase Activity of Alkaline Phosphatase

In order to correlate surface-bound biomolecule activity with effective concentration, it was necessary to measure solution-phase enzyme activity at a variety of biomolecule concentrations. Thus, an experiment was performed to determine the relationship between Anti IgG-AP concentration and observed activity, defined as the slope of a fluorescence vs. time plot. This experiment was performed at both pH 8.0 and pH 9.0 in the Tris buffer described above. A 1.00 mL solution containing 40 µM 4-methylumbelliferyl phosphate in buffer was prepared in a cuvette. After collecting one minute of background fluorescence data, Anti IgG-AP was added by pipette to reach a variety of concentrations. The solution was mixed by repeatedly aspirating the solution with a pipette over the course of 5 to 10 seconds. After this mixing time, which represented time = 0 for the enzyme reaction, fluorescence measurements were taken at the peak emission wavelength of 4-methylumbelliferone for 30 minutes. The slope of the resulting plot of fluorescence signal vs. time could be correlated with the quantity of enzyme-antibody conjugate in solution.

4.2.2 Covalent Protein Immobilization Method Development

The general method used in the protein immobilization optimization phase of this experiment was kept constant. It is described here and alterations to this method will be noted where applicable. A model immunoassay with rabbit immunoglobulin G as the antigen, and anti rabbit immunoglobulin G-alkaline phosphatase (produced in goat) as the detection antibody was used for these experiments. This simplified immunoassay involved the essential steps of the procedure, but avoided the extra antigen binding step that would be required in a sandwich ELISA.

First, Sylgard films were cured in cuvettes by mixing Sylgard 186 components in a 9:1 ratio of base to curing agent by mass, adding hexanes to provide a 2:1 (mass of polymer to volume of solvent)
mixture, and 60 µL of this mixture was deposited using a needle and syringe into each plastic UV-transparent cuvette (BrandTech, Essex, CT). The film was spread across the bottom of the cuvette by tapping on a hard surface (Figure 4-5). The cuvette films were cured for 36 hours before further use.

**Figure 4-5**: Sylgard 186 cast in a PMMA semi-micro cuvette. The polymer is located at the bottom of the cuvette in a layer approximately 1 – 2 mm thick.

Surfaces that were intended for covalent functionalization were then chemically treated to incorporate the necessary functional groups by a combination of siloxane chain exchange and acid hydrolysis (Figure 4-6 and Figure 4-7). Linear functionalized siloxanes, poly(methyloctylsiloxane-co-dimethylsiloxane (PMOS-PDMS) and poly((4-carbomethoxy)butylmethyl-co-dimethyl)siloxane (PCBMS-PDMS) were synthesized as described previously (Section 3.2.6). PMOS-PDMS was dissolved at 37.5% (m/v) in solvent, which was a mixture of 9:1 hexanes to dichloromethane. PCBMS-PDMS was dissolved at 37.5% (m/v) in hexanes. For both PMOS-PDMS and PCBMS-PDMS, 1 mL of the prepared oligomer solution was mixed with 40 µL of concentrated hydrochloric acid. A 100 µL aliquot of the polymer-acid solution was then added to the cured Sylgard 186 PDMS films in the cuvettes. Siloxane chain exchange was allowed to proceed for 30 minutes. After the treatment was completed, the films were
Figure 4-6: The process of incorporating an ester-functionalized siloxane oligomer into cured Sylgard PDMS by chain exchange. The wavy bonds indicate that polymer chains continue beyond the scope of the diagram. For simplicity, only a small siloxane oligomer is shown.

Figure 4-7: Acid-catalyzed hydrolysis using heat causes the methyl esters to be removed, leaving a carboxyl-functionalized polymer near the siloxane surface. The wavy bonds indicate that polymer chains continue beyond the scope of the diagram. For simplicity, only a small siloxane oligomer is shown.

carefully washed with isopropanol to remove the chain exchange solution and allowed to dry for 15 minutes in air.

Following chain exchange to incorporate either ester or octyl-functionalized siloxane, 400 µL of 5 M hydrochloric acid were added to each cuvette and the hydrolysis of the PCBMS-PDMS esters was
allowed to continue at 35°C for 24 hours. After this time, the films were rinsed at least three times with distilled water to remove residual acid. The chemical treatment of the films was completed by activating the carboxylic acid groups produced during hydrolysis using a 400 µL solution of 1.0 mg/mL $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (EDAC.HCl) and 0.1 mg/mL $N$-hydroxysuccinimide (NHS) (both from Sigma-Aldrich) in either distilled water or pH 6.0 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma Aldrich). The treatment lasted two hours at room temperature, after which the cuvettes were rinsed again with distilled water.

Protein binding was completed on films that were treated through the chemical modifications above, and some that were composed of unmodified Sylgard 186. For the chemically treated films, for which the aim was covalent immobilization, protein attachment was completed immediately following activation, using the activated carboxylic acid groups to bond with amines on the protein. For untreated Sylgard 186, the goal was to adsorb proteins onto the polymer surface to compare the success of non-covalent attachment with the covalent method on the carboxyl-functionalized siloxane. All protein attachment steps were the same in both cases. Three different protein binding schemes were employed: i) direct binding of Anti IgG-AP to the surface, ii) binding of IgG to the surface, followed by affinity binding of Anti IgG-AP, and iii) binding of Protein A to the surface, followed by non-covalent attachment of IgG and then non-covalent attachment of Anti IgG-AP (Figure 4-8). The proteins used in the surface-binding step (adsorption and covalent) included rabbit immunoglobulin G (IgG) (Sigma-Aldrich) at 5.0 µg/mL, protein A (from Staphylococcus aureus) (Sigma-Aldrich) at 50 µg/mL, and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Anti IgG-AP) at 5.0 µg/mL. Each cuvette was treated using 400 µL of protein solution, which was made up in either PBS (described above), or 12.0 mM phosphate buffer at pH 8.0. The linkage step took place overnight at 4°C. Following this treatment, the films were rinsed three times in PBS containing 0.1% (v/v) Tween 20 (PBS-T). Each rinse was 10
Figure 4-8: Various protein attachment procedures were used, depending upon the experiment: a) attachment of IgG-AP directly to the modified surface, b) attachment of IgG to the surface for model immunoassay, and c) attachment of Protein A to the surface for directional immobilization of IgG and model immunoassay.

minutes in length in a 1.00 mL volume. After the detergent rinses were completed, the films were thoroughly rinsed with distilled water.

For films that were undergoing further protein binding steps, it was necessary to block any remaining covalent bonding and/or adsorption sites on the cuvette. This was accomplished by a 1 hour treatment in phosphate buffer (0.100 M Na$_2$HPO$_4$, 0.150 M NaCl, pH 7.2) containing 20 g/L bovine serum albumin, and 0.05% Tween 20. Each cuvette was treated in a 1.00 mL solution at room temperature and rinsed with distilled water following treatment.

Non-covalent protein binding of IgG (to Protein A), and Anti IgG-AP (to IgG) was completed following the blocking step. For IgG, 400 µL of 5.0 µg/mL antibody in 12.0 mM phosphate buffer at pH 8.0 were used, while, for IgG-AP, 400 µL of 2.0 µg/mL conjugate in Tris buffer at pH 8.0 (10.0 mM Trizma HCl, 5.0 mM MgCl$_2$, 0.10 mM ZnCl$_2$) were used. In either case, this treatment was 1 hour in length at room temperature. After binding, three PBS-T washes using 1.00 mL of buffer per wash for 10
minutes each were completed. If necessary, further non-covalent protein binding could take place at this point. PBS-T was used as before to rinse the surface after binding.

Before activity testing, each cuvette was filled with Tris buffer at pH 9.0 (10.0 mM Trizma HCl, 5.0 mM MgCl₂, 0.10 mM ZnCl₂) to maintain enzyme activity prior to testing. When activity testing was underway, 1 mL of 40 μM 4-methylumbelliferyl phosphate in Tris buffer at pH 9.0 (10.0 mM Trizma HCl, 5.0 mM MgCl₂, 0.10 mM ZnCl₂) was added to each cuvette in turn and the cuvette was placed in the multi-chamber spectrometer set-up described in Section 4.2.1.3.1. The fluorescence signal was monitored for 30 minutes at room temperature at 445 nm.

4.2.2.1 Effect of Siloxane Chain Exchange Conditions on Immobilized Protein Activity

In initial experiments (Chapter 3), 3 1-hour treatments were used during chain exchange to attach the PCBMS-PDMS onto the surface of Sylgard. To determine the necessary level of chain exchange for maximum biomolecule activity, multiple conditions were compared: a single 30 minute treatment with 100 μL of the polymer and acid solution, a single one hour treatment with 100 μL, and the original method of 3 1-hour treatments with 75 μL of the solution. These conditions were tested for PCBMS-PDMS-functionalization and surfaces modified with PMOS-PDMS served as a control for changes to the surface not caused by carboxylic acid formation.

Anti IgG-AP was covalently attached to the surface once chain exchange, hydrolysis, and activation were completed. Activity testing in pH 8.0 Tris buffer with 40 μM MUP as the substrate was completed to determine the approximate quantity of IgG-AP attached. This was completed both before and after the surface was washed with PBS-T. This analysis allowed a determination of strongly bound biomolecule vs. weakly adsorbed biomolecule amounts.
4.2.2.2 Effect of Acid Hydrolysis Conditions on Immobilized Protein Activity

The effect of the acid hydrolysis (ester conversion to carboxylic acid) step was compared based on the quantity of active antibody enzyme conjugate (Anti IgG-AP) immobilized. This experiment was completed by the usual biomolecule attachment procedure with minor changes: 3 x 1 hour treatments with 75 µL of polymer solution (PMOS-PDMS and PCBMS-PDMS) were used in the chain exchange step, and either distilled water or 5 M HCl was used in the acid hydrolysis step. After activation, Anti IgG-AP was attached directly to the siloxane surface. Activity was tested with Tris pH 8.0 buffer and 40 µM MUP after IgG-AP attachment, both before and after washing with PBS-T.

4.2.2.3 Optimization of Carboxylic Acid Activation Conditions

Carboxylic acid activation can be completed under a variety of conditions. Variables that can be adjusted include the buffer type, buffer pH, activating agent concentrations, activation temperature, and treatment time. Based on a literature search, common treatment conditions were identified and many of these were tested to determine the optimal conditions for this experiment (Table 4-1). Since the activation temperature was always room temperature when it was specified, this variable was not optimized.

4.2.2.3.1 Buffer Type and pH Optimization

An important consideration for carboxylic acid activation is the choice of buffer and pH of treatment. For this optimization experiment, distilled water, 20 mM MES, and 20 mM phosphate buffer were used to dissolve the activating agents, EDAC.HCl and NHS at 1 mg/mL and 0.1 mg/mL respectively. The conditions tested are summarized in Table 4-2. All available conditions were tested using ester-functionalized polymers, while select conditions were tested using octyl-functionalized materials.
Table 4-1: A summary of carbodiimide/succinimide carboxylic acid activation conditions used in the literature for attachment of proteins to solid supports. A dashed line indicates that a variable was not specified. “R. T.” indicates room temperature and “sulfo-NHS” indicates that N-hydroxysulfosuccinimide was used.

<table>
<thead>
<tr>
<th>EDAC Concentration (mg/mL)</th>
<th>NHS Concentration (mg/mL)</th>
<th>Buffer Composition</th>
<th>Treatment Temperature</th>
<th>Treatment Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>2</td>
<td>Water</td>
<td>--</td>
<td>10 s – 30 min</td>
<td>Patel et al. 2007</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>Water, pH 3.5</td>
<td>R. T.</td>
<td>Overnight</td>
<td>Vermett et al. 2003</td>
</tr>
<tr>
<td>0.063 – 6.3</td>
<td>0.096 – 9.6</td>
<td>MES, pH 6.0</td>
<td>R. T.</td>
<td>2 hrs</td>
<td>Puleo et al. 2002</td>
</tr>
<tr>
<td>77</td>
<td>12</td>
<td>--</td>
<td>--</td>
<td>4 hrs</td>
<td>Li et al. 2013</td>
</tr>
<tr>
<td>0.8</td>
<td>12</td>
<td>10 mM MES, pH 6.1</td>
<td>R. T.</td>
<td>1 hr</td>
<td>Croll et al. 2004</td>
</tr>
<tr>
<td>0.08</td>
<td>0.01</td>
<td>Water</td>
<td>R. T.</td>
<td>30 min</td>
<td>Shi et al. 2015</td>
</tr>
<tr>
<td>77</td>
<td>12</td>
<td>--</td>
<td>--</td>
<td>30 min</td>
<td>Li et al. 2015</td>
</tr>
<tr>
<td>38</td>
<td>11 (sulfo-NHS)</td>
<td>--</td>
<td>R. T.</td>
<td>30 min</td>
<td>Sungkanak et al. 2010</td>
</tr>
<tr>
<td>38</td>
<td>none</td>
<td>Imidazole, pH 6.0</td>
<td>--</td>
<td>20 min</td>
<td>Kang et al. 2009</td>
</tr>
<tr>
<td>19</td>
<td>12</td>
<td>DMSO</td>
<td>R. T.</td>
<td>1 hr</td>
<td>Lim et al. 2015</td>
</tr>
<tr>
<td>77</td>
<td>12</td>
<td>Water</td>
<td>--</td>
<td>15 min</td>
<td>Gutierrez-Zuniga et al. 2016</td>
</tr>
<tr>
<td>4</td>
<td>11 (sulfo-NHS)</td>
<td>100 mM MES, pH 4.7</td>
<td>R. T.</td>
<td>15 min</td>
<td>Vashist et al. 2011</td>
</tr>
<tr>
<td>38</td>
<td>6</td>
<td>--</td>
<td>--</td>
<td>30 min</td>
<td>Fodey et al. 2011</td>
</tr>
<tr>
<td>3.8</td>
<td>0.6</td>
<td>PBS, pH 7.4</td>
<td>R. T.</td>
<td>2 hr</td>
<td>Lin et al. 2014</td>
</tr>
<tr>
<td>0.4</td>
<td>11.5 (sulfo-NHS)</td>
<td>Phosphate, pH 7</td>
<td>R. T.</td>
<td>15 min</td>
<td>Soukka et al. 2001</td>
</tr>
</tbody>
</table>

Table 4-2: Conditions tested during activation buffer optimization. “Polymer Functionality” indicates the siloxane attached during acid equilibration.

<table>
<thead>
<tr>
<th>Polymer Functionality</th>
<th>Solution Composition</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl, Ester</td>
<td>Distilled water</td>
<td>n/a</td>
</tr>
<tr>
<td>Ester</td>
<td>20 mM MES</td>
<td>5.5</td>
</tr>
<tr>
<td>Octyl, Ester</td>
<td>20 mM MES</td>
<td>6.0</td>
</tr>
<tr>
<td>Ester</td>
<td>20 mM MES</td>
<td>6.5</td>
</tr>
<tr>
<td>Ester</td>
<td>20 mM phosphate</td>
<td>6.5</td>
</tr>
<tr>
<td>Octyl, Ester</td>
<td>20 mM phosphate</td>
<td>7.0</td>
</tr>
</tbody>
</table>

In this experiment, the 3 x 1 hour treatment method was used for chain exchange. Anti IgG-AP was attached directly to the siloxane surface following activation and the cuvettes were tested for alkaline
phosphatase activity in pH 8.0 Tris buffer with 40 µM MUP as a substrate both before and after washing with PBS-T.

4.2.2.3.2 Activation Molecule Concentration Optimization

The concentrations of EDAC.HCl and NHS in the activation solution could also impact the amount of activated ester present on each film surface. This could in turn affect the levels of attached and active antibody-enzyme conjugate (Anti IgG-AP). In this experiment, the general method described above was followed, and the concentrations of EDAC.HCl and NHS in pH 6.0 MES were varied in the activation step (Table 4-3).

**Table 4-3:** EDAC and NHS concentrations used in the activation solution optimization.

<table>
<thead>
<tr>
<th>EDAC Concentration (mg/mL)</th>
<th>NHS Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

The activation conditions were compared using PMOS-PDMS-functionalized surfaces, to control for changes in the surface that were not carboxylic acid-specific, and contrasted with attachment in the absence of an activating step. The success of the various methods was based on activity measurements of the attached Anti IgG-AP in 40 µM MUP in Tris buffer at pH 8.0 both before and after washing the films with PBS-T.

4.2.2.3.3 Activation Time Optimization

A study of the duration of the activation step was carried out similarly to Section 4.2.2.3.2. The standard surface treatment method was used, as described above. During the activation step, the films
were treated in 1 mg/mL EDAC.HCl, and 0.1 mg/mL NHS in 20 mM MES at pH 6.0 for either 10
minutes, 2 hours, or 4 hours. Anti IgG-AP was attached to the surface and activity testing was carried out
as described previously.

4.2.2.4 Effect of Solution Antibody Concentration on Immobilized Activity

The concentration of antibody used in the covalent immobilization step was also optimized. This
experiment was carried out by the standard method above, with some exceptions. The chain exchange step
was completed with 3 x 1 hour treatments using 75 µL in each treatment. Both ester and octyl-
functionalized siloxanes were used for comparison. In the covalent antibody immobilization step, a
variety of Anti IgG-AP concentrations were used between 0.5 µg/mL and 10 µg/mL in phosphate buffered
saline. The activity of alkaline phosphatase on the resulting films was tested as usual, except that 44.4 µM
MUP was used in Tris pH 8.0.

4.2.2.5 Effect of Antibody Solution Volume on Immobilized Activity

The original protocol for these experiments used a 400 µL treatment solution for the antibody
binding steps. However, this volume was more than what was required to cover the surface of the siloxane
polymer in the cuvette, and this could allow other reactions to occur on the cuvette plastic surface.
Antibody immobilization was a particular concern since the goal was to immobilize antibodies on the
siloxane surface for a maximum product uptake rate. Thus, the effect of varying the volume of this
treatment step was investigated. In reducing the volume of the treatment solution, the concentration of the
antibody was increased by a corresponding amount.

This experiment was done using a model ELISA, rather than with direct conjugate
immobilization. After chemical treatment steps were completed as described in the standard method,
Rabbit IgG was immobilized on the films in one of three conditions: i) 400 µL volume, 5 µg/mL
concentration, ii) 200 µL volume, 10 µg/mL concentration, and iii) 100 µL volume, 20 µg/mL concentration. The smallest volume that was capable of covering the siloxane surface was 100 µL. The immobilization solution was made in 12 mM phosphate pH 8.0, containing 0.05% Tween 20, which, it will be shown later, reduced non-covalent binding of the antibodies to the polymer surface. Following this step, the surface was washed with PBS-T, blocked with BSA blocking solution, Anti IgG-AP was bound at 2.0 µg/mL, and the surface was washed again. After this process, activity measurements were carried out using 40 µM MUP in Tris pH 9.0.

4.2.3 Comparison of Immobilization Methods

4.2.3.1 Initial Activity Comparison

The attachment of Protein A followed by binding of Rabbit IgG was compared with direct immobilization of IgG on three immobilization surfaces: unmodified Sylgard, PMOS-PDMS modified Sylgard, and PCBMS-PDMS-modified Sylgard. PMOS-PDMS modified Sylgard acted as a control for attachment to the modified siloxane that was not specific to carboxyl functionality, while the comparison with unmodified Sylgard represented an alternative (adsorption-based) attachment scheme. This comparison was carried out as described above in the general method. For the unmodified Sylgard data set, cuvettes were prepared with cured Sylgard 186 films, but not subjected to any chemical treatment. Thus, they were treated immediately with Rabbit IgG, which was allowed to adsorb to the surface at 4°C overnight. The covalently immobilization of Rabbit IgG on ester-modified Sylgard was carried out as described in the general method. For Protein A-mediated binding samples, the binding protein was immobilized from PBS at 50 µg/mL at 4°C overnight. IgG was bound to these Protein A functionalized cuvettes in a solution at 5 µg/mL in 12 mM phosphate pH 8.2 for 1 hour at room temperature. All other
steps were carried out as described earlier. Activity testing was completed with 40 µM MUP in Tris pH 9.0.

4.2.3.2 Comparison of Immobilization Methods in Buffer with Detergent

The presence of surfactants in the covalent immobilization buffer is known to decrease the quantity of adsorbed proteins. In order to compare the nature of the protein attachment to siloxane on unmodified and ester modified materials, an ELISA experiment was completed under these conditions. IgG was immobilized in 12 mM phosphate pH 8.0, with half the samples containing 0.05% (by volume) Tween 20 as well. Washing and blocking the surface, as well as Anti IgG-AP binding, and a final wash step, were carried out as described in the general method. Finally, activity testing using 40 µM MUP in Tris pH 9.0 was completed.

4.2.3.3 Stability of ELISA Using Various Immobilization Methods

The stability of the cuvettes produced through different biomolecule attachment methods was analyzed by completing a model ELISA using cuvettes that were stored in a refrigerator for multiple weeks after initial biofunctionalization. The experiment consisted of the necessary chemical treatments, followed by immobilization of the first biomolecule (IgG or Protein A), a wash step, blocking, second biomolecule binding (IgG or Anti IgG-AP), a wash, third biomolecule immobilization if necessary (Anti IgG-AP), a final wash if necessary, and activity testing using 40 µM MUP in Tris pH 9.0 (see above, Figure 4-8). Surface treatment methods that were tested included: untreated Syglard with adsorbed IgG, ester-functionalized siloxane with covalently bound IgG, and ester-functionalized siloxane with covalently bound Protein A. Immobilization in the presence and absence of Tween 20 was also tested for covalent immobilization conditions. For Protein A conditions, a set of films was stored after the
immobilization of Protein A, while another set was stored after IgG was bound to Protein A. This led to a large number of test groups, as shown in Table 4-4.

Table 4-4: Testing conditions for the analysis of the stability of modified cuvettes over time. “Storage” indicates the step after which the cuvette were stored in the refrigerator for later testing.

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemical Surface Functionalization</th>
<th>Surface Bound Protein</th>
<th>Detergent in Immobilization Buffer</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>IgG</td>
<td>No</td>
<td>After IgG attachment</td>
</tr>
<tr>
<td>B</td>
<td>Ester</td>
<td>Protein A</td>
<td>No</td>
<td>After Protein A attachment</td>
</tr>
<tr>
<td>C</td>
<td>Ester</td>
<td>Protein A</td>
<td>No</td>
<td>After IgG attachment</td>
</tr>
<tr>
<td>D</td>
<td>Ester</td>
<td>Protein A</td>
<td>Yes</td>
<td>After Protein A attachment</td>
</tr>
<tr>
<td>E</td>
<td>Ester</td>
<td>Protein A</td>
<td>Yes</td>
<td>After IgG attachment</td>
</tr>
<tr>
<td>F</td>
<td>Ester</td>
<td>IgG</td>
<td>No</td>
<td>After IgG attachment</td>
</tr>
<tr>
<td>G</td>
<td>Ester</td>
<td>IgG</td>
<td>Yes</td>
<td>After IgG Attachment</td>
</tr>
</tbody>
</table>

The films were stored containing 1 mL of BSA blocking solution, covered with Parafilm and maintained at 4°C in the refrigerator. After removal on the required day, the treatment was continued as described in the general method. Stability tests were performed at 0, 3, 6, and 9 weeks after initial treatment.

4.3 Results and Discussion

4.3.1 Examination of Protein Quantitation Methods

4.3.1.1 Protein Immobilization and Analysis by ATR-FTIR

After overnight adsorption of BSA on Sylgard films, ATR-FTIR spectra were obtained. The results of these experiments were inconsistent (Figure 4-9).
Figure 4-9: ATR-FTIR spectra of siloxane films exposed to bovine serum albumin solution at a range of concentrations from 0 to 0.83 mg/mL.

The concentration of protein in the adsorption solution did not correlate with the magnitude of the absorbance peak. When compared to the spectrum for the Sylgard film that was exposed to buffer, the films exposed to the three lower concentrations of BSA (0.27 mg/mL, 0.42 mg/mL, and 0.56 mg/mL) exhibited the expected Amide I (1650 cm\(^{-1}\)) and Amide II (1550 cm\(^{-1}\)) vibrations.\(^{66}\) The intensities of these peaks, while low, were consistent with the expected results for the adsorption of BSA on surfaces.\(^{81}\) The featureless nature of the amide I band could indicate a disordered secondary structure, consistent with adsorption to a surface.\(^{66}\) In contrast, the next highest BSA concentration (0.70 mg/mL) produced a spectrum almost identical to that of the buffer-exposed film. This observation illustrates one of the challenges of ATR-FTIR for surface analysis. It was possible that the BSA adsorption was not uniform across the film surface and this measurement could have been taken from a region of the film with a
minimal BSA concentration, resulting in the lack of protein signal. It was also possible that the protein adsorption was inconsistent between films, but this could not be determined with certainty from these results. Another challenge in interpreting these results was that the quality of contact between the ATR crystal and the film had an effect on the spectrum intensity, which was difficult to avoid. If this equally affected all peaks in the spectrum, it may have been possible to normalize the spectra to reduce this source of variability.

The adsorption solutions used here contained high protein concentrations (mg/mL) relative to what was feasible with the antibody solutions (µg/mL), and this, along with the limitations described, meant that ATR-FTIR was not a useful quantitative technique for optimizing protein attachment to siloxanes. This observation was confirmed in a further experiment, which attempted to repeat the procedure with anti rabbit immunoglobulin G-alkaline phosphatase at 2 µg/mL, and in which no characteristic protein absorption peaks were seen in the ATR-FTIR spectrum.

4.3.1.2 Contact Angle Measurements of Protein Immobilization on Siloxane Surfaces

After overnight BSA adsorption on Sylgard 184 films and washing the surfaces with PBS-T, advancing contact angle measurements were taken using distilled water. As shown in Figure 4-10, the adsorption of BSA from solutions containing less than 10 mg/mL of protein did not result in a significant change in contact angle. However, films that were exposed to BSA solutions at 10 and 20 mg/mL exhibited a significantly lower advancing contact angle relative to films that were exposed to buffer alone. For the surface exposed to 20 mg/mL BSA, the advancing contact angle was approximately 102° ± 5°, a decrease of 18° ± 6°. While this change was significant, other researchers have found that layers of BSA immobilized on hydrophobic surfaces produced contact angles of approximately 55°, when adsorbed from a 20 mg/mL solution. It is possible that PDMS does not adsorb proteins to the same degree as other materials, meaning that less BSA was adsorbed. The literature results quoted above were also completed
Figure 4-10: Advancing contact angle measurements for Sylgard 184 films exposed to bovine serum albumin solution concentrations between 0 and 20 mg/mL overnight. Each point represents the average of three replicate measurements and the error bars represent the standard deviation (n = 3).

on a heterogeneous membrane surface, which may have had an impact on the water contact angle.\textsuperscript{82} Finally, advancing contact angles tend to be affected more strongly by hydrophobic regions on a material.\textsuperscript{83} Since protein adsorption would lead to hydrophilic regions, the advancing angle would be expected to be show a smaller change than a static or receding angle measurement.

In the literature, a solution concentration of 2.5 mg/mL of BSA was sufficient to produce monolayer protein coverage on a poly(methylmethacrylate) surface.\textsuperscript{84} These previous data do not correspond with the results reported here, since the contact angle only began to change measurably at 10 mg/mL BSA. However, the different surface properties make any direct comparison difficult; PDMS is well-known for its tendency to non-specifically adsorb proteins due to its hydrophobicity,\textsuperscript{85} while poly(methylmethacrylate) is not.\textsuperscript{24} It is therefore possible that higher concentrations of BSA could be required to produce a monolayer on PDMS. Another possibility is that the surface tension of the modified
film is affected more strongly by multilayer coverage, which could be occurring at higher protein concentrations.

These results demonstrated that the useable concentration range for contact angle analysis of protein immobilization was in the mg/mL range, approximately one thousand times the concentrations used in immunoglobulin immobilization experiments. As a result, contact angle measurements were not useful in optimizing the antibody-functionalization process. However, 20 mg/mL was the BSA concentration used to block protein adsorption on the surfaces of siloxanes following the immobilization of the capture antibody during immunoassays. From these contact angle results, it was shown that this concentration of blocking protein resulted in a significant modification of the surface properties of the siloxane film, which was consistent with successful blocking.

4.3.1.3 Enzyme Activity for Quantitation of Immobilized Protein

4.3.1.3.1 Role of Buffer pH in Alkaline Phosphatase Enzyme Activity

When alkaline phosphatase or alkaline phosphatase conjugate activity was characterized in solution using 4-methylumbelliferyl phosphate, a typical enzyme activity plot was obtained, demonstrating linear behaviour, consistent with pseudo first order kinetics, in the initial phase. (Figure 4-11) This initial phase lasted at least 10 minutes for the enzyme concentrations tested. The slope of the linear portion of this type of plot, representing the initial rate, was used to compare activity under different conditions. Using Tris and carbonate buffers and a variety of pH conditions, it was found that pH 9.0 Tris buffer produced the greatest activity for a fixed alkaline phosphatase concentration (5 ng/mL) (Figure 4-12). This does not fully agree with the literature, which suggests that pH values above 9 should result in higher activity.86,87 However, the optimum is known to vary based on substrate type and concentration,
Figure 4-11: 40 μM 4-methylumbelliferyl phosphate cleavage to 4-methylumbelliferone by 1 ng/mL Anti Rabbit Immunoglobulin G-Alkaline Phosphatase in Tris buffer at pH 8.0. The fluorescence signal produced by 4-methylumbelliferone was monitored at 445 nm using a 365 nm LED for excitation.

Figure 4-12: The average slope of the fluorescence vs. time plot obtained for solution phase alkaline phosphatase (AP) (5 ng/mL) with 4-methylumbelliferyl phosphate in a variety of buffers. The error bars represent the standard deviations of the data set (n=3).

among other factors. When 4-MUP was mixed in pH 9.0 Tris buffer containing no alkaline phosphatase, no cleavage was observed based on the fluorescence intensity at 445 nm (data not shown), indicating that the substrate cleavage at pH 9.0 can be attributed to the catalysis by the enzyme.
4.3.1.3.2 Solution-Phase Activity of Anti Immunoglobulin G-Alkaline Phosphatase

Both pH 8.0 and pH 9.0 Tris buffer were used for a more detailed characterization of Anti IgG-AP conjugate activity in solution (Figure 4-13). Despite being below the optimal pH, the characterization was completed at pH 8.0 because some early immobilization studies were performed in this condition. The data from this experiment demonstrated again that pH 9.0 was a more optimal pH for alkaline phosphatase activity. The linear relationship between the slope of the kinetics plot and the conjugate (IgG-AP) concentration confirmed that the enzyme conjugate reaction rate was proportional to conjugate concentration.

![Graph showing enzyme activity at pH 8.0 and pH 9.0](image)

**Figure 4-13:** Activity characterization of Anti Rabbit Immunoglobulin G-Alkaline Phosphatase in solution using 40 µM MUP as substrate in Tris pH 8.0 and pH 9.0 buffer. The average slopes were calculated from the fluorescence at 445 nm vs. time (hours) plot. For pH 8.0, the error bars represent the standard deviation of the set (n=3). For pH 9.0, only one set of experiments was completed.

This was the expected result, based on enzyme kinetics theory. The linear regression equations for these plots were later used to determine effective surface immobilized conjugate concentrations for Anti IgG-AP in further experiments.
4.3.2 Covalent Protein Immobilization Method Development

4.3.2.1 Effect of Siloxane Chain Exchange Conditions on Immobilized Protein Activity

The role of the siloxane chain exchange procedure in antibody-enzyme conjugate immobilization was investigated by performing immobilization on surfaces that were functionalized with PCBMS-PDMS and hydrolyzed to produce carboxylic acid groups. It was found that ester-functionalized surfaces bound more active Anti IgG-AP than octyl-functionalized surfaces (Figure 4-14).

![Graph](attachment:image.png)

**Figure 4-14:** Activity of Anti IgG-AP attached in the covalent linkage step to octyl or ester-modified Sylgard in cuvettes. Films were compared on the basis of the length of the chain exchange step and the type of functional siloxane introduced in the process. Activity was tested in pH 8.0 Tris buffer both before and after washing the films with PBS-T. The error bars represent the standard deviation of each data set (n=3).

This result corresponds to the expected mechanism of attachment of biomolecules to the two functionalized surfaces. Octyl functionalized siloxanes should not contain sites for covalent biomolecule attachment whereas the ester-functionalized materials are expected to contain covalent linkage sites. Based on these results, it appeared that extending the chain exchange treatment beyond 30 minutes did not have an effect on the degree of active conjugate immobilized. This indicated that the chain exchange reaction reached completion within the first half hour and that further treatment was unnecessary. This
agreed with the findings of other researchers, who found that a 30 minute chain exchange treatment was sufficient for surface modification. The same researchers observed that the use of isopropanol, rather than methanol, as the chain exchange solvent led to increased brittleness and cloudiness of the siloxane. Since methanol did not dissolve the functionalized oligomers used in this study, it would be ideal to use the shortest equilibration time possible to reduce damage to the film. Thus, a single 30 minute treatment was used for further siloxane chain exchange procedures.

4.3.2.2 Effect of Acid Hydrolysis Conditions on Immobilized Protein Activity

The effect of hydrolysis of the methyl ester functional group on the siloxane surface in the presence of 5 M hydrochloric acid was determined in terms of the amount of immobilized active Anti IgG-AP. Control films were treated in neutral water during the acid hydrolysis step to determine the effect of acid catalysis. Since activated esters formed from carboxylic acids were required for the covalent attachment process, it was predicted that catalyzing hydrolysis of the methyl ester would increase the quantity of protein attached to the surface. As expected, it was found that the use of hydrochloric acid led to significantly more Anti IgG-AP activity on the modified Sylgard surface following washes with PBS-T. There was no significant decrease in Anti IgG-AP activity on the ester-modified and acid hydrolyzed surface following the detergent washing step, indicating that the antibody-enzyme conjugate was strongly bound to this surface. By comparison, the ester-modified surface that was not hydrolyzed in acid displayed a loss of approximately 40% of the conjugate activity following washing (Figure 4-15). Octyl-modified films, which acted as a control for the effect of hydrolysis on the siloxane backbone, showed significantly less Anti IgG-AP activity on the surface, whether or not acid catalysis was included. A t-test showed that the difference in immobilized, active Anti IgG-AP between hydrolysis in water and in 5 M HCl for ester-functionalized films was significant (t=3.5199, 4 degrees of freedom, 95% confidence). This
Figure 4-15: The relative IgG-AP activity present on siloxane films that were functionalized with octyl or ester-containing siloxane and subsequently hydrolyzed in either water or 5 M HCl. Activity was tested both before and after washing the surfaces with PBS-T in pH 8.0 Tris buffer. The error bars represent the standard deviation of the data set (n=3).

Information meant that the acid hydrolysis step, which should cleave the methyl ester group to produce carboxyl functionality, was an important part of the biomolecule attachment procedure.

4.3.2.3 Optimization of Carboxylic Acid Activation Conditions

4.3.2.3.1 Buffer Type and pH Optimization

In the scientific literature on covalent linkage through carboxyl groups, a wide variety of solutions are used for carbodiimide-mediated activation (see Table 4-1). Along with the fact that a variety of pH conditions have been employed, it is clear that there is no standard method for the activation process. Despite this lack of agreement, the pH of the activating solution is important since the protonation state of the reactants (carboxylic acid, succinimide, and carbodiimide) could affect the process. In fact, it is known that the activation reaction with EDAC.HCl is most efficient in acidic environments (e.g. pH = 4.7 - 6). It was important to optimize the activation conditions based on the particulars of each experiment since a set of rules governing the best conditions was not available. Distilled water, phosphate buffer, and
MES buffer were all tested for use as activation solutions since they appeared most commonly in the literature. The pH values tested for each buffer fall within their optimal buffering ranges.

In this experiment, high activity from Anti IgG-AP was seen on surfaces treated with ester-functionalized siloxanes, relative to those treated with octyl-functionalized siloxanes, after washing with PBS-T. This was consistent with the ability of the ester-modified polymer to covalently immobilize, or strongly adsorb, proteins. The use of distilled water, or MES buffer at pH 6.0 and 6.5, seemed to lead to higher levels of activity, while phosphate buffer produced similar or lower activity levels (Figure 4-16).

![Bar chart](image)

**Figure 4-16:** Anti IgG-AP activity on siloxane films functionalized with ester or octyl polymer and activated in a variety of solutions, including distilled water, MES buffer, and phosphate buffer. The activity was tested both before and after washing each film with PBS-T. The error bars represent the standard deviation of the data set (n=3).

The large error bars for all samples demonstrate that activity levels were very inconsistent between films, especially prior to washing. This was partially attributed to the surface area of the polymer, which may have been slightly inconsistent. Possibly the largest source of variability, however, was the newly-assembled multi-chamber spectrometer. There were still inconsistencies in the signal obtained from the four chambers and a large amount of variability in signal upon repeated insertions of the same fluorescent
cuvette. These difficulties were resolved in other experiments by standardizing the spectrometer chambers using a solution of 4-methylumbelliferone and changing the integration times for each accordingly, as well as using supports to stabilize the cuvettes within the chamber. Nevertheless, it was determined that the use of distilled water or MES buffer (pH 6.0 or 6.5) led to improved levels of activity relative to other conditions tested. Since EDAC-NHS linking chemistry is expected to be more effective at acidic pH values, this result was consistent with the literature. It was decided to use MES at pH 6.0 in future experiments, since the pH of a buffer can be controlled between and during experiments, unlike distilled water.

4.3.2.3.2 Activation Molecule Concentration Optimization

Since EDAC.HCl and NHS worked in tandem to activate a carboxyl-functionalized surface, it was important to optimize the relative concentrations of the two components. Many publications have used a range of concentrations and ratios for this step, showing again that there is no consensus on the best way to execute this treatment (Table 4-1). Based on the Anti IgG-AP activity data obtained in this experiment, no significant differences in immobilized active conjugate resulted from the use of different concentrations or ratios of EDAC.HCl and NHS, so the previously used procedure (1 mg/mL EDAC.HCl, 0.1 mg/mL NHS) was retained (Figure 4-17). The octyl-functionalized films showed reduced activity relative to the ester-functionalized films. In addition, the use of water alone in place of activating chemistry resulted in lower average levels of activity on the films, as well as a very high degree of variability. While biomolecules are known to strongly adsorb to carboxyl-functionalized surfaces that have not been activated, this experiment showed that higher activity levels for Anti IgG-AP were observed when activating chemistry was used.
4.3.2.3.3 Activation Time Optimization

In activating the carboxyl groups for biomolecule attachment, the duration could be an important factor. Activation could have been insufficient if the reaction time was too short, while undesirable side reactions of the water-sensitive activating groups, such as hydrolysis, could occur if the reaction was too long. In this experiment, it was found that 10 minutes of activation reaction was insufficient to obtain maximum activity on the surfaces from Anti IgG-AP, while both 2 and 4 hours provided similar results (Figure 4-18). This suggested that the activation reaction reached completion between 10 minutes and 2 hours of treatment time and did not decay substantially until more than four hours had passed. This result was consistent with the work of Palazon et al., who found that activation was complete in less than 30 minutes and decayed soon afterwards (30 minutes or 2 hours, depending on activation conditions), leaving no remaining evidence of activated ester at 24 hours. As a result of these experiments, two hours was used for activation in further experiments.
Figure 4-18: Anti IgG-AP activity on siloxane films functionalized with ester or octyl polymer and activated for various amounts of time. The activity was tested both before and after washing the films with PBS-T. The error bars represent the standard deviation of the data set (n=3).

4.3.2.4 Effect of Solution Antibody Concentration on Immobilized Activity

The concentration of antibody used in the surface immobilization step was important, balancing the need for sufficient biomolecule to allow a successful immobilization with the cost of using antibodies at high concentrations. Microgram per milliliter concentrations are usually recommended for this purpose, but the exact concentration required depends on the experiment.

In this analysis, it was found that Anti IgG-AP solution concentrations below 1 µg/mL on the ester-modified film resulted in surface activity as low as that observed for the conjugate on the octyl-modified film, but as the concentration increased, the observed activity increased on the ester-modified films (Figure 4-19). While the maximum immobilized activity appeared to occur at 10 µg/mL or greater conjugate concentration, it was decided that 5 µg/mL antibody concentration represented an appropriate balance between high activity and minimal cost per cuvette. In contrast, it was observed that octyl-modified films immobilized approximately the same low level of active conjugate regardless of the Anti
Figure 4-19: The relative amount of IgG-AP activity immobilized on films modified with octyl and ester functional groups. For the ester-functionalized films, the error bars represent the standard deviation of the set (n=3, except n=2 for 10 µg/mL Anti IgG-AP). For the octyl-functionalized films, only one measurement was completed at each concentration.

IgG-AP concentration used in the binding solution. This might suggest that available adsorption sites on this material were occupied at this point and further immobilization was not possible, or that the octyl-modified surface was not as amenable to protein immobilization.

4.3.2.5 Effect of Antibody Solution Volume on Immobilized Activity

An ELISA using IgG as the analyte and Anti IgG-AP as the detection antibody was used to determine the effect of the surface immobilization solution volume on the immunoassay signal. The volume of the antibody immobilization solution did not appear to have an effect on the antibody-enzyme conjugate activity of the subsequent ELISA (Figure 4-20). Given that the capture antibody (IgG) concentration was increased proportionally with each decrease in volume, the total mass of antibody in the immobilization solution remained constant, which may explain the lack of trend in the data. In addition, detergent was used in the covalent immobilization buffer, meaning that antibodies would not be expected to adhere to the cuvette plastic. It is possible that the antibody immobilization volume may
4.3.3 Comparison of Immobilization Methods

4.3.3.1 Initial Activity Comparison

A comparison between covalent immobilization, adsorption, and affinity-based binding was carried out for the model immunoassay system consisting of Rabbit IgG and Anti IgG-AP. The immobilization of IgG directly on siloxane, compared to affinity binding through Protein A, produced varied results depending on the surface being used (Figure 4-21). On unmodified Sylgard, on which only adsorption was expected, the use of Protein A as an intermediate layer resulted in a 70% reduction in immunoassay activity, relative to samples in which IgG was directly adsorbed. This suggested that IgG adsorbed on the Sylgard surface more strongly than did Protein A. PMOS-PDMS modification resulted in lower levels of IgG-AP activity in the immunoassay, with or without Protein A. Finally, ester modified surfaces produced similar ELISA signals whether or not Protein A was used as an intermediary. This may
Figure 4-21: Comparison of signal generated by ELISA on surfaces modified by different methods: unmodified Sylgard 186, Sylgard modified with PMOS-PDMS, and Sylgard modified with PCBMS-PDMS. Direct immobilization of IgG, versus binding through Protein A, was also compared. The error bars represent the standard deviation of the values (n=3).

mean that covalent immobilization resulted in comparable amounts of IgG on the surface, whether the IgG was directly immobilized or bound to Protein A. This result suggested that stronger bonding of protein to the surface could occur with the ester-modified material. One reason for the similar results for attachment schemes with and without Protein A may be that this assay is truly an antigen down experiment, rather than a sandwich. The orientation of the antibody on the surface would therefore be less critical than in a sandwich assay. Finally, it was shown by this experiment that unmodified Sylgard bound approximately the same amount of active ELISA antibodies as ester-modified Sylgard and, interestingly, that the octyl modification actually reduced the amount of bound active protein relative to unmodified Sylgard.

4.3.3.2 Comparison of Immobilization Methods in Buffer with Detergent

A dramatic difference was seen when comparing the presence and absence of detergent in the immobilization buffer for unmodified and ester-modified siloxane materials. On unmodified Sylgard,
almost no immunoassay activity was observed when the IgG immobilization was attempted in the presence of Tween 20. On the other hand, PCBMS-PDMS-treated Sylgard showed significant activity, even when immobilized in the presence of Tween 20 (Figure 4-22).

![Figure 4-22:](image)

**Figure 4-22:** Comparison of signal generated by ELISA on ester-modified and unmodified Sylgard, showing the effect of Tween 20 in the IgG surface immobilization buffer. The error bars represent the standard deviation of the values (n=5).

The 70% decrease in activity for the ELISA on PCBMS-PDMS in which IgG was immobilized in the presence of Tween 20 could have been caused by disruption of non-covalent interactions with the surface. Another possibility was that Tween 20, which contained hydroxyl functionality, may have reacted with the activated esters on the siloxane surface, using covalent bonding sites that would otherwise have attached proteins. However, high levels of enzyme activity were present on these films, even when detergent was used, indicating that strong attachment did occur. This experiment provided evidence that covalent bonding to the surface occurred when PCBMS-PDMS was used.

4.3.3.3 Stability of ELISA Using Various Immobilization Methods

Although attachment to ester-modified siloxanes did not significantly increase the binding of IgG to the polymer surface, relative to unmodified Sylgard 186, it was possible that this process would
enhance the long-term stability of the biomolecules. The stability of the siloxane-immobilized proteins during long-term storage was investigated using an ELISA method that was performed under various immobilization and storage conditions (Table 4-4). The use of physical adsorption to unmodified Sylgard as well as immobilization on ester-modified Sylgard (PCBMS-PDMS) was compared. Other factors of interest were the use of Protein A as an orientation-specific binding mediator, the presence of detergent (Tween 20) in the immobilization buffer, and conditions of storage (after Protein A immobilization, or after IgG immobilization). Cuvettes in each test group were stored with 1 mL of blocking buffer in a refrigerator until analysis.

There were notable differences in the ELISA outcomes depending upon the conditions used for cuvette preparation. Most importantly, there appeared to be no significant ELISA activity loss in cuvettes prepared by adsorption of IgG, following 9 weeks of storage (Figure 4-23).

![Figure 4-23: Stability experiment for a model ELISA system in which Rabbit IgG was immobilized on unmodified Sylgard 186 without Tween 20 in the buffer. The location of the dash symbol indicates the point in the ELISA at which the samples were stored prior to testing. The error bars represent the standard deviation of each set (n=5).](image)

This suggested that the antibodies adsorbed on the Sylgard surface remained active and were not significantly desorbed from the material during storage. In comparison to the samples immobilized on
ester-modified Sylgard (without Tween), there was no significant benefit to the use of the chemical attachment procedure (Figure 4-24).

![Graph](image)

**Figure 4-24:** Stability experiment for a model ELISA system in which Rabbit IgG was immobilized on PCBMS (ester) without Tween 20 in the buffer. Samples compared direct attachment of IgG and the use of Protein A (PrA) as a mediator in the IgG attachment process. The location of the dash symbol indicates the point in the ELISA at which the samples were stored prior to testing: some samples were stored after Protein A attachment, while others were stored after IgG attachment. The error bars represent the standard deviation of each set (n=5).

The addition of Tween 20 to the immobilization buffer had a large negative effect on the stability of IgG attached directly to PCBMS-PDMS-modified surfaces, but no effect on the initial activity was observed (Figure 4-25). Storage of Protein A modified cuvettes before or after the attachment of IgG had no significant effect on the stability of the cuvette activity.

The most important conclusion of this experiment was that the stability of adsorbed and covalently immobilized antibodies in cuvettes was similar over the nine-week experiment, meaning that covalent immobilization may not have imparted any useful advantages for our ELISA system. Solid-phase adsorbed antibodies are known to be stable for multiple weeks when stored in buffer solution in cold conditions.
Figure 4-25: Stability experiment for a model ELISA system in which Rabbit IgG was immobilized with Tween 20 in the buffer (it was expected that Tween 20 should reduce protein adsorption). PCBMS-modified materials were used to immobilize Protein A (PrA) as a mediator in the IgG attachment process. The location of the dash symbol indicates the point in the ELISA at which the samples were stored prior to testing: some samples were stored after Protein A attachment, while others were stored after IgG attachment. The error bars represent the standard deviation of each set (n=5).

As a result, it was not surprising that antibody activity was not impaired by the 9-week storage time on some surfaces. For longer term storage, many protocols recommend storing antibody-coated microwell plates dry, and since desorption would then not be a risk, the method of antibody binding would not be expected to impact storage under these conditions. Since the use of adsorbed antibody did not seem to impair the activity or stability of the coated cuvettes and this protocol involved less preparation, reagents, and time, it was used in further ELISA procedures.

4.4 Conclusions

Multiple methods of analysis for the immobilization of proteins on siloxane materials were investigated. While contact angle and ATR-FTIR procedures were successful under some conditions, the sensitivity and reliability of these methods was inferior to the use of enzyme assays. Using the alkaline
phosphatase enzyme assay protocol, many aspects of the covalent immobilization procedure for antibodies on ester-modified siloxane materials were optimized.

While the covalent nature of the immobilization could not be confirmed, evidence was provided that the ester-modified surface led to a stronger immobilization: Protein A was effectively immobilized on the ester-functionalized surface, but not on unmodified Sylgard. In addition, the inclusion of Tween 20 in the coating buffer during the immobilization of antibodies on unmodified Sylgard led to an almost complete loss of ELISA activity, while significant activity was retained on the ester-modified surface coated under the same conditions. However, it was found that the covalent immobilization method did not improve the stability of antibody-coated cuvettes over the course of two months, meaning that the use of adsorbed antibody may be sufficient in many cases.

It is important to note that, while the covalent immobilization protocol was optimized, the variables of the adsorption procedure (time, temperature, buffer conditions) were not fully investigated. High levels of ELISA activity were observed despite this lack of optimization. However, in the future, it would be useful to test these variables to determine if even higher activity levels can be achieved.

Based on the results of the Protein A-mediated ELISA, the use of the orientation-specific mediator did not impart any advantages in terms of signal or stability for the assay used here. However, in a sandwich ELISA, the use of an oriented immobilization procedure could improve the assay results. Under such conditions, the use of the ester-modified surface would be advantageous since higher levels of ELISA activity were observed for Protein A-bound IgG under these conditions. Future work on this system could involve the search for protocols in which Protein A-mediated immobilization would be an advantage.
A model ELISA has been demonstrated to function similarly on ester-modified and unmodified PDMS in semi-micro cuvettes. Given the similar stability and activity results obtained, surfaces functionalized by adsorption were carried on to the development of the SPME-linked ELISA in the following work.

4.5 Acknowledgements

The protein attachment experiments would not have been possible without the assistance of Katie Flynn, an undergraduate summer student who performed many of the studies. Her enthusiasm and curiosity were a great asset in this project. A number of other undergraduate students also contributed to the development of this area of the project: Sara Toja Ortega (ATR-FTIR), Aitor Bermejo Arteagabeitia (contact angles), Tiffany Chai (preliminary investigation of dye assays of protein), Jeffrey McCarthy (preliminary characterization of surface-immobilized enzyme activity), Stephanie Fong (initial experiments on surface-immobilized ELISA), and Timothy Hutama (preliminary investigation of Protein A immobilization). In addition, I would like to acknowledge the assistance and support of Dr. Igor Kozin, for training and access to the undergraduate instrument room (ATR-FTIR, UV-Vis), as well as David Simon and Dr. David Zechel, who assisted with SDS-PAGE gels during my experiments in enzyme-antibody conjugation.

4.6 List of References


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Chapter 5

Development and Applications of a Solid Phase Microextraction ELISA System

5.1 Introduction

A wide variety of waterborne pathogens can contaminate sources of drinking water in both developed and developing countries and such contamination can lead to outbreaks of diseases including diarrhea, Legionnaire’s disease, cholera, typhoid, and many others. Because of the wide distribution of water supplies, the presence of these pathogens in drinking water systems can lead to large-scale disease outbreaks affecting huge populations, as was the case when Cryptosporidium parvum contaminated the drinking water supply in Milwaukee, Wisconsin, infecting 403,000 people and causing 54 deaths in 1993. To prevent such widespread outbreaks, as well as the many smaller incidents of contamination that occur on a regular basis, it is imperative to analyze the microbiological quality of drinking water.

Due to the numerous species of potential pathogens in water, the costs associated with monitoring each individual species are prohibitive in terms of both time and money. Since many waterborne pathogens enter the water supply through fecal contamination, total coliforms and Escherichia coli, which are reliably present in such contamination, are used as water quality indicators. The concentration of these indicator species must be less than 1 colony forming unit (CFU) per 100 mL water sample to be considered safe to drink. Methods of detecting these indicator organisms were reviewed in Chapter 1. Despite the general success of the indicator analysis methods, challenges still exist in drinking water quality testing. The existing indicator analysis methods are slow relative to the public health challenges that may emerge due to pathogenic water contamination. The approved water analysis methods
all require a culturing step to amplify the number of bacteria in the water sample, a process that takes a minimum of 18 hours. In the interim, contaminated water may be distributed to homes and consumed, exposing many people to disease-causing pathogens. The conventional microbiological testing methods must be performed in approved laboratories by trained technicians, which presents a challenge, particularly for small, remote communities where it is very unlikely to have a local laboratory capable of such analysis. Consequently, water samples may need to be shipped to larger urban centres, potentially adding days to the time before results are obtained. For these reasons, it would be advantageous to develop water analysis methods that can be completed rapidly, eliminating the culturing step, and on-site, with minimal need for laboratory facilities or operator training. The development of portable waterborne pathogen sensors that can produce results rapidly, while requiring minimal operator skill, would be an ideal solution to these problems.

Beyond the indicator bacteria, many other pathogens can pose a disease risk when they contaminate drinking water. Among this variety of microbiological contaminants, there are many that are not reliably indicated by the presence of *E. coli* or coliforms in the water sample. Some pathogens, such as *Legionella pneumophila*, are not associated with fecal contamination, meaning that the use of a fecal indicator is not an appropriate substitute for direct testing. Even the pathogens that are associated with fecal matter can pose challenges, however, due to factors such as different survival in environmental waters or in disinfection processes. Despite these challenges, the cost to monitor all of these pathogens by culture methods would be prohibitive and, for some pathogens, the tests are less successful or slower than those for the indicators. The development of a biosensor device that can be used to detect many types of pathogens would provide an additional benefit, relative to existing methods.

Immunosensors would be ideal candidates for developing rapid, easy-to-use tests for a wide range of pathogens. The use of antibodies as the biorecognition elements would allow a high degree of
flexibility in terms of the pathogenic analytes used by a particular sensor, since antibodies can be
developed for nearly any foreign substance that produces an immune response.

**5.1.1 Immunoassay Methods**

Immunoassays are highly specific and sensitive techniques, capable of detecting low
concentrations of analytes in complex samples.\(^1^2\) These techniques can be used to detect a wide variety of
contaminants, provided an antibody to the analyte of interest can be produced. This is straightforward in
the case of pathogens, since these infectious organisms usually produce an immune response in infected
hosts.\(^1^3\) Most immunoassays employ a solid phase, which facilitates washing to remove unbound assay
components while retaining bound components.\(^1^4\) The original immunoassays that were developed used
radioactive labeling techniques for signal transduction.\(^1^5\) The considerations of safety and practicality led
researchers to develop enzyme-labeled immunoassays, such as ELISA, over the next several years,\(^1^5\) since
enzyme-substrate reactions can provide signal amplification without the necessity for radioactive safety
measures and can be detected by simple spectrophotometric systems.\(^1^6\)

A small number of formats comprise the majority of solid phase immunoassays used today:
direct, indirect, sandwich, and competitive.\(^1^7\) Direct and indirect detection refer to the role of the
recognition antibody in the assay. In the direct method, the antigen-recognizing antibody is conjugated
directly to a label, while in the indirect method, a secondary labeled species must be added to detect the
antigen-binding antibody.\(^1^7,1^8\) Sandwich assays involve the use of at least two antibodies; the first is
immobilized on a solid surface and binds the antigenic analyte from solution, and the second recognizes
the bound analyte. Since at least two antibodies must bind to different sites on the analyte, sandwich
assays function most effectively for large antigens, such as proteins and cells.\(^1^4\) This format provides low
limits of detection, since the signal from the assay is proportional to the concentration of analyte, and
enhanced specificity, since two different antibodies must bind in order to obtain a positive result.\(^1^8,1^9\)
Competitive assays are an alternative format that facilitates the detection of small molecule analytes. In these experiments, the unknown quantity of analyte competes with a known amount of labeled analyte for binding to a limited number of antibodies. The presence of unlabeled analyte creates competition for the binding sites and this results in a reduction of signal from the label, which can be analyzed and used for quantitation. This method requires the analysis of a decreasing signal from a “bright” background, which creates challenges in obtaining low limits of detection. Considering these factors, the sandwich assay is a preferable format for pathogen analysis.

Although ELISA is a widely accepted analytical method, new variations are being developed to improve the stability, reliability, and detection limit. New analyte-binding substances, such as aptamers, are being produced. Aptamers can be selected to be stable under a range of non-physiological conditions and can be produced reproducibly without living organisms. Liquid handling is also moving beyond the pipettes and microtiter plates associated with conventional immunoassays. Microfluidic platforms are being used to reduce the volume of analyte solution required for analysis, as well as to increase the solid phase surface area to liquid volume ratio, providing enhanced sensitivity. The use of lateral flow strips aims to produce portable versions of conventional assays. The coupling of immunoassays with a solid phase extraction pre-treatment or immunomagnetic separation has also been used to increase sensitivity by removing interfering compounds and concentrating the analyte of interest. Homogeneous assays are also being investigated for their desirable properties, since the lack of washing steps in such assays leads to reduced assay times and labour requirements. Many options are also available for labeling and signal transduction in immunoassays: radioisotopes, fluorophores, chemiluminescent species, enzymes, nanoparticles, metal ions, and more. While assay techniques are improving, immunoassays for pathogen detection are characterized by detection limits such as 10 CFU/mL, 100 CFU/mL, or greater, all of which are well above the required drinking water quality detection limit of 1 CFU/100
mL. Immunoassays, which are adaptable and relatively fast to perform, would still be ideal candidates for adaptation into pathogen-detecting biosensor designs. Therefore, methods of increasing the sensitivity of ELISA should continue to be investigated.

5.1.1.1 Factors in ELISA Performance

ELISA performance is determined by factors such as sensitivity, specificity, accuracy, and precision and these are in turn related to assay components including buffer composition (ionic strength and pH), solid phase material, antibody (concentration, specificity, coating density), antigen, enzyme conjugate, washing protocols, substrate, and signal transduction method. All of these components require optimization during the development of a new ELISA procedure since the conditions that are ideal for one system may inhibit signal generation or lead to false positives in another. The signal generation step, which includes the choice of enzyme conjugate, substrate, and signal transduction method, can have a significant impact on the performance attributes of the ELISA.

ELISA enzyme labels are almost exclusively dominated by horseradish peroxidase or alkaline phosphatase, although β-galactosidase, catalase, and acetylcholinesterase have also been used. Horseradish peroxidase is the preferred choice in most assays due to its small size, which allows multiple enzyme labels to be conjugated to a single antibody, and its higher specific enzyme activity. Because of these two attributes, peroxidase-labeled assays usually have greater signal amplification and therefore greater sensitivity. While alkaline phosphatase has an inferior specific enzyme activity, it characteristically retains linear reaction rates over longer time scales, allowing improvements in sensitivity by incorporating longer reaction times. An overall challenge in the use of enzyme labels is the potential for inhibition, which can slow the reaction rate. Due to the important role of the metal cofactors, zinc and magnesium, in the enzyme active site, alkaline phosphatase is inhibited by substances including cyanides, arsenate, and cation chelators. The inhibition of phosphatase by
inorganic phosphate is an example of product inhibition,\textsuperscript{38} which is an enzyme regulation pathway. One way to improve the sensitivity of ELISA would therefore be to remove the products of the enzyme reaction from the solution, decreasing the product inhibition effect.

ELISA signal transduction is usually accomplished using a microwell plate reader equipped for the optical detection method of interest. For colourimetric assays, clear microwell plates are used; broad spectrum light is transmitted through each well from the bottom and the intensity of the transmitted light is read from the top of the well.\textsuperscript{16} Absorbance measurements depend upon the factors described by the Beer-Lambert Law, in which absorbance ($A$) is equal to the product of the molar extinction coefficient ($\varepsilon$), the path length of the cell ($b$), and the concentration of the absorbing molecule ($c$) (Equation 5-1).

\textbf{Equation 5-1} \hspace{1cm} A = \varepsilon bc

Fluorescence assays are performed using white or black microwell plates with clear well bases, and both the excitation source and detector are located either above or below the well.\textsuperscript{16} Fluorescence measurements are governed by the relationship between emission intensity ($F$) and the intensity of the excitation source ($I_o$), quantum yield ($\phi$), the molar extinction coefficient, ($\varepsilon$), the path length of the cell ($b$), and the concentration of the fluorescing molecule ($c$), where $k$ is a proportionality constant (Equation 5-2).

\textbf{Equation 5-2} \hspace{1cm} F = kI_o \phi \left(1 - 10^{-\varepsilon bc}\right)

At low fluorophore concentrations, in which less than 2\% of the excitation light is absorbed, this equation can be approximated to show that fluorescence intensity is proportional to the molar extinction coefficient, path length, and concentration of the fluorescent molecule (Equation 5-3).

\textbf{Equation 5-3} \hspace{1cm} F = kI_o \phi (\varepsilon bc)$
Thus, fluorescence-based ELISA depends on the same factors as colourimetric detection, with the added dependence on excitation light intensity and quantum yield. The sensitivity of a fluorescent ELISA, then, can be improved by methods including increasing the excitation light intensity, increasing the product concentration, either by producing more product or concentrating existing product, choosing a molecule of higher molar absorptivity and quantum yield, or increasing the path length. Beyond increasing the ELISA sensitivity, reducing the noise associated with measurements due to factors including solution turbidity and fluorescent matrix components\(^{12}\) could result in improved ELISA performance through more reproducible results.

A subset of the factors limiting ELISA performance could be addressed by removing the coloured or fluorescent product molecules of the enzyme reaction from the bulk solution and concentrating these in a phase of lower volume where optical detection may occur. Such a process could increase the product concentration, reduce product inhibition, and negate variations of the optical properties of the sample matrix. If the performance limitations brought about by these factors could be reduced, assays of higher sensitivity and precision could be developed.

5.1.2 Immunosensors for Environmental Analysis

Immunosensors are a category of biosensors that utilize antibodies as the recognition element. The development of immunosensors aims to transfer immunoassay techniques from the laboratory to the clinic or field location, requiring changes to the assay design for portability, simplicity, and transducer integration. For the detection of microbial contaminants, the ideal characteristics of a biosensor include: accurate results, rapid response times, sensitivity to pathogens at infective doses, specificity to the target organism, reproducibility, robustness, and ease of use.\(^{39}\) A variety of signal transduction methods can be employed for immunosensors, including electrochemical,\(^{40}\) mechanical,\(^{39,41}\) and optical detection.\(^{39,42}\) Optical detection is most frequently accomplished using fluorescently labeled antibodies, since these are
more stable in solution and allow more rapid detection.\textsuperscript{43,44} Enzyme-labeled immunosensors are occasionally combined with optical analysis,\textsuperscript{45} but electrochemical detection is currently more common.\textsuperscript{46,47} However, if a convenient assay format was developed, enzyme labeled optical assays should provide higher sensitivity than fluorescently labeled antibodies, due to the signal amplification afforded by the enzyme reaction. Ideally, if the fluorescent product molecules of the enzyme could be concentrated within an optical detection matrix, such as a waveguide, the fluorescent signal could be further amplified.

5.1.2.1 Fluorescent Immunosensors

Optical detection methods for immunosensors include absorbance, fluorescence, fluorescence resonance energy transfer (FRET), chemiluminescence, surface plasmon resonance, and Raman spectroscopy.\textsuperscript{48} Fluorescence sensing can be applied using a variety of labeling schemes. Recognition antibodies can be directly conjugated to organic or nanoparticle-based fluorescent labels and bound labeled antibodies can be detected following a washing step.\textsuperscript{48} Fluorescence resonance energy transfer (FRET) is based on the proximity of two chromophores: a donor and acceptor. When the donor is excited and is located within close proximity to the acceptor, the excitation energy can be transferred to the acceptor, causing emission or quenching by the acceptor.\textsuperscript{48} This methodology prevents the need to remove unbound antibodies allowing the production of homogeneous assays since the binding process itself changes the optical emission properties.\textsuperscript{48} While all fluorescence-based immunosensors necessarily require costly and time-consuming labeling protocols, this additional step provides a corresponding advantage relative to label-free sensors, which contend with a high degree of non-specific binding-related signal.\textsuperscript{49} Fluorescent signal transduction would therefore be preferable when selecting a technique for pathogen detection from water samples, where many matrix components may cause non-specific binding and low detection limits are required.
Fluorescence-based immuno sensors have been used to detect pathogens in a number of formats, including waveguide-based detection. Researchers at the United States Naval Research Laboratory have developed two biosensor systems that employ fluorescent detection of antibody recognition events.\textsuperscript{50,51} The first is an “array biosensor” in the form of a microscope slide functionalized with an array of capture antibodies, and combined with fluidic channels that direct a contaminated sample over the capture array. Fluorescently labeled recognition antibodies bind to captured pathogens and the optical signal is detected using a light source that propagates excitation light through the slide to the captured sandwich structures, where the evanescent wave excites the fluorophores.\textsuperscript{52} The array format allows multiplex sensing of a series of pathogens at concentrations as low as 970 CFU/mL.\textsuperscript{51,53} While the detection limits for these systems were impaired for solutions containing other pathogens or in food samples,\textsuperscript{51,53} the authors suggest that a short period of culturing could provide a pre-enrichment step prior to the use of the biosensor and this would raise the assay sensitivity.\textsuperscript{51} The second device developed by the Naval Research Laboratory is called the RAPTOR and has been commercialized by Research International.\textsuperscript{54} This device is similar to the array sensor described above, except that the immunosensing process takes place on the surface of an optical fibre waveguide, where the evanescent wave excites the labeled fluorescent antibodies bound to the surface.\textsuperscript{50} The process takes only 15 minutes and has been used to detect pathogens including \textit{E. coli} from diluted human feces at \(10^4\) CFU/mL\textsuperscript{55} and \textit{Bacillus anthracis} at 100 CFU/mL.\textsuperscript{56} There have been a lack of recent advances in performance for these devices, however. A similar fibre optic sensor was employed to perform a sandwich assay, achieving detection limits of 1000 CFU/mL in buffer for \textit{E. coli, Salmonella enterica,} and \textit{Listeria monocytogenes}.\textsuperscript{57} A lateral flow device that detects fluorescence quenching has also been developed. In this example, capture antibodies were immobilized on quantum dots on the device and the presence of pathogens bound to the capture antibodies prevented graphene oxide-based quenching of the fluorescent quantum dots. The optical signal
from this test were analyzed in a lateral flow reader and the device was shown to detect 10 CFU/mL of *E. coli* in buffer and 100 CFU/mL in bottled water and milk.\textsuperscript{25}

While these immunosensors demonstrate selectivity to the pathogen of interest, the presence of complex matrices continues to challenge the sensitivity of the detection system. Even in the presence of laboratory quality buffers, the detection limits of current fluorescent immunosensors do not meet the requirements of drinking water quality standards.

### 5.1.3 Solid Phase Microextraction

The examples above show that both traditional immunoassays and more advanced immunosensors based on fluorescent molecule detection fail to attain the detection limits required for the analysis of drinking water for pathogens. As a result, there remains a need to develop additional methods of signal amplification to improve the sensitivity of immunoassay techniques. The concentration of the fluorescent signaling molecules in a smaller volume is one approach to increasing the observed intensity. This approach could be used to concentrate the fluorescent products of an enzyme reaction in a solid phase that is optically clear, providing more sensitive fluorescence measurements, along with consistent optical clarity relative to solution detection, and the potential to decrease product inhibition of the enzyme.

A common technique that is used to extract analytes from a complex matrix into a smaller volume phase is solid phase microextraction (SPME), an analyte extraction technique that was developed by the group of Janusz Pawliszyn and commercialized by Supelco.\textsuperscript{58,59} In the conventional SPME sample preparation process, a polymer coated fibre is inserted into a sample and analytes partition into the polymer film until equilibrium is reached; the extracted analytes can then be desorbed and analyzed inside a gas chromatograph.\textsuperscript{60} The distribution equilibrium that describes the partitioning of an analyte between the SPME extraction film and the sample matrix is described by Equation 5-4, where $K_{fs}$ is the ratio of the
equilibrium analyte concentration in the extraction film \((C_f)\) to the equilibrium analyte concentration in the sample \((C_s)\).\(^{50}\)

\[
K_{fs} = \frac{C_f}{C_s}
\]

When the SPME film is hydrophobic and analytes are in an aqueous solution, \(K_{fs}\) can be correlated with the octanol-water distribution constant, \(K_{ow}\).\(^{60,61}\) By this correlation, hydrophobic analytes in aqueous solutions are predicted to partition into hydrophobic films, leading to \(K_{fs}\) values greater than 1. This relationship can be tuned for a wide variety of analytes of interest through functionalized polymers, mesoporous silicates, metal organic frameworks, molecularly imprinted polymers, macromolecules, and immunosorbents.\(^{62-66}\)

While the original function of SPME was as a sample preparation technique, from which analytes would later be desorbed for analysis, many modern sensors make use of SPME to concentrate analytes for detection within the extraction film. This method has been applied to mass-sensitive,\(^{67,68}\) electrochemical,\(^{69,70}\) and optical detection systems.\(^{71-76}\) Fluorescence-based detection specifically has been used to characterize the SPME process for analytes within a polymeric extraction matrix.\(^{77,78}\) Only rarely, however, has fluorescence detection been applied to a solid phase microextraction-based sensor. Recent examples include a system with Nile red dye immobilized within a PDMS film that underwent an emission wavelength shift upon absorption of ethanol by the film\(^{79}\) and the Endetec pathogen detection system, which monitors the uptake of the fluorescent product of an enzyme reaction within a PDMS structure.\(^{80,81}\) Given the rarity of examples of immunosensors that utilize fluorescent detection, particularly from enzyme reactions, and limited amount of work in the area of fluorescent molecule detection in solid phase microextraction materials, there is an opportunity for the development of a novel
pathogen detection system based on these methods that may improve upon the properties of conventional immunoassays for water analysis.

5.1.4 Research Objectives

The goal of this project was to demonstrate the combination of an enzyme-linked immunosorbent assay (ELISA) with solid phase microextraction (SPME). The concentration of the enzyme reaction products within the siloxane polymer matrix is predicted to enhance the sensitivity of the ELISA, bringing the detection limit closer to that required for applications in drinking water quality (Figure 5-1).

![Diagram of ELISA pathogen detection system with solid phase microextraction of the enzyme product. The capture antibodies were immobilized on the siloxane polymer and, after binding of the alkaline phosphatase-labeled detection antibody, pyrene phosphate was cleaved by the enzyme to form 1-hydroxypyrene and inorganic phosphate. 1-Hydroxypyrene partitioned into the siloxane polymer layer due to its hydrophobicity and was detected using a customized optical system with a 365 nm excitation source.]

Figure 5-1: The proposed ELISA pathogen detection system with solid phase microextraction of the enzyme product. The capture antibodies were immobilized on the siloxane polymer and, after binding of the alkaline phosphatase-labeled detection antibody, pyrene phosphate was cleaved by the enzyme to form 1-hydroxypyrene and inorganic phosphate. 1-Hydroxypyrene partitioned into the siloxane polymer layer due to its hydrophobicity and was detected using a customized optical system with a 365 nm excitation source.
To couple ELISA and SPME, it is necessary to use an ELISA substrate that undergoes a change in hydrophobicity during the enzyme reaction. For this purpose, pyrene phosphate was chosen as the alkaline phosphatase substrate, since the cleavage of the substrate yields 1-hydroxypyrene, which is highly hydrophobic (pyrene phosphate $K_{ow} = 7.08 \times 10^{-3}$, 1-hydroxypyrene $K_{ow} = 1.01 \times 10^4$, predicted using EPI Suite). The cleavage of pyrene phosphate to 1-hydroxypyrene was previously used in our group for the purposes of a sterility test, so its SPME properties are known. However, for the purposes of this experiment, was necessary to optimize the formulation of the siloxane polymer layer into which the 1-hydroxypyrene was extracted, as well as the optical system used for the fluorescent detection of the product molecules. The optimal conditions for the enzyme reaction were also determined. Once the parameters of the SPME ELISA were investigated, the relative sensitivity of this assay was compared to a conventional solution-detected ELISA system. Finally, the SPME ELISA was demonstrated for the detection of a model fecal indicator, *E. coli* ATCC 25922.

5.2 Materials and Methods

5.2.1 Fluorescence Spectrometer System for SPME Measurements

Two fluorescence spectrometers were used for the SPME characterization. The first included only one cuvette chamber and was described in Chapter 3. The second worked on a similar principle, with some key feature changes. The detector was an Ocean Optics USB2000+ custom CCD array spectrometer (Ocean Optics, Dunedin, FL) and it was capable of handling bundled collection fibres from 16 identical cuvette chambers (Figure 5-2). Only four of the sixteen chambers were used for these experiments. Each chamber included two 365 nm LEDs, focused on the chamber at angles of 67.5° from the normal (Figure 5-3). The focal length of the optical components was 2 mm from the lens. The cuvette could be raised and lowered within the chamber to accommodate both solution-phase and siloxane film-phase fluorescence.
Figure 5-2: 16-chamber spectrometer system. a) An overall view of the instrument, showing the computer-controlled spectrometer and sample chambers. b) A sample chamber is shown with its collection fibre in place. Wires to the left and right of this fibre connect to the LEDs. c) A sample chamber from the opposite direction is shown with a cuvette in place for in-polymer measurements. d) A close-up view of the OEM2000Plus Ocean Optics spectrometer. Top left is the collection fibre bundle and top right is the excitation LED assembly.

measurements. A computer running proprietary software from Endetec controlled the spectrometer and the LEDs for each chamber. This software controlled the sequential illumination of the LEDs in each chamber and recorded time-resolved spectra in a data file.
**Figure 5-3**: Top view of cuvette in one chamber of the 16-chamber spectrometer. The two LEDs send excitation beams into the polymer at angles of 67.5° relative to the normal. An optical fibre collects emission light through a lens and along the normal.

### 5.2.2 Optimization of Waveguide Polymer Film

A series of PDMS film formulations were cast in PMMA semi-micro cuvettes (BrandTech, Essex, CT) to determine the optimal film formulation for SPME fluorescence measurements. Sylgard 184 and 186 were purchased from Paisley Products (Toronto, ON) and prepared at a 9:1 (m:m) base to curing agent ratio in 20 mL scintillation vials. Both prepared materials were dissolved in hexanes at 2:1 and 4:1 (m:v) ratios (*i.e.* 2 g Sylgard to 1 mL hexanes or 4 g Sylgard to 1 mL hexanes). The polymer-solvent mixture was stirred to achieve a uniform consistency, then the vial containing the mixture was capped and allowed to rest at ambient conditions until any air bubbles in the material has diffused out (10 – 30 minutes), leaving a clear polymer-solvent mixture. Following this waiting time, the mixture was taken up into a 1 mL plastic syringe, the tip of which was then cleaned with a tissue so that a 21-gauge (0.8 mm x 25 mm) needle could be attached. The syringe and attached needle were used to deposit either 60 or 80 µL of polymer mixture into the bottom of each plastic cuvette, being careful to avoid coating the sides of the
cuvette with polymer. This resulted in a range of masses of PDMS between 40 and 64 mg in the cuvettes (Table 5-1).

**Table 5-1:** The Sylgard 184 and 186 formulations with hexanes used to prepare PDMS layers in cuvettes resulted in films with a range of calculated masses.

<table>
<thead>
<tr>
<th>Dilution Factor (mass of polymer (g) to volume of hexanes (mL))</th>
<th>Mixture Volume Deposited (µL)</th>
<th>Final Mass of Sylgard in Cuvette (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>53</td>
</tr>
<tr>
<td>4:1</td>
<td>60</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>64</td>
</tr>
</tbody>
</table>

For Sylgard 186 films, the high viscosity meant that it was necessary to tap the cuvettes on a hard surface repeatedly to spread the polymer mixture across the bottom of the cuvette. Sylgard 184 was less viscous, so this additional step was unnecessary. Also as a result of the high viscosity of Sylgard 186, only the 2:1 dilution factor was used, since the 4:1 dilution left the material too viscous to spread evenly in the cuvette. After the polymer was deposited and evenly distributed at the bottom of the cuvette, all cuvettes were allowed to cure at room temperature for at least 36 hours (Figure 5-4). To test the optical properties and reproducibility of these films, a solution of 1 mL of 40 µM 1-hydroxypyrene (made in lab) in 10 mM Tris buffer at pH 9.0 was added to the cuvette and the extraction of the fluorophore into the film was monitored. The buffer composition was 10 mM Tris.HCl (Sigma-Aldrich), 5.0 mM MgCl₂ (Sigma-Aldrich), and 0.10 mM ZnCl₂ (Sigma-Aldrich) in distilled water. The fluorescence signal within the polymer was monitored using the system described above using a 365 nm LED for excitation and collecting the emission at 409 nm over 30 minutes.
**Figure 5-4:** 40 mg of Sylgard 186 cast in a PMMA semi-micro cuvette. The polymer is located at the bottom of the cuvette in a layer approximately 1 – 2 mm thick.

### 5.2.3 Optical Alignment of Waveguide Film for Fluorescence Analysis

The plastic cuvette-holding chambers in the multi-chamber spectrometer (Figure 5-2) were similar in design to the earlier one-chamber model. However, the cuvette opening was slightly larger in this model, leading to inconsistent cuvette placement. It was therefore necessary to develop a system to hold the cuvette within the spectrometer chamber in a consistent position. For in-film fluorescence measurements, it was also necessary to align the siloxane film in the cuvette at the optimal height relative to the optical components (Figure 5-5). The initial design for the cuvette mount was a sawed-off plastic cuvette piece 1.34 cm tall. This was combined with pieces of paper (cardstock, 1 cm by 2 cm in size) inserted vertically around the cuvette for more stable positioning. A second cuvette mounting system was integrated to include the required height for optical alignment as well as positioning stability. It was produced by 3-D printing poly(lactic acid) from a Solid Works design (Eric Marcotte) at the Kingston Frontenac Public Library (Kingston, ON). This part consisted of a square mount with two arms that extended to fit around a plastic semi-micro cuvette (Figure 5-6).
Figure 5-5: Side-on view of the SPME fluorescence system. The cuvette is raised within the spectrometer chamber so that the Sylgard PDMS layer is aligned with the optical components, consisting of two 365 nm LEDs and a collection fibre that guides light to the spectrometer. The height of the cuvette mount was important in aligning the polymer layer with the optical system.

Figure 5-6: 3-D printed cuvette mount designed to increase stability of the cuvette during in-polymer measurements.

The size and fit of the mount were refined using sandpaper so that the fit of the cuvette was snug, but removable. Sandpaper was also used to refine the height of the mount for each spectrometer chamber. To further refine the height alignment, a set screw was placed in the base of each cuvette chamber. To do this, a hole was drilled in the plastic base of each chamber to fit a ¼" screw tap. The hole was tapped with
a ¼”-20 tap and a set screw was inserted. This set screw could be moved up and down to make fine adjustments to the height of the cuvette within the chamber. To determine the effect of the set screw, a series of fluorescence spectra were collected with a cuvette containing 1-hydroxypyrene in a PDMS film in place. Between each collected spectrum, the set screw was rotated by ¼ turn using a hex key, providing a height increase of approximately 0.0125 inches.

It was necessary to normalize the signal obtained from each chamber in this instrument, both in terms of the intensity (controlled by the integration time for each chamber) and the peak wavelength (different for each chamber based on the fibre placement in the spectrometer). For in-polymer normalization, a 1 mL solution of 10 µM 1-hydroxypyrene in water was left in a Sylgard-coated cuvette (coated with 40 mg Sylgard film - see section 5.2.2) overnight to allow uptake of the fluorescent molecule by the polymer. This cuvette was then inserted repeatedly into each chamber of the system and the intensity and peak wavelength results were used to normalize the chambers.

5.2.4 Characterization of SPME ELISA Substrate – Pyrene Phosphate

5.2.4.1 Hydrolysis of Pyrene Phosphate at Alkaline pH Conditions without Enzyme

Since alkaline phosphatase catalyzes the hydrolysis of a wide range of phosphate esters,84 it can be used in combination with a variety of substrates. For SPME ELISA, a new alkaline phosphatase substrate, pyrene phosphate, was synthesized by Dr. Ray Bowers in our lab. Since alkaline phosphatase functions most effectively in basic pH conditions, it was necessary to determine the pH stability of the pyrene phosphate substrate. For this purpose, solutions were prepared containing 40 µM pyrene phosphate in Tris buffer (10 mM Trizma HCl, 5.0 mM MgCl₂, 0.10 mM ZnCl₂) at pH 7.5, 8.0, 8.5, and 9.0. These solutions were prepared from an aqueous stock of 4.0 mM pyrene phosphate immediately prior to testing each sample. The resulting 1 mL solution of 40 µM pyrene phosphate in buffer was added to a PMMA
cuvette containing a 40 mg Sylgard 184 film (as described in section 5.2.2), and the cuvette was placed in the fluorescence spectrometer using a mount to align the film with the optical components. Kinetic measurements were completed by measuring the appearance of fluorescence at 409 nm within the polymer film due to 1-hydroxypyrene extraction from the solution. A 1500 ms integration time was used for all samples and the experiments were completed at room temperature. Two samples were tested in each of the four different pH buffers. Using the resulting kinetic data, the initial slope of the fluorescence vs. time plot at 409 nm was used to compare the conditions.

5.2.4.2 Effect of pH on Cleavage of Pyrene Phosphate by Alkaline Phosphatase

The pH dependence experiment was repeated in the presence of the ELISA enzyme label, alkaline phosphatase. For this purpose, a solution of 41 µM pyrene phosphate in Tris buffer (as described in Section 5.2.4.1) was prepared at a volume of 975 µL in a cuvette containing a 40 mg Sylgard 184 film. After beginning fluorescence monitoring at 409 nm in the spectrometer, 25 µL of a 0.2 U/mL alkaline phosphatase (bovine intestinal, Sigma-Aldrich) solution were added to reach a final enzyme concentration of 0.005 U/mL and a pyrene phosphate substrate concentration of 40 µM. The pipette used to add the enzyme was used to mix the solution in the cuvette by repeatedly aspirating the liquid. The cuvette chamber cap was then put in place to exclude external light and fluorescence measurements continued for at least 5 minutes. A 1500 ms integration time was again used for all samples and the experiments were completed at room temperature.

This experiment was repeated using a bound enzyme system. For this set of trials, rabbit immunoglobulin G (Sigma-Aldrich) was dissolved at 5 µg/mL in 12 mM phosphate buffer at pH 8.0. An aliquot of 400 µL of this solution was added to each of 8 cuvettes containing a 40 mg Sylgard 184 film and the antibodies were allowed to bind overnight at 4°C. The following day, the films were washed three times with 0.1% (v/v) Tween 20 (Acros Organics, New Jersey) in phosphate buffered saline (PBS) at pH 230
The composition of the PBS buffer was 137 mM NaCl (Acros Organics), 2.7 mM KCl (Fisher Scientific), 10 mM Na$_2$HPO$_4$ (Fisher Scientific), and 1.8 mM KH$_2$PO$_4$ (Shawinigan Chemical, Shawinigan, QC). Each wash consisted of 1 mL of the 0.1% Tween 20 PBS solution and lasted 10 minutes. After washing, the films were rinsed with distilled water and then a 1 mL solution of 20 g/L bovine serum albumin (BSA) (Sigma-Aldrich) in buffer at pH 7.2 was added for 1 hour to block any remaining adsorption sites. The blocking buffer consisted of 0.1 M Na$_2$HPO$_4$, 0.15 M NaCl, and 0.05% (v/v) Tween 20. Following blocking, a 400 µL solution of 2 µg/mL anti rabbit immunoglobulin G-alkaline phosphatase (Anti IgG-AP) (Sigma-Aldrich) in Tris buffer at pH 8.0 (10 mM Trizma HCl, 5 mM MgCl$_2$, 0.1 mM ZnCl$_2$) was added to each cuvette and allowed to bind to rabbit IgG on the films for 1 hour. After this binding process, the films were washed again three times using 0.1% Tween 20 PBS as described above. Following washing, the cuvette films were rinsed with distilled water and the cuvettes were filled with the corresponding Tris buffer (pH 7.5 to 9.0) that would later be used for activity testing. When each cuvette was ready for testing, the existing buffer was disposed of and the cuvette was tapped upside down to remove as many droplets of buffer as possible. A 1 mL solution of 40 µM pyrene phosphate in the required Tris buffer was then added to the cuvette and it was inserted into the spectrometer chamber for testing. During the test, the cuvette was raised on a mount so that the optical components aligned with the PDMS film and fluorescence was monitored at 409 nm using an integration time of 1500 ms. All experiments were conducted at room temperature.

**5.2.5 Comparison of Conventional and SPME ELISA for a Model System**

The properties of the SPME ELISA were compared to a conventional system with the enzyme reaction product detected in solution using a model ELISA system. This experiment consisted of rabbit IgG adsorbed on the PDMS (Sylgard 184) surface, which was recognized by Anti IgG-AP. The presence
of the enzyme-labeled antibody could then be detected by adding the conventional substrate, 4-methylumbelliferyl phosphate, or the SPME substrate, pyrene phosphate (Figure 5-7).

**Figure 5-7**: The ELISA systems compared in this study both used rabbit IgG bound to a PDMS surface, which was recognized by Anti IgG-AP. The conventional ELISA system used 4-methylumbelliferyl phosphate as the alkaline phosphatase substrate and detected the 4-methylumbelliferone product by fluorescence at 445 nm in solution. In the SPME ELISA, pyrene phosphate was cleaved to produce 1-hydroxypyrene, which was extracted into the siloxane polymer where it was detected by fluorescence at 409 nm.
For this set of trials, samples containing different concentrations of rabbit IgG were compared using a constant concentration of Anti IgG-AP. Samples containing rabbit IgG that were not exposed to Anti IgG-AP were also tested.

Rabbit IgG was dissolved at concentrations between 0 and 0.5 µg/mL in 12 mM phosphate buffer at pH 8.0. An aliquot of 400 µL of this solution was added to each cuvette containing a 40 mg Sylgard 184 film and the antibodies were allowed to bind overnight at 4°C. The following day, the films were washed three times with 0.1% (v/v) Tween 20 PBS at pH 7.4. Each wash consisted of 1 mL of the 0.1% Tween 20 PBS solution and lasted 10 minutes. After washing, the films were rinsed with distilled water and then a 1 mL solution of 20 g/L BSA in blocking buffer at pH 7.2 was added for 1 hour to occupy any remaining adsorption sites. Following blocking, a 400 µL solution of 2 µg/mL Anti IgG-AP in Tris buffer at pH 8.0 (10 mM Trizma HCl, 5 mM MgCl₂, 0.1 mM ZnCl₂) was added to each cuvette and allowed to bind to rabbit IgG on the films for 1 hour. After this binding process, the films were washed again three times using 0.1% Tween 20 PBS as described above. Following washing, the cuvette films were rinsed with distilled water and then filled with Tris buffer at pH 9.0. When each cuvette was ready for testing, the existing buffer was disposed of and the cuvette was tapped upside down to remove as many droplets of buffer as possible. A 1 mL solution of 40 µM pyrene phosphate or 4-methylumbelliferyl phosphate (Sigma-Aldrich) in Tris buffer at pH 9.0 was then added to the cuvette and it was inserted into the spectrometer chamber for testing. During the SPME test, the cuvette was raised on a mount so that the optical components aligned with the PDMS film and fluorescence was monitored at 409 nm using an integration time of 1500 ms. For the conventional substrate, the mount was not used so that fluorescence in the solution phase could be detected at 445 nm. All experiments were conducted at room temperature.
5.2.6 Detection of *E. coli* Using SPME ELISA

A demonstration of the SPME ELISA system for waterborne pathogen detection was undertaken using *E. coli* 25922 as the analyte. In order to investigate the degree of capture by the immobilized capture antibody on the siloxane film surface, half of the cuvettes used in the experiment were cultured following exposure to *E. coli* while the other half were tested in the ELISA procedure (Figure 5-8).

![Diagram of the preliminary E. coli ELISA](image)

**Figure 5-8:** A schematic diagram of the preliminary *E. coli* ELISA used in this project. *E. coli* antibodies produced in goat were immobilized on a siloxane polymer surface and were used to bind *E. coli* from solution. These analytes were detected by first adding an *E. coli* antibody produced in goat with a biotin label, and then binding alkaline phosphatase-labeled avidin to the biotin functionality. The presence of alkaline phosphatase was detected using pyrene phosphate as an AP substrate.

These experiments were performed using carboxyl-functionalized siloxane films in cuvettes, which were prepared as follows. First, 40 µg Sylgard 186 films were cast in a series of PMMA cuvettes and allowed to cure for at least 36 hours. PCBMS-PDMS was prepared as described previously (Section 3.2.6.2) and dissolved at 37.5 % (m/v) in isopropanol (*e.g.* 75 µL of PCBMS-PDMS in 125 µL of isopropanol) immediately after hydrosilylation. Just before an experiment was performed, concentrated hydrochloric acid was added at 4% (v/v) (*e.g.* 4 µL of concentrated hydrochloric acid for 100 µL polymer solution). A 100 µL aliquot of the polymer, solvent, and acid mixture was then added to each Sylgard
186-coated cuvette and the reaction was allowed to proceed for 30 minutes. After this time, the cuvettes were carefully washed with isopropanol three times to remove unbound siloxane. Following chain exchange, 400 µL of 5 M hydrochloric acid were added to each cuvette and the hydrolysis of the PCBMS-PDMS esters was allowed to continue at 35°C for 24 hours. After this time, the films were rinsed at least three times with sterile water to remove residual acid. The resulting carboxylic acid groups were activated using a 400 µL solution of 1 mg/mL N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDAC.HCl) (Sigma-Aldrich) and 0.1 mg/mL N-hydroxysuccinimide (NHS) (Sigma-Aldrich) in sterile pH 6.0 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich) at 20 mM. The activation treatment lasted two hours at room temperature, after which the cuvettes were rinsed with sterile water.

Following the formation of the activated surface, 400 µL of 5 µg/mL of Goat Anti E. coli (polyclonal, for E. coli serotypes O and K, ThermoFisher Scientific) in sterile phosphate buffer (pH 8.0, 12 mM, 0.05% (v/v) Tween 20) were added to each cuvette and allowed to bind to the surface overnight at 4°C. The next day, the films were washed three times with 1 mL of sterile PBS containing 0.1% Tween 20, with each wash being 10 minutes in duration. After the washes were completed, the films were rinsed with sterile water. Next, each cuvette surface was blocked with 1 mL of 20 g/L BSA in sterile blocking buffer for one hour at room temperature. After blocking was completed, the solution was removed and replaced with a 1 mL solution of E. coli 25922 at the required concentration (expected concentrations were between 0 and 10⁶ colony forming units per mL) in sterile water. One hour of binding at room temperature was allowed before the analyte solution was disposed in the biohazardous waste. The films were then washed three times with PBS containing 0.05% Tween 20, by the previously described method.

At this point, half the samples (one at each concentration) were carried forward to the ELISA method and half were cultured, as described below. The next step for the ELISA samples was the addition of a biotinylated detection antibody. For this purpose, 400 µL of 5 µg/mL of biotin labeled goat Anti E.
coli (polyclonal, for serotypes O and K, ThermoFisher Scientific) in sterile PBS were added to each cuvette and allowed to bind for 1 hour at room temperature. After this binding step, the cuvettes were washed three times with 0.05% Tween 20 in sterile PBS, as described previously. Finally, 400 µL of the ELISA label, avidin-alkaline phosphatase conjugate, (ThermoFisher Scientific) were added to each cuvette. The solution was prepared by diluting the conjugate at 1:1000 (v:v), the minimum working dilution recommended by the manufacturer, in sterile Tris buffer at pH 8.0 (10 mM Trizma HCl, 5 mM MgCl₂, 0.1 mM ZnCl₂). After 1 hour of binding at room temperature, the solution was removed and the cuvettes were rinsed three more times with sterile PBS containing 0.05% Tween 20. Cuvettes were then rinsed with sterile water and filled with sterile Tris buffer at pH 9.0 at room temperature until activity testing was initiated. For the activity tests, 1 mL of sterile Tris buffer at pH 9.0 containing 40 µM pyrene phosphate was added to each cuvette. Fluorescence measurements were immediately started in the Ocean Optics spectrometer system, with cuvette mount in place to measure the emission at 409 nm within the polymer film. These measurements were continued for 30 minutes for each cuvette.

For the culture samples, one cuvette exposed to each E. coli concentration was cultured using a standard E. coli culture medium containing 12.7 µM pyrene-β-D-glucuronide, a β-glucuronidase (enzyme characteristic of E. coli) substrate (Figure 5-9).

![Figure 5-9: β-Glucuronidase, an enzyme characteristic of most E. coli strains, consumes the substrate pyrene-β-D-glucuronide to produce 1-hydroxypyrene and glucuronic acid.](image-url)
The initial fluorescence spectrum of the films in these culture cuvettes was recorded and then the cuvettes were placed in an incubator at 35°C. These cuvettes were removed at various times for fluorescence measurements over a total of 18 hours. The fluorescence at 409 nm was measured using an integration time of 1500 ms in the spectrometer equipped with a mount to raise the height of the cuvette so that the film was aligned with the optical components. A positive control cuvette was also included in this group, consisting of a cuvette containing the Sylgard 186 PDMS film, but not exposed to any prior chemical or antibody treatments. A 500 µL aliquot of water containing approximately 100 CFU of *E. coli* 25922, and 500 µL of the culture medium containing 12.7 µM pyrene-β-D-glucuronide were added to this cuvette, its initial in-polymer fluorescence spectrum was measured, and it was cultured and analyzed in the same way as the culture samples.

To confirm the *E. coli* concentrations used in this experiment, a reference count was performed using the Colilert Quantitray test (Idexx, Westbrook, ME). For this test, a presumed 100 CFU *E. coli* aliquot was added to the Colilert broth, the solution was mixed and poured into a Quantitray plate, which was sealed, and the sealed plate was incubated overnight at 35°C. The next day, the number of positive wells was counted and the result was compared with the quantitation chart provided by the company.

### 5.3 Results and Discussion

#### 5.3.1 Optimization of Waveguide Polymer Film

It was necessary to optimize the formulation of the Sylgard PDMS layer deposited in the cuvettes for fluorescence measurements based on a number of factors: rapid uptake rates of 1-hydroxypyrene, consistent film quality, minimal air bubble inclusion, and consistent fluorescence signal. Previous work in our group had shown that Sylgard 186 was superior to Sylgard 184 in terms of the uptake kinetics of 1-hydroxypyrene. However, Sylgard 186 is substantially more viscous (124 Pa.s vs. 5.1 Pa.s for Sylgard
184, which resulted in a deposition process that was more prone to inconsistency. It was difficult to remove air bubbles from the film, for example, and challenging to spread the material evenly across the cuvette base. In comparing equivalent formulations of Sylgard 184 and 186 (2:1 dilution and 60 μL deposited = 40 mg film) for this system, 1-hydroxypyrene was taken up at a more consistent rate in 184 than 186. In addition, the average rate of 1-hydroxypyrene uptake in Sylgard 184 was faster than in 186 (Figure 5-10).

**Figure 5-10:** Uptake kinetics of 1-hydroxypyrene in 40 mg Sylgard 184 and Sylgard 186 films measured using the fluorescence intensity at 409 nm inside the polymer film.

These findings, which contradicted our earlier results, may have been due to changes to the optical system and the use of thicker polymers. Based on these results, it was decided that a Sylgard 184 formulation would be preferred.
Four different film formulations were tested using Sylgard 184, summarized in Table 5-1 above. These were 2:1 and 4:1 polymer to solvent dilutions, with 60 and 80 µL of the mixture deposited into each cuvette. There was a large amount of variability in the resulting 1-hydroxypyrene fluorescence measurements between cuvettes produced under the same conditions (Figure 5-11).

**Figure 5-11:** Average fluorescence intensity of 1-hydroxypyrene (at 409 nm) inside Sylgard 184 films after 30 minutes of exposure to a 40 µM 1-hydroxypyrene solution. The error bars represent the standard deviation for replicate cuvettes tested under each condition (n=3).

Some of the chamber-to-chamber variability can be attributed to the fact that the film alignment with the optical components of the system was not fully optimized at this point in the research. However, some film formulations produced more reproducible results than others. Specifically, the films produced using 60 µL of 2:1 diluted Sylgard 184 had relatively high signals from 1-hydroxypyrene in all the chambers. This could be attributed to films with consistent thickness and optical properties. Once this optimization was completed, the decision was made to use 60 µL of 2:1 diluted Sylgard 184 in further SPME experiments. It was also clear that further refinement of the optical alignment system was necessary so
that consistent fluorescence measurements could be made in all four chambers of the multi-chamber spectrometer.

5.3.2 Optical Alignment of Waveguide Film for Fluorescence Analysis

While the cuvette mounts that were made from sawed off pieces of plastic cuvettes were effective in raising the PDMS film-containing cuvettes to the correct height for in-polymer fluorescence measurements, the fluorescence intensity measured for the same film inserted into the cuvette chamber multiple times varied by almost 50% of peak intensity (Figure 5-12).

![Fluorescence Intensity vs Wavelength](chart.png)

**Figure 5-12:** The fluorescence peak at 409 nm for a Sylgard film containing 1-hydroxypyrene measured three times in the same spectrometer chamber. The variability in intensity was due to the unstable cuvette mounting system.

It was therefore necessary to design a more stable cuvette alignment system and this was accomplished by 3-D printing a cuvette mount that included arms to hold the cuvette more securely in place. Using the 3-D
printed mounts produced more consistent signals in most trials (Figure 5-13), although some measurements still demonstrated variability.

**Figure 5-13:** The fluorescence peak at 409 nm for a Sylgard film containing 1-hydroxypyrene measured three times in the same spectrometer chamber. This measurement was repeated for all four chambers (Ch 1 to 4). These measurements were completed with the new 3-D printed cuvette mount. The peaks are deliberately offset by 3 nm to simplify interpretation. Replicates: n=3.

This variability indicated that the alignment of the film with the optical components in each chamber was still inconsistent. To provide fine adjustments to the vertical alignment, a set screw was added beneath the cuvette mount. As the set screw was rotated, the resulting 1-hydroxypyrene peak within the film could be measured as a function of set screw height (Figure 5-14). As the set screw was raised, the optical components were aligned near the top surface of the film, resulting in low fluorescence signals, the centre of the film, resulting in maximum fluorescence, and below the film, where no fluorescence was observed. From the data, it was observed that there was a very small optimal height range, corresponding to approximately 0.0125 inches or 0.3 mm. The use of a set screw to make these very fine adjustments
Figure 5-14: The 1-hydroxypyrene fluorescence intensity in a Sylgard film as a function of the set screw position under the cuvette mount. Replicates: n=1.

...was demonstrated to be important since this level of accuracy was not achieved in the 3-D printing or by sanding the cuvette mount after printing. Using the set screw, the height of each chamber’s cuvette mount was optimized, leading to a chamber-to-chamber variability of less than 10% of the peak intensity for a 1-hydroxypyrene-containing film (Figure 5-15).

All of these optimizations and adjustments made it possible to perform four simultaneous measurements, which improved the possible throughput for the planned SPME ELISA. Since conventional ELISAs are carried out in 96-well plates, the ability to analyze multiple samples at once should be an important consideration going forward.
Figure 5-15: Fluorescence spectra of 1-hydroxypyrene in a PDMS film placed three times in each of four chambers of the multi-chamber fluorescence spectrometer. The spectra are deliberately offset by 3 nm for each chamber to simplify interpretation. Replicates: n=3.

5.3.3 Characterization of SPME ELISA Substrate – Pyrene Phosphate

5.3.3.1 Hydrolysis of Pyrene Phosphate at Alkaline pH Conditions without Enzyme

Phosphate substrates may be susceptible to hydrolysis under basic conditions, which conflicts with the need for high pH environments for the functioning of alkaline phosphatase. The relationship between substrate hydrolysis and protein activity for pyrene phosphate was therefore characterized. The appearance of fluorescent 1-hydroxypyrene within the PDMS film was monitored at 409 nm for each pH condition. Despite the absence of alkaline phosphatase enzyme, significant 1-hydroxypyrene fluorescence signals were obtained in all pH conditions. The slopes of the fluorescence vs. time plots were used to make a quantitative comparison between the conditions tested and these results showed that the apparent
rates of 1-hydroxypyrene appearance seemed to depend upon the pH of the solution, although the correlation was not strong (Figure 5-16).

**Figure 5-16:** a) Fluorescence vs. time plots at 409 nm for solutions of pyrene phosphate at various pH conditions monitored within PDMS films in cuvettes. The data was smoothed using a moving average of 5 points. b) Effect of the buffer pH on the apparent rate of hydrolysis of pyrene phosphate. Since the fluorescence signal for the 1-hydroxypyrene product was measured within the PDMS film, the slope of the fluorescence vs. time plot depended on both the rate of hydrolysis and the rate of extraction. Replicates: n=2.

The rate of hydrolysis appeared to increase up to pH 8.5 and decrease at pH 9.0. It is important to note that the slopes shown here are a combination of the rate of hydrolysis of the substrate and the rate of uptake of the product into the siloxane polymer used for monitoring. It appears that two competing factors may have led to these results: the increased rate of hydrolysis at higher pH values, and the decreasing rate of uptake at higher pH values due to the lower degree of protonation of the 1-hydroxypyrene product above its pKₐ (8.7-8.8). Using a pKₐ of 8.75, it was estimated that 1-hydroxypyrene is 64% protonated at pH 8.5 and 36% protonated at pH 9.0. The deprotonated 1-hydroxypyrene anion would be more hydrophilic and therefore less readily extracted from the water into the siloxane film.
5.3.3.2 Effect of pH on Cleavage of Pyrene Phosphate by Alkaline Phosphatase

It was also necessary to observe the effect of pH on pyrene phosphate cleavage in the presence of alkaline phosphatase. This enzyme is known to function best at alkaline pH values, so pH conditions between 7.5 and 9.0 were investigated. As before, the fluorescence at 409 nm was measured within the PDMS layer, corresponding to the concentration of 1-hydroxypyrene extracted from solution. The apparent rate of cleavage of the pyrene phosphate substrate in the presence of alkaline phosphatase increased as a function of pH, based on the slope of the fluorescence vs. time plots (Figure 5-17a).

**Figure 5-17:** Effect of buffer pH on observed alkaline phosphatase activity using pyrene phosphate as the substrate. a) Solution phase alkaline phosphatase. b) PDMS-immobilized Anti IgG-AP bound to IgG. Fluorescence was monitored within the PDMS film at 409 nm and the initial slope of the fluorescence vs. time plot was used for comparison. Replicates: n=2.

In this case, three processes may have contributed to the observed slope plotted in Figure 5-17a: the cleavage of pyrene phosphate by alkaline phosphatase (expected to increase with pH), the hydrolysis of pyrene phosphate by hydroxide ions in the solution (expected to increase with pH), and the extraction of 1-hydroxypyrene into the PDMS film, which is affected by the equilibrium of the 1-hydroxypyrene protonation state (pK_a = 8.7 to 8.8). The apparent rate of pyrene phosphate cleavage in the presence of
alkaline phosphatase is higher at pH 9.0 than pH 8.5, suggesting that the activity increase afforded to the enzyme at the higher pH more than compensates for the decreased extraction of 1-hydroxypyrene due to its deprotonated state. Buffer pH values above 9.0 were not tested because experiments using the solution phase substrate (4-methylumbelliferyl phosphate) showed reduced activity under these conditions (see Section 4.3.1.3.1).

This pH dependence experiment for alkaline phosphatase was repeated for a surface-bound system consisting of Rabbit IgG adsorbed to a PDMS film, to which anti rabbit immunoglobulin G-alkaline phosphatase (IgG-AP) was then allowed to bind. Similarly to the solution-phase alkaline phosphatase, the surface-bound antibody-enzyme conjugate demonstrated increasing apparent activity at higher pH conditions (Figure 5-17b).

Based upon the results of the solution-phase and bound alkaline phosphatase pH dependence studies, it was decided that a pH 9.0 buffer for ELISA activity analysis could be used for pyrene phosphate, as it was for the conventional 4-methylumbelliferyl phosphate substrate.

5.3.4 Comparison of Conventional and SPME ELISA for a Model System

In comparing the conventional ELISA, with detection in solution, to the SPME ELISA, there were a number of factors that could not be controlled, making a completely fair comparison impossible. While alkaline phosphatase possesses broad substrate specificity, the different structures of 4-methylumbelliferyl phosphate and pyrene phosphate may lead to slightly different enzyme-substrate affinities as well as different catalytic rates. In addition, the optical system being used for fluorescence measurements was optimized for measurements in the polymer film, not in solution. Finally, while the concentrations of the two substrates were the same, their optical properties were not. The substrates have distinct excitation spectra, molar absorptivities, and quantum yields. All these factors meant that no truly controlled comparison of the two substrates could be completed. Nevertheless, it was useful to compare
the properties of the two ELISA systems, including their sensitivities, kinetics, and limits of detection, in order to determine whether the SPME process leads to better performance characteristics. These attributes were compared by performing a model ELISA, consisting of Rabbit IgG as the analyte and Anti IgG-AP as the detection system, at varying concentrations of Rabbit IgG.

When examining the kinetics of the two ELISA experiments at the same IgG concentration, experiments using 4-methylumbelliferyl phosphate as the substrate had a noticeable delay in the appearance of fluorescence after the addition of the substrate, whereas this delay was not observed for the SPME ELISA (pyrene phosphate) experiments (Figure 5-18).

**Figure 5-18:** The appearance of fluorescence during a model ELISA using a) 4-methylumbelliferyl phosphate (monitored at 450 nm) and b) pyrene phosphate (monitored at 409 nm). The reactions were monitored over 20 minutes and the initial fluorescence reading was subtracted from all measurements so that each experiment started from the origin. The “Ab Control” sample contained rabbit IgG without Anti IgG-AP.

For conventional ELISA samples, the slope was calculated from the rate of change of fluorescence vs. time was calculated after the 10 minute delay had elapsed. The fluorescence for the conventional ELISA
was monitored at a position approximately 1.5 cm above the film-immobilized antibodies at the base of the cuvette. Thus, the conversion of 4-methylumbelliferyl phosphate to fluorescent product occurred at the bottom of the cuvette and the 4-methylumbelliferone product had to diffuse through the solution to reach the height of the optical components. By comparison, the 1-hydroxypyrene product was produced close to the surface of the siloxane polymer, where it was extracted and detected by the optical system. This proximity effect may have been enhanced by two factors. First, enzyme active sites typically have lower dielectric constants than aqueous solutions, leading to pKₐ perturbation of ionizable groups in favour of the neutral form. It is therefore possible that the environment experienced by the newly cleaved 1-hydroxypyrene favoured the neutral form of the molecule, rather than the anionic form, and this could have subsequently allowed it to be extracted into the siloxane polymer more rapidly. Second, the fluorescent products were detected within the waveguiding siloxane film, through which the excitation and emission waves propagated. A protein layer, expected to have a refractive index of between 1.4 and 1.5, was present on the PDMS film. This value is close to or greater than the refractive index of Sylgard 184 (1.4118 at 589 nm) and could lead to extension of the waveguiding layer from the polymer into the layer of protein (Figure 5-19).

Figure 5-19: The refractive index of an immobilized protein layer is between 1.4 and 1.5, which is similar to or higher than that of the Sylgard 184 waveguide core. Light propagating through the waveguide will experience total internal reflection at the protein-solution boundary, rather than the PDMS-protein boundary, extending the waveguide into the protein layer.
As the 1-hydroxypyrene was formed and diffused through the protein layer to the PDMS film, it may have been detected as being in the waveguide before it was actually extracted into the polymer. These two factors, along with the proximity of the enzyme reaction to the optical components, could account for the more rapid response observed for the SPME ELISA. This rapid response alone could be an advantage for the SPME ELISA compared with the conventional system, however most ELISAs are performed in 96 well plates with detection from above or below the well, which would eliminate the diffusion delay for solution-phase product detection.

To determine the limit of detection for the conventional and SPME ELISA systems, a calibration curve was prepared for each of the two systems, consisting of a range of concentrations of Rabbit IgG detected by a constant Anti IgG-AP concentration. This was combined with multiple measurements of the signal at an IgG concentration near the detection limit in order to determine the standard deviation of these results. In the calibration plots for 4-methylumbelliferyl phosphate, the conventional substrate, and pyrene phosphate, the SPME substrate, it was observed that the sensitivity of the ELISA was approximately four times higher for the SPME system (Figure 5-20). This could be attributed to factors including the proximity of the enzyme-labeled antibodies to the optical components in the SPME assay, as well as the enhanced fluorescence signal produced by concentrating the fluorescent products in the lower volume polymer film. However, we could not control for the fluorescence properties of the two ELISA substrates. In particular, the excitation wavelength was optimized for 1-hydroxypyrene, which may have reduced the sensitivity to 4-methylumbelliferone.

For both 4-methylumbelliferyl phosphate and pyrene phosphate, 0.01 µg/mL IgG was chosen as a concentration near the detection limit for further investigation of the assay sensitivity. A total of five assays were carried out at this concentration, as well as five tests on a blank sample (0 µg/mL IgG) using
Figure 5-20: The analyte concentration dependence of a model immunoassay using Rabbit IgG as the analyte and Anti IgG-AP as the detection antibody. The alkaline phosphatase labeled antibodies were detected by the fluorescence of a) 4-methylumbelliferone at 450 nm in solution and b) 409 nm of 1-hydroxypyrene extracted into the PDMS film. Replicates: n=1.

The limit of detection was calculated using the method of Loock and Wentzell\(^\text{92}\) through Equation 5-5, where \(y_{LOD}\) is the signal at the limit of detection, \(\bar{y}_{\text{blank}}\) is the average blank signal, \(t_{\alpha, k-1}\) is the t value for a given confidence interval \((1 - \alpha)\) with \(k-1\) degrees of freedom (where \(k\) is the number of replicate measurements), and \(s_y\) is the standard deviation of a sample measurement near the limit of detection.

\[
y_{LOD} = \bar{y}_{\text{blank}} + t_{\alpha, k-1} s_y
\]

For the conventional ELISA, the average blank signal was 106 rfu/hr and the standard deviation of the 0.01 µg/mL IgG sample measurements was 464 rfu/hr. Using a t value of 2.132 (\(\alpha = 0.05\), \(k = 5\)), the signal at the limit of detection was calculated to be 1096 rfu/hr. Using the equation of the calibration curve, the limit of detection in terms of analyte concentration was determined to be 3.7 ng/mL IgG. The same procedure could be used for pyrene phosphate. The average blank signal was 1823 rfu/hr, and the standard deviation of the 0.01 µg/mL IgG samples was 3311 rfu/hr. Using these values, the limit of
detection was calculated to be 8882 rfu/hr, or 7.1 ng/mL IgG. One of the five samples measured at 0.01 µg/mL exhibited a fluorescence vs. time slope that was approximately 50% of the value of the next lowest measured slope. If this value was excluded from the calculation, the standard deviation of the positive samples decreased to 2197 rfu/hr, resulting in a limit of detection of 6508 rfu/hr or 4.7 ng/mL IgG. Even with this value excluded, the SPME ELISA did not reach the limit of detection of the conventional system. While some of the sample-to-sample deviation can be attributed to the variation in protein attachment between replicate samples, a portion of the variability is likely due to the inconsistent optical alignment with the PDMS film and slight inconsistencies in the film thickness. Thus, at this point, the goal of achieving a lower detection limit for ELISA has not been achieved for the model protein system, despite the higher sensitivity demonstrated.

5.3.5 Detection of *E. coli* Using SPME ELISA

A non-pathogenic *E. coli* strain was used to test the SPME ELISA system for waterborne pathogen detection. Based on two reference Colilert tests, a presumed 100 CFU *E. coli* aliquot contained 85 ± 15 CFU. In the samples that were cultured after the capture of *E. coli*, no positive results were noted over the first 7 hours of the test. After 24 hours, however, the samples that contained 8500 CFU/mL and 850 000 CFU/mL, as well as the positive control sample, (*E. coli* spike without capture or washing steps) had fluorescence peaks at 405 nm, indicating the presence of 1-hydroxypyrene in the siloxane films. However, the 0 CFU/mL and 85 CFU/mL samples did not show evidence of *E. coli* in the cuvettes (Figure 5-21). This result may indicate the capture limit of the test: below a specific concentration (between 85 and 8500 CFU/mL) of bacteria, the probability of one or more *E. coli* cells being captured by the antibodies is low, leading to negative results. This same concentration limit was observed for ELISA samples. In these tests, the slope of the fluorescence versus time plot at 405 nm was used to compare the concentration of enzyme label in each cuvette (Figure 5-22).
**Figure 5-21:** The fluorescence signals inside the siloxane films of cuvettes functionalized with anti *E. coli* antibodies and exposed to various concentrations of *E. coli* 25922. These samples were cultured in a medium containing pyrene-β-D-glucuronide substrate and incubated, with occasional monitoring of the fluorescence signal within the polymers. In the positive control sample, 85 CFU *E. coli* was added along with culturing medium and no washing steps were performed, meaning that it controlled for the viability of the injected bacteria. a) The fluorescence measured over 24 hours. b) Fluorescence after 24 hours. Replicates: n=1.

**Figure 5-22:** The slopes of fluorescence vs. time relationships for anti *E. coli* antibody-functionalized cuvettes that were exposed to various concentrations of *E. coli* 25922, followed by ELISA detection using a biotin-labeled antibody and alkaline phosphatase-labeled biotin. The fluorescence slope represents the extraction of the 1-hydroxypyrene ELISA product into the siloxane film at the base of each cuvette. Replicates: n=1.
Again, the cuvettes spiked with 0 CFU/mL and 85 CFU/mL *E. coli* showed similar signals and the samples spiked with 8500 CFU/mL and 850 000 CFU/mL showed higher but similar signals. The similarity of the negative sample and the 85 CFU/mL sample can be explained by the corresponding results from the culture assay: below some concentration of bacteria (between 85 and 8500 CFU/mL), the probability of capturing at least one cell is low and produces negative results.

This “capture limit” could be a major hindrance to the further development of ELISA for waterborne pathogen detection, since drinking water must be analyzed using techniques that can detect a single colony forming unit in a 100 mL water sample. In order to improve upon this, it would be necessary to increase the probability of capture by antibodies. Agitating the sample to increase the rate of mixing might assist with this, but more effective capture techniques may also be required. This could be accomplished using antibody-functionalized magnetic beads, for example. If the water sample could be agitated with the *E. coli* antibody-functionalized beads in suspension, the probability of capture would increase. These beads could then be separated from the bulk sample and retained in the water during subsequent washing steps using a magnet. It may also be possible to capture and concentrate *E. coli* cells by means of filtration: the water sample could be passed through a submicron pore filter, which should retain the cells, then the direction of water flow could be reversed to remove the captured cells. If the reverse water volume is lower than the initial water volume, while removing most cells from the filter, a concentrating effect could be achieved. Finally, the concentration of the bacteria in the water could be increased by using an intermediate culturing step prior to the ELISA. It is possible that a short culturing step (at least 4 hours) could increase the *E. coli* concentration sufficiently to be detected, given the capture limit found here.

Although a full detection limit analysis was not performed, the SPME ELISA performed here was capable of detecting 8500 CFU/mL, but not 85 CFU/mL. It is therefore likely that the detection limit for
the assay lies between these two concentrations, without any optimization having been performed. This compares favourably with conventional ELISA methods, which report detection limits of $1 \times 10^3$ to $1 \times 10^4$ CFU/mL. While this is inferior to the state of the art biosensor-based pathogen detection methods, the most sensitive of which was able to detect 1 CFU/mL, refinement of the optical system for reproducibility, and optimization of the assay parameters should improve this performance measure.

Another challenge with this assay was the high level of background slope exhibited by the negative ELISA sample. A number of possible reasons for this high background were considered. Increasing the number of Tween 20-PBS washes following avidin-AP binding did not have an effect on the background level. The avidin-AP concentration could also be lowered, but this would need to be balanced against the strength of the positive sample signals. Some aspect of the surface treatment, such as the protein blocking agent or the chemical modification, may also have caused higher levels of non-specific avidin-AP binding. Finally, it is possible that the high background signals were due to hydrolysis of pyrene phosphate in the absence of alkaline phosphatase. The avidin-AP concentration, surface treatment steps, and pyrene phosphate stability would all need to be considered to improve the results of future tests. Nevertheless, this preliminary *E. coli* detection assay by ELISA showed promise for the SPME method and more work is needed to characterize its response as well as optimize the signals obtained.

### 5.4 Conclusions

An SPME-ELISA assay was developed, a process that included refining the optical system, characterizing the SPME-enabled substrate and enzyme-substrate interactions, as well as investigating the performance of the assay.
The optical alignment system for the detection of fluorescence within a thin polymer layer was improved through the development of a cuvette mounting system and fine adjustment method. The reproducibility of in-polymer fluorescence measurements was demonstrated to be improved based on these developments. It was found that the lower viscosity of Sylgard 184, combined with a 2:1 (m:v) dilution in hexanes to produce a 40 mg film, provided the most ideal PDMS casting conditions in terms of the reproducibility of the optical measurements. Despite these improvements, greater film reproducibility would assist in decreasing the noise in the polymer fluorescence measurements, which would improve the performance of the system overall.

The characteristics of the newly synthesized pyrene phosphate substrate were demonstrated for use in the ELISA system. It was found that the rate of hydrolysis of pyrene phosphate in the absence of enzyme appeared to increase as a function of pH, while increasing pH also led to the deprotonation of 1-hydroxypyrene. In the presence of enzyme, the observed rates of alkaline phosphatase activity increased as a function of pH up to the maximum buffer pH tested, 9.0. This pH was therefore used during the enzyme activity analysis step in all ELISA experiments. Further work could investigate the role of pH in hydrolysis of pyrene phosphate in order to determine if this phenomenon can be slowed or stopped. Doing so would reduce the level of background fluorescence observed in the assays.

A model ELISA, which detected Rabbit IgG using Anti IgG-AP compared an ELISA using the conventional 4-methylumbelliferyl phosphate substrate (fluorescence detected in solution) with the newly developed SPME ELISA using pyrene phosphate (fluorescence detected in polymer). The experiment showed enhanced fluorescence kinetics and sensitivity for the SPME ELISA, but failed to demonstrate an improved detection limit relative to the conventional system. Improvements to the optical alignment system and polymer casting to decrease the noise associated with the fluorescence measurements could change this observation.
Finally, the SPME ELISA was used to detect a model pathogen, *E. coli* 25922, using a sandwich assay. This test showed that the SPME ELISA did not reach the detection standard achieved by the most advanced current biosensors, but did have an estimated detection limit of 8500 CFU/mL or below. Since this assay was completed without optimization, adjustments to parameters of the test, as well as the aforementioned optical system refinement, could lead to improvements in this value.

5.5 Acknowledgements

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Chapter 6

Conclusions and Future Perspectives

6.1 Conclusions

We demonstrated the development and application of chemically modified polysiloxanes for environmental sensor devices. The modification of siloxanes was accomplished through a variety of strategies: synthesis of siloxane oligomers from functionalized monomers, hydrosilylation to incorporate functional groups into existing siloxane oligomers, and post-curing surface modification. Several post-curing surface modifications were demonstrated: siloxane chain exchange to incorporate functional oligomers into a poly(dimethylsiloxane) film, acid catalyzed hydrolysis to introduce carboxylic acid functionality, activated ester formation using carbodiimide and succinimide chemistry, and protein immobilization. The solid phase microextraction properties of these modified materials have been implemented in sensing schemes for volatile organic compounds (VOCs), and in an immunoassay system, which was shown to detect *E. coli*.

6.1.1 Volatile Organic Compound Sensing Using Modified Siloxane Materials

Siloxane films including methyl, phenyl, and naphthyl-functional groups were synthesized and applied to the detection of volatile organic compounds by solid phase microextraction using two methods: a Fabry-Perot cavity device and a visible wavelength refractometer. These sensing methods were shown to work on the principles of film deformation and refractive index change, respectively, resulting from the uptake of analyte molecules into the bulk of the siloxane film by partition from the gas phase. The
functional groups incorporated into the siloxanes influenced both the optical and analyte-uptake properties of these films. A summary of the key conclusions is provided here:

1) Dimethyl (PDMS) and diphenyl-functionalized (PDPS-PDMS) siloxanes were coated in Fabry-Perot interferometer cavities and applied to the detection of m-xylene and cyclohexane. These devices were shown to detect these VOCs through the swelling-based deformation of the reflection elements that comprise the cavity. Measurements of the sensor response to the two analytes showed similar sensitivities when PDMS and PDPS-PDMS were used, suggesting that the chemical functionality of the film was not an important factor. The detection limits for m-xylene (190 to 590 ppm) and cyclohexane (840 to 2100 ppm) were higher than would be required for indoor air quality monitoring, (ACGIH Threshold Limit Values: 100 ppm for cyclohexane and m-xylene\(^1\)) but could have applications in solvent spill detection.

2) A high refractive index siloxane film that contained naphthyl, phenyl, and methyl functionalities (PMNS-PDPS-PDMS) had a RI of 1.5846 at 635 nm, substantially higher than the RI of Sylgard 184 PDMS (1.4225 at 632.8 nm \(^2\)). This provided a high refractive index contrast with solvents including m-xylene and cyclohexane, and increased the sensitivity of the film for VOC analysis by approximately an order of magnitude relative to previous work in our group,\(^3\) though the limits of detection (4.0 x 10\(^2\) ppm for m-xylene and 4.0 x 10\(^3\) ppm for cyclohexane) were still not sufficient for air quality regulations. Non-linear refractive index responses were observed at low solvent concentrations, which will limit further improvements in these detection limits.

3) It was shown that these label free VOC detection methods did not reach limits of detection applicable to indoor air quality analysis. This, combined with the challenges of identifying analytes in complex mixtures by label free sensing, demonstrate the advantages of label-based
detection. VOC sensors of this type will likely need to be applied in arrays of partially selective individual sensors or in screening devices to be used in practical applications.

6.1.2 Chemical Modification of Siloxane Polymers for Biomolecule Attachment

Bulk and surface modification methods were used to prepare PDMS for protein attachment. Both schemes aimed to introduce ester functionality, which could later be hydrolyzed and used to form amide bonds with proteins. These modifications have been characterized and optimized through a variety of surface analysis methods. The effects of these modifications on the solid phase microextraction properties of siloxanes have also been investigated. A summary of the key conclusions is provided here:

6.1.2.1 Bulk-Modified Siloxanes

1) The bulk modification of linear siloxanes was used to incorporate a methyl ester functional group, forming the ester-functionalized linear siloxane oligomer (PCBMS-PDMS). Following film casting and curing, acid-catalyzed hydrolysis was used to remove the methyl ester from PCBMS-PDMS to form carboxylic acid functional groups. Using EDAC.HCl dye analysis, it was observed that 5 M H$_2$SO$_4$ at 45°C for 4-6 hours produced the greatest number of carboxyl functional groups.

2) The bulk-modified PCBMS-PDMS material prevented solid phase microextraction of 1-hydroxypyrene into a PDMS waveguide layer. While the use of a heterogeneous (partially covered) PCBMS-PDMS surface avoided this problem, the heterogeneous surfaces were difficult to produce consistently, leading to the need to pursue other modification methods.
6.1.2.2 Surface Modification of Siloxanes by Chain Exchange

1) Ester functional groups were also incorporated into cured Sylgard PDMS materials using chain exchange in the presence of acid. The incorporation of PDMS-PMS and PCBMS-PDMS were both successful, based on ATR-FTIR spectra. It was found that the incorporation of PCBMS-PDMS, which had already undergone hydrosilylation to add the ester group, was preferable to hydrosilylation of PDMS-PMS after chain exchange due to incompatibility between the plastic cuvettes, solvent, and hydrosilylation catalyst. Based on contact angle measurements, continuing the chain exchange process for more than 30 minutes did not produce more extensive surface modification.

2) The hydrolysis of methyl ester functional groups incorporated by chain exchange was monitored using contact angle measurements. Acid hydrolysis of the ester in the presence of 5 M HCl at 35°C for either 6 or 24 hours resulted in significant increases in the hydrophilic character of the surfaces, which was attributed to the formation of carboxylic acid groups.

3) 1-Hydroxypyrene was successfully extracted from aqueous solution by the chain exchange-modified films, which maintained uptake properties similar to unmodified Sylgard PDMS. This method was more repeatable than the bulk modification followed by heterogeneous film casting described earlier.

6.1.3 Attachment of Proteins to PDMS and Ester-Modified PDMS Surfaces

The protein immobilization process on unmodified PDMS and carboxyl-modified PDMS was investigated and the conditions of the immobilization were optimized. The amount of immobilized protein was determined by enzyme assays, although contact angle and ATR-FTIR methods were also used for characterization. A summary of the key conclusions is provided here:
1) Contact angle and ATR-FTIR results showed changes in the surface properties of the siloxane materials following the immobilization of proteins. An increase in surface hydrophilicity was observed when the unmodified PDMS films were exposed to solutions of at least 10 mg/mL bovine serum albumin. ATR-FTIR spectra of PDMS films to which BSA had been adsorbed showed small characteristic amide peaks. These results were important in confirming the effect of BSA blocking on the surface, but insufficiently sensitive for characterizing the immobilization of antibodies.

2) The use of alkaline phosphatase labels on the immobilized antibodies resulted in more sensitive measurements of the quantity of bound antibody (less than 1 ng/cm$^2$ detected). The enzyme assay protocol was used to optimize a number of parameters associated with the immobilization of antibodies on the carboxyl-modified surface, including: i) the chemical modification steps (chain exchange and acid hydrolysis), ii) the composition of the carboxylic acid activation solution (buffer pH, carbodiimide and succinimide concentration, treatment time), and iii) the protein immobilization step (antibody concentration, solution volume).

3) Although the covalent nature of the immobilization to carboxyl-modified siloxanes could not be definitively confirmed, the immobilization on this modified surface was shown to function by a different mechanism than adsorption to unmodified PDMS: i) Protein A immobilization was successful on the carboxyl-modified surface, but not on unmodified Sylgard, and ii) the presence of Tween 20 in the antibody immobilization buffer minimized antibody adsorption on unmodified Sylgard, while significant activity was observed on the carboxyl-modified surface.

4) Despite the stronger binding offered by the carboxyl-modified surface, antibody immobilization was found to be equally effective on the unmodified PDMS material in terms of both the quantity
of immobilized antibody and long-term stability. For most applications, the use of adsorbed antibody on unmodified PDMS may be sufficient. Exceptions to this would include situations where a detergent was present in the immobilization buffer, where Protein A or another affinity linker that did not adsorb effectively was being used, or where adsorption of a particular antibody did not result in sufficient immobilized material.

5) For the model ELISA, the use of Protein A as an orientation-specific affinity linker did not provide advantages in terms of the ELISA sensitivity or stability. This may be because the ELISA being carried out in this project was essentially an “antigen-down” ELISA, where Rabbit IgG acted as the antigen, rather than the capture antibody. It is possible that a true sandwich ELISA would benefit more from orientation-specific immobilization.

6.1.4 Development of Solid Phase Microextraction Enzyme-Linked Immunosorbent Assay

A solid phase microextraction enzyme-linked immunosorbent assay was developed with the aim of improving the detection limit of ELISA for applications in waterborne pathogen detection. This process involved refinements to the optical analysis system, characterization of the SPME-capable enzyme substrate, and evaluating the assay performance. A summary of the key conclusions is provided here:

1) Semi-micro cuvettes with a polymer film in the bottom of the channel were shown to be effective for SPME ELISA. This required optimization of the film formulation, and the optical alignment. Optimization of the alignment required the testing of a variety of cuvette mounting systems and the eventual design involved a 3-D printed cuvette mount and set screw to improve the in-polymer fluorescence measurements by providing alignment stability and making fine adjustments possible.
2) The characteristics of the new ELISA substrate, pyrene phosphate, were investigated for use in the detection system. The optimal pH for the ELISA was 9.0, which was determined to provide the greatest difference between the rate of pyrene phosphate hydrolysis by the enzyme and the background rate of hydrolysis in the absence of enzyme. pH 9.0 buffer was therefore used in all SPME ELISA experiments.

3) The performance of a model ELISA was compared using a conventional substrate (4-methylumbelliferyl phosphate, fluorescence detected in solution) and the SPME substrate (pyrene phosphate, fluorescence detected in polymer). While more rapid kinetics resulted in a higher sensitivity for the SPME ELISA, the conventional ELISA produced a slightly better detection limit (3.7 ng/mL compared to 7.1 ng/mL IgG) owing to greater signal reproducibility. Additional optimization of the optical system would likely result in a lower detection limit for the SPME ELISA.

4) The SPME ELISA was used to detect a model pathogen, *Escherichia coli* 25922, using a sandwich assay. The preliminary (estimated) detection limit for this test was less than 8500 CFU/mL, based on a small sample set. This does not meet the standard of currently available biosensors, although it must be noted that no optimization of this assay has yet been performed. The ELISA also appeared to have an elevated background signal, which could obscure small positive results and must be corrected going forward.

### 6.1.5 General Conclusions

Siloxanes have been shown to have versatile properties that can be applied to sensor design. Poly(dimethylsiloxane) has a refractive index high enough to act as a waveguide, can be cast in a variety of shapes, is compatible with adsorption of antibodies, and can also extract analytes from a sample matrix.
Modifications to the siloxane material can be made in a number of ways, including through the extent and type of cross-linking, the use of functionalized monomers, and through surface chemistry. These changes in properties have been demonstrated to affect the refractive index of the material, the binding of biomolecules such as proteins, and the extraction properties of the material.

Bulk and surface modifications of siloxanes have been demonstrated to have complementary applications. Bulk modifications can be achieved by altering the chemical functionality of the siloxane oligomers and these have been shown to impact the SPME and optical properties of the materials. Surface modifications of the cured films were also useful for the production of sensor devices, allowing protein attachment and affecting the surface energy of the films. It was shown to be important to select the type of modification with care, since some modifications can have consequences on many properties simultaneously. For example, adding additional titanium tetraisopropoxide crosslinker increased the refractive index of a material and enhanced curing, but may have been detrimental to SPME applications. The use of bulk ester-modified siloxanes when only surface functionalization was required also inhibited the SPME properties of the material. In this case, performing surface-only modifications provided the necessary functional groups without a noticeable effect on optical or extraction properties.

The non-specific extraction properties of siloxanes were demonstrated to be a drawback to their application to label-free sensors. Therefore, siloxane SPME-based sensing methods must rely on a signal transduction method that imparts the required level of specificity. When combined with a label, the non-specific extraction properties of siloxanes become an advantage: allowing label molecules to be extracted from solution with minimal optimization of the polymer or label.

The properties of labeled and label-free sensing mechanisms have been demonstrated through the detection of VOCs by refractive index and swelling measurements, and the detection of biomolecules and bacteria using ELISA. While label-free sensing is more straightforward to perform, since it does not
require the design of labeling schemes, the utility is compromised by a lack of specificity and sensitivity. Labeled sensing methods require more time to develop, including the identification of a sensing mechanism that is effective for the analyte of interest and performance of any necessary labeling reactions, but result in more sensitive and specific analyte measurements. For environmental analysis, which often involves low analyte concentrations in complex matrices, label-based sensing is thus the practical choice.

6.2 Future Perspectives

This project demonstrated the development of modified siloxane materials for applications in environmental sensor design. These materials were applied to the detection of volatile organic compounds and waterborne pathogens. While some promising preliminary results were obtained, there are many directions that could be taken in future projects. The main areas for further investigation are improving the properties of the siloxane materials for various sensing applications, more detailed investigation of the chemical and biological modification of the siloxane materials, and improving the quality of the signals in the SPME ELISA. A summary of the proposed future work is provided here:

1) The low sensitivity of the naphthyl and phenyl-functionalized siloxanes to volatile organic compounds, particularly at low concentrations, prevents their use for indoor air quality analysis. This may have been due to the mechanisms of solvent uptake that are involved: filling of free volume in the siloxane material, which always leads to an increasing refractive index, and swelling of the material, which leads to a refractive index intermediate between the pure polymer and the pure analyte. The isolation of one of these mechanisms would provide a sensor with a more consistent response over a range of concentrations. Although increasing the titanium cross-linker content in the films led to higher starting RI values, this high level of cross-linking may be
responsible for the delayed onset of swelling. Further experiments should investigate whether reducing the cross-linker concentration, or changing the type of cross linker to a tri or tetrafunctional silane\(^5\) or a hydrosilylation-based system,\(^6,7\) would facilitate more consistent swelling of the films. This could allow the film to respond in a way that mimics the mixing of two liquids, with the refractive index of the mixture depending on the volume fractions of the components.

2) The chain exchange process for siloxane surface modification was shown to be a promising technique to incorporate useful functional groups into siloxane polymers. This method was demonstrated here for the surfaces of thick waveguides, but should be investigated for the bulk modification of thin films. These films could be applied to sensors and post-curing modifications could alter the optical properties and specificity of the materials.

3) It would be useful to further characterize the chain exchange process to understand the mechanism of the modification and if any improvements could be made to the procedure to increase the degree of modification. For example, the use of X-ray photoelectron spectroscopy could give more surface-sensitive information regarding the chemical composition of the material surface and this could be combined with further ATR-FTIR characterization to determine the presence of particular functional groups. The depth of modification could be characterized using a fluorescently-labeled siloxane oligomer or refractive index analysis could be used to assist in label-free characterization.

4) The use of harsh hydrolysis conditions to remove the methyl ester from PCBMS-PDMS leads to concerns around the degradation of the siloxane backbone. It would be ideal to use a protecting group that could more easily be converted to a carboxylic acid after hydrosilylation. For example, \(\text{t-butyl esters are more readily hydrolyzed under mild acidic conditions.}^8\) While previous attempts
were made by members of our group to synthesize \( t \)-butyl-4-pentenoate, these were not successful in producing high yields of the desired product. The incorporation of the \( t \)-butyl ester groups through chain exchange would need to be done carefully, however, as the method involves concentrated acid that could prematurely hydrolyze the \( t \)-butyl ester. This synthetic procedure would require further optimization and the compatibility of the reagents with chain exchange would need to be determined.

5) While the antibody immobilization procedure was optimized for the chemically modified siloxanes, the adsorption procedure did not undergo extensive investigation. Further studies into the ideal conditions for this process, including protein concentration, adsorption time, temperature, and buffer conditions, could further improve the concentration of active antibodies introduced by adsorption.

6) Affinity-based immobilization of antibodies should be investigated further. While Protein A was used in the model ELISA here, it is possible that the use of such a direction-specific linker would be more beneficial in a true sandwich ELISA, where the antibody orientation is critical for antigen capture. Avidin-biotin affinity immobilization could also be investigated, particularly if biotin can be specifically incorporated into the constant region of the antibody, providing direction-specific immobilization.³

7) The use of the stronger immobilization procedure on the carboxyl-modified siloxane surface did not lead to improved ELISA results based on activity or stability. However, the system was only compared using a model ELISA. It is possible that this immobilization chemistry could be of greater use in other systems. For example, an analyte that does not adsorb effectively to siloxanes may be immobilized on the chemically modified surface. In addition, if Protein A was required as
a direction-specific linker, the use of the carboxyl-modified siloxane could play a useful role in increasing the amount of bound antibody.

8) Despite the improvements made to the in-polymer fluorescence detection system for SPME ELISA, the main limitation with this system remains the variability in the optical signals. Poor optical alignment of cuvettes in the cuvette mount and inconsistent siloxane film morphology will both give rise to variable rates of uptake and inconsistent fluorescence signals. It remains challenging to cast all siloxane films in a precise fashion, since some of the film usually cures onto the sides of the plastic cuvette. Ideally, a rectangular prism-shaped film of reproducible thickness should be formed. To accomplish this, it may be necessary to treat the interior surface of the cuvette, apart from the bottom surface, to prevent siloxane adherence. Alternatively, the films could be cast and cured outside the cuvette, cut to the correct size and then later adhered to the cuvette using a small amount of freshly prepared siloxane or a chain exchange procedure.

9) The high background signals obtained in the SPME ELISA are believed to originate from two sources: in all cases, the hydrolysis of pyrene phosphate in high pH buffer leads to the production of 1-hydroxypyrene without the presence of enzyme, and in the E. coli ELISA, additional background signal was thought to originate from the non-specific binding of avidin to the solid support. It appeared that most of the background signal was due to the hydrolysis of pyrene phosphate. Changing the method of storage of pyrene phosphate, from water to an aprotic organic solvent, may decrease any hydrolysis that occurs while the stock solution is stored prior to use. It may also be useful to investigate more stable phosphate derivatives with the required hydrophobic product of the enzyme reaction. This may be a difficult balance, since the polycyclic aromatic hydrocarbon is both very hydrophobic and stabilizes the deprotonated form of 1-hydroxypyrene.
10) The *E. coli* ELISA requires significant further optimization. Many options for ELISA-based detection of this pathogen are possible, although sandwich assays are a logical starting point since *E. coli* is a cell with many antigenic surface proteins. However, the detection system could involve avidin-biotin affinity between the detection antibody and the enzyme label, an enzyme labeled detection antibody, or an enzyme labeled secondary antibody. An enzyme labeled secondary antibody would be expected to increase the sensitivity of the ELISA since many of these antibodies could bind to each detection antibody. Improvements to this test could bring SPME ELISA closer to applications in water analysis by lowering the detection limit while providing versatile recognition of pathogens.

11) Other analytes could also be detected by the SPME ELISA system. Pesticide, protein toxin, and pathogen detection should be explored for environmental sensing applications, since these systems require highly sensitive and specific detection schemes.

### 6.3 List of References


