Towards Construction of a Biosensor for the Detection of Early-Stage Ovarian Cancer: Evaluation of Novel DNA Aptamers for the Capture of Biomarker HSP10

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

The current test for ovarian cancer is associated with many false positives and false negatives. With ovarian cancer being the deadliest gynaecological cancer, the diagnostic inadequacy of the current test creates an imperative need for a method that can detect for early-stage ovarian cancer. Fortunately, HSP10 has recently emerged as a potential biomarker for early-stage ovarian cancer. Four DNA aptamers were evaluated for their binding affinity towards HSP10 using two methods. The first method employed use of gel electrophoresis, a common method to study interactions, mainly bind-and-release, between biomolecules. The second employed novel surface chemistry with the EMPAS. With the combination of both methods, a candidate DNA aptamer was selected for surface chemistry immobilization, moving towards building a crude biosensor. This is the first steps towards creating a novel biosensor which can provide women with a screening test that has the ability to mitigate deaths associated with ovarian cancer.
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Sincerely,

Jenise B. Chen

2016
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AU</td>
<td>absorbance units</td>
</tr>
<tr>
<td>ARXPS</td>
<td>angle-resolved X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>BAW</td>
<td>bulk acoustic wave</td>
</tr>
<tr>
<td>CA-125</td>
<td>cancer antigen 125</td>
</tr>
<tr>
<td>CAG</td>
<td>contact angle goniometry</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>Cpn10</td>
<td>chaperonin 10</td>
</tr>
<tr>
<td>cps</td>
<td>counts per second</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylforamidane</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>enterokinase</td>
</tr>
<tr>
<td>EMPAS</td>
<td>electromagnetic piezoelectric acoustic sensor</td>
</tr>
<tr>
<td>EPF</td>
<td>early pregnancy factor</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>His-tag</td>
<td>histidine tag</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSP10/60</td>
<td>heat shock protein 10/60</td>
</tr>
<tr>
<td>IMAC FF</td>
<td>immobilized metal-ion affinity chromatograph fast flow</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple clonal site</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTA</td>
<td>Na,Nα-bis(carboxymethyl)-l-lysine hydrate</td>
</tr>
<tr>
<td>OC125</td>
<td>ovarian cancer 125 monoclonal antibody</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ODTTS</td>
<td>octyldecyltrichlorosilane</td>
</tr>
<tr>
<td>OTS</td>
<td>octyltrichlorosilane</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFP-TTTA</td>
<td>pentafluorophenyl 13-trichlorosilyl-tridecanoic acid 2,2,2-trifluoroethyl</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
</tbody>
</table>
RNA  ribonucleic acid
rpm  revolutions per minute
RSD  relative standard deviation
SAM  self-assembling monolayer
SAW  surface acoustic wave
SELEX  systematic evolution of ligands by exponential enrichment
SDS  sodium dodecyl sulfate
SH-SAW  shear horizontal surface acoustic wave
SPR  surface plasmon resonance
TBE  Tris-Borate-EDTA buffer
tmMNC-38  thiol-modified MNC-38 DNA aptamer
Tris  tris(hydroxymethyl)aminomethane
TSM  thickness shear mode
UV/Vis  ultraviolet/visible light
XNA  xeno nucleic acid
XPS  X-ray photoelectron spectroscopy

*Note: The abbreviation “NTA” is a more commonly used for the compound N,N-bis(carboxymethyl)glycine. In this manuscript, the compound used in partner with this abbreviation is Na,Nα-bis(carboxymethyl)-l-lysine hydrate.
Chapter 1.
Introduction

1.1 Ovarian Cancer: the underlying problem

The hallmark of cancer is governed by an uncontrolled growth of cells that occurs when normal signalling, replication, and overall homeostasis within the cells are lost. The resulting factor is a manifestation of cellular aberration that hampers the function of the surrounding normal tissue. Over time, the mutated mass will continue to progress in growth and without any intervention, the cancer will cause a fatal outcome for the individual. Cancer can occur in any part of the body, in any cell type, as it is a result of a series of changes to the cellular phenotype. The progression of cancer is frequently broken down into four main stages. Stage I, the earliest, is defined by the tumour being contained within its location. Stage II signifies a larger tumour mass and minimal delocalization, while Stage III represents local spreading of the cancer to lymph nodes and surrounding tissue. On the other end of the spectrum, Stage IV is labelled when cancer metastasis has occurred and survival prognosis at this point in time is generally grim. As a general consensus, early detection is vital for enhancing survival prognosis for any type of cancer. Ideally, it would be the most beneficial for the patient to detect the cancer at Stage I, where treatment is the most potent and effective. Unfortunately this is not the case for ovarian cancer, as most cases are only detected in Stages III-IV of the disease where metastasis has usually occurred. This is a result of the onset of ovarian cancer being rather quiescent, yet insidious; many of the symptoms not specific to this type of cancer. As a result, ovarian cancer has adopted the alias of the silent killer.

Ovarian cancer, as the name implies, is a type of cancer that manifests in the ovaries of the female reproductive system. The ovaries can be broken down into three basic components: the epithelium, the stroma, and the germ cells. The epithelium consists of the cells that encase the ovary and reside on the outside periphery of the organ. The stroma pertains to the cells that
reside within the ovary and are involved in the structural integrity of the tissue. The germ cells, which also reside within the ovary, are the cells that pertain to the ovum or egg released from the ovary during ovulation. Cancer can arise in any of these components, but epithelial ovarian cancer remains to be the most common, consisting of approximately 90% of all ovarian cancers. However, regardless of the type of ovarian cancer, the survival rate for this disease remains abysmal.

Approximately a quarter million women worldwide is affected by ovarian cancer, accounting for the deaths of over 140,000 women annually. Surprisingly, the five-year survival rate for women with ovarian cancer is only 45% compared to the 99% for women with breast cancer. The reason for this apparent discrepancy is due to the inconspicuous onset of ovarian cancer, which lacks specific symptoms and signs. As a result, only 15% of all ovarian cancers will be diagnosed at an early stage. When symptoms finally develop, women will have already entered the late stages of the cancer, and metastasis will have already occurred. At this point, prognosis for survival falls well below 10%. However, studies have shown that prognosis of the disease can exceed 90% survival if detected early. Unfortunately to date, there is still a lack of a reliable marker that can detect for an early-stage ovarian cancer tumour. With ovarian cancer being the deadliest gynaecological cancer with the highest mortality rate in the developed world, there is a growing need to detect for this cancer in its early stages. Not only is detection for early-stage ovarian cancer significantly needed, but the detection can also be applied to many other deep visceral cancers with similar challenges.

1.2 CA-125 Biomarker: the current unreliable test

Thirty-five years ago, Robert C. Bast and colleagues developed a monoclonal antibody for ovarian cancer in which they named OC125. This led to the discovery of cancer antigen 125 (CA-125), which the antibody was found to be specific for. From the time it was discovered until now, CA-125 is still widely used as a biomarker for ovarian cancer as it is considered to be the gold standard for detection, mainly for epithelial-derived ovarian cancer as it is the most
CA-125 is a transmembrane glycoprotein, meaning that it contains carbohydrate groups, and is expressed on the surface of epithelial ovarian cancer cells. It may also be released into the bloodstream, which is what the ovarian cancer assay tests for using the OC125 antibody. In other words, this test relies on the presence of the CA-125 in blood in order for it to make a diagnostic assumption.

Although the CA-125 assay is the current most trusted test for ovarian cancer, it is actually not ideal for providing a diagnostic conclusion by itself, especially at the early-stage. In the more recent years, increasing amounts of literature discuss the continuing issues that are associated with the CA-125 assay. For diagnostic purposes, the assay must be used in conjunction with an abdominal ultrasound. The main use of this assay is actually to observe and regulate the response ovarian cancer has towards its treatment, as well as to test for possible reoccurrence of the cancer after treatment. A higher serum level of CA-125 would be indicative of a lack of response to the cancer treatment or that the cancer has returned. However, there are several problems associated with this test. The first is that it is mainly used as an evaluation of ovarian cancer progression or regression. Second, the CA-125 test also cannot be used for any screening purposes because CA-125 is not found to be elevated at early stages of ovarian cancer in almost all cases. The biomarker is also found to be elevated in many different types of cancers, rendering not specific to ovarian cancer. It is also elevated in benign cases, such as pregnancy or during the menstrual period, all of which result in many false positives. There have also been cases where individuals with ovarian cancer showed low serum levels of CA-125, resulting in false negatives. Statistical findings show that approximately only 80% patients with advanced stages of ovarian cancer and 50% of patients in the early-stages will have elevated CA-125 serum levels. Over the last few decades, major efforts have been put into evaluating the efficacy of this tumour biomarker, with considerable success in the end. However, there is still a general agreement upon a need to provide a better, more accurate and specific method for detecting this disease, especially in its early stages. As a result of past findings and the current abysmal prognosis for ovarian cancer, the test is deemed to be unreliable for screening purposes and there is an urgent need to develop a method that possesses both high sensitivity and selectivity for biomarkers to substitute this current test.
1.3 HSP10: a new potential ovarian cancer biomarker

Heat shock protein 10 (HSP10) is the molecular homologue (Figure 1) to the bacterial protein known as GroES, which was first to be discovered. Both of these proteins in mammalian and bacterial cells are found in the matrix of the mitochondria. These 10 kDa proteins work in conjunction with their 60 kDa counterpart (HSP60 in mammalian cells or GroEL in bacteria) and combined, are essential in orchestrating correct protein folding and assembly as a chaperone complex (Figure 2). It has been found that HSP10, in particular, is formed and released when induced by stressful conditions, such as heat. These biomolecules are released into the cytoplasm of the cell under higher temperatures due to the fact that most proteins lose structure and function when temperatures are elevated.

Figure 1. 3D structure of the human HSP10/HSP60 complex. A) Profile view of complex. B) Top and bottom view of complex. (Image from Ref. 24)
HSP10 has recently emerged as a potential biomarker for ovarian cancer\textsuperscript{26} as it is seen to promote tumour formation through suppressing apoptosis of malignant cells.\textsuperscript{27} HSP10 has been found to be elevated in the immediate tissues surrounding the tumour within the ovary\textsuperscript{26} and therefore, taking a blood sample from the immediate area would prove acceptable as a possible screening method. HSP10 is not present at elevated levels in healthy individuals, rendering it a plausible biomarker with high specificity and low possibility of eliciting false positives.\textsuperscript{28,29} It is known that HSP10 is involved in cell proliferation during pregnancy and is found to be released at these early stages, thus it is also known as Early Pregnancy Factor.\textsuperscript{26,30} It is also seen to be released from proliferating liver cells during regeneration,\textsuperscript{31,32} as well as several other pathological diseases, such as large bowel carcinoma and chronic obstructive pulmonary disease.\textsuperscript{31,33,34} Knowing these facts, HSP10 may be upregulated as well during tumour formation, allowing it to be a great biomarker for detecting early stages of ovarian cancer. However, further understanding of the mechanism behind the release HSP10 still needs to be explored.\textsuperscript{30,31}
1.4 Aptamers: biomolecular probes for detection

First introduced in the early 1990s by two different research groups, aptamers are described as synthetic and short sequences made up of RNA, DNA, and more recently in the mid-2000s, even XNA.\textsuperscript{35,36} RNA aptamers were the first type to be explored, before the introduction of DNA-based aptamers. Due to RNA being single stranded by nature, they provide a wider range of three-dimensional structures, thereby allowing for higher selectivity and binding to the target.\textsuperscript{36,37} On the other hand, DNA aptamers are more stable under different conditions compared to RNA, especially under biological conditions.\textsuperscript{38} XNA is a type of nucleic acid that consists of an unnatural sugar backbone. These types of aptamers have properties that can enhance bioavailability and pharmacokinetics towards better therapeutic applications.\textsuperscript{36} Generally, aptamers possess many great advantages over natural biological probes such as antibodies and enzymes.\textsuperscript{39} This is because they can be selected or designed in such a way to be highly specific towards a target.\textsuperscript{40} Aptamers are also relatively small in molecular size (Figure 3), highly chemically stable\textsuperscript{40,41}, can be commercially produced at a rapid rate, and undergo conformational changes that can be tailored to specific transduction techniques.\textsuperscript{41,42} Due to these reasons, aptamers have been proven to be an enhanced choice over its natural protein predecessors.\textsuperscript{42}

![Figure 3. Three-dimensional molecular models comparing the size difference between the IgG antibody and thrombin aptamer.](image from Ref. 43)
1.4.1 SELEX

Systematic Evolution of Ligands by Exponential enrichment or SELEX is a method independently developed in 1990 by Ellington and Turek. This technique uses large combinatorial libraries of oligonucleotides through an iterative process to select for a sequence \textit{in vitro} that binds to the target analyte (Figure 4). As it is an iterative process, selection of the final aptamer may take anywhere from a few weeks up to several months. The process begins with a random sequence library acquired from an initial library constructed from combinatorial-chemically synthesized DNA. Conventionally, aptamer sequences are screened for non-specific binding through a process called \textit{counter selection}. Sequences that bind at this stage are discarded, and the rest are introduced to the target analyte. The unbound sequences after this stage are discarded, while the specifically bound sequences are eluted from the target. With DNA sequences, PCR is performed for amplification. If the sequences are RNA, an additional step for \textit{in vitro} reverse transcription is performed before amplification. The sequences are further transcribed to generate a new set of sequences that is then run through the cycle again. The process is usually repeated approximately 20 times to allow for full enrichment of the aptamer towards the specified target. However, although the conventional SELEX process is effective, it is very labour-intensive and requires a large amount of time. As a result, there are many other types of SELEX processes described and currently being researched on as well.
1.4.2 Applications in Biosensing

One of the forefront technologies in the biosensing world are aptasensors, which, as the name implies, are a particular group of biosensors that probe for a target analyte using aptamers.\textsuperscript{42} Aptamers have increasingly become a popular tool in novel diagnostic technologies due to their ability to provide high affinity and selective binding towards a very wide range of targets. Since their initial engineering in the 1990s, aptamers have the ability to significantly revolutionize the field of developing rapid selection technologies due to their high binding affinity to target molecules.\textsuperscript{40} Advances in aptamer research coupled with biosensor technology continue to expand and grow with excellent progress. As the research becomes more in-depth, the ability for these types of sensors to become an essential tool in healthcare and biomedical applications looks promising.\textsuperscript{44}
1.5 Biosensors

Figure 5. Schematic diagram showing the comparison between a typical sensor (top) versus a biosensor (bottom). A sensor provides a signal in response to a given input, while biosensors consist of a bio-recognition site of high selectivity that serves as the input. (Image adapted from Ref. 45)

The first biosensor emerged in 1953, when Leland C. Clark Jr. desired to measure the oxygenation levels in red blood cells of whole blood.\textsuperscript{46,47} Albeit crude at first, many iterations of this sensor such as the addition of a semi-permeable membrane (cellophane at the time, now Teflon) and the glucose oxidase enzyme\textsuperscript{48} allowed for the extensive progression of the biosensor field. The word biosensor combines the words biological and sensor and was first coined by Karl Cammann in 1977, but was not formally acknowledged by IUPAC until 1997.\textsuperscript{46,49,50} As its name suggests, biosensors are devices that consist of a biological component and are capable of analytically sensing the occurrence of complex biochemical interactions, usually on a solid surface.\textsuperscript{51} The difference between a typical sensor versus a biosensor lies within their ability to detect specific interactions (Figure 5). While a sensor has a very large range of possible input quantities – such as mechanical, chemical, as well as biological – selectivity usually remains rather low. Biosensors, on the other hand, are valued as a desirable alternative when focusing on
high selectivity for a particular analyte. With the consistent development of biosensors, the term now encompasses many different subcategories and types. One can appreciate this field garnering a rapidly increasing audience over the decades as general technology and its advancement became more widespread (Figure 6). The constant growing interest in the biosensor realm is built upon the attraction for rapid, easy, and robust methods for testing, yet harbouring the ability to provide label-free and specific analyses. In addition, the application for biosensors within the field of medicine and healthcare can prove to be immensely beneficial for improvement, if not revolutionary. This is evidently seen as a majority of literature pertaining to biosensors gear their applicability towards healthcare, as it is a field that continues to face new and old challenges, as well as to change and grow with the discoveries made in science and research.

![Biosensor Publications](image)

**Figure 6.** Graphical representation of the search term "biosensor" over the period of 1980 to 2011 using the Web of Knowledge. (Image from Ref. 51)

1.6 Acoustic Wave Sensors, Piezoelectric Effect, and Quartz

Acoustic waves are a result of longitudinal wave propagation through a medium that can be manipulated through compression and decompression. This effect has been exploited for use in acoustic wave sensors as a method for surface interaction detection. Similar to the oscillation of a coiled spring, when a force is applied in one direction, acoustic waves are generated in an
analogous fashion, albeit more complex. When a force is applied onto a solid, inevitable strain results, in which particles within the surface are displaced to cause deformation. This displacement of particles propagates a wave across the solid, in which the directionality of this phenomenon is termed polarization. The effect of an applied force coupled with electrical polarization (polarization stimulated by an applied electric field) results in the accumulation of an electric charge, a phenomenon termed as piezoelectricity. The direct piezoelectric effect is known as the generation of an electric polarization as a result of an applied mechanical strain. On the other hand, when the solid substrate is introduced to an electric field to cause polarization, the substrate can be mechanically strained. This is known as the converse piezoelectric effect. Both piezoelectric effects occur only when the substrate lacks a point of central symmetry.

Figure 7. Illustration of a quartz crystal cut at 35°10' relative to the optical axis (Z-axis) to produce an AT-cut quartz wafer. (Reprinted with permission from Ref. 54. Copyright 2003 American Chemical Society.)
One of the more prevalent substrates used in acoustic wave sensors is silicon dioxide (SiO$_2$), also known as crystalline quartz. Quartz offers many advantages, the first being low in cost relative to lithium tantalate and lithium niobate, both of which are commonly used piezoelectric substrates. A second advantage quartz offers is that it allows for selection of temperature dependence based on the cut angle and direction of the wave propagation. The angle at which quartz is cut is important to its piezoelectric properties, and in turn will instigate a characteristic vibration mode as a result of the difference in symmetry and properties between each type of cut. AT-cut quartz (Figure 7) is one of the more commonly used quartz cuts due to its excellent stability in temperature and capability to operate in the MHz frequency range.

1.6.1 SAW and BAW Sensors

Surface acoustic wave (SAW) and bulk acoustic wave (BAW) devices are two general categories of sensors that exploit the piezoelectric properties of quartz. The two types differ in the mode of which the wave travels through the quartz medium. The mode of travel for the wave depends on the particle displacements induced by electrical polarization. Thus, particles can be displaced either perpendicular or parallel to the sensing surface. SAW devices generate longitudinal waves that are perpendicular to the sensing surface. These types of sensors are capable of operating at high frequencies, generally between 25 – 500 MHz. However, because these waves traverse perpendicularly, they become attenuated when operating in liquid media due to the creation of compressional waves. Therefore, SAW devices are not suited for biological applications. Fortunately, with the proper cut of crystal to allow for appropriate alignment of particles, these waves can be oriented horizontally, allowing for a dramatic reduction in the attenuation when in contact with liquid media. This is known as the shear horizontal surface acoustic wave (SH-SAW) sensor. BAW devices, on the other hand, propagate waves through the thickness of the substrate, causing particle displacement to be parallel to the surface. Much like the SH-SAW sensor, the waves do not propagate energy into liquids, allowing for operation in this type of medium without massive attenuation. BAW devices operate at moderate frequency levels, with a range of approximately 5 – 30 MHz. Although they do not achieve quite the same sensitivity as SAW devices, they are seen to operate better in liquid media due to its low noise level and therefore, will be the focus from herein.
1.6.2 EMPAS

The Electromagnetic Piezoelectric Acoustic Sensor (EMPAS) is a BAW-type device that was designed and built in the Thompson Lab in 2003. It couples the advantages of two sensors, the Thickness Shear Mode (TSM) acoustic wave sensor, also known as the Quartz Crystal Microbalance (QCM), and Magnetic Acoustic Resonator Sensor (MARS), to achieve exceedingly high sensitivity. The EMPAS is designed to detect the resonance frequency generated from the quartz disc. This resonance is induced by an oscillating electromagnetic field from a copper coil located 30 µm under the quartz disc (Figure 8). Because the EMPAS can generate ultra-high frequencies (up to 1 GHz), it is therefore very sensitive to structural changes, viscoelastic, and mass loading properties (Figure 9). The advantage of this detection method over the previous systems is that it is label-free and there is no metal electrode necessary, allowing for high frequency measurements, which ultimately provides for higher analytical sensitivity. Unlike the TSM, the lack of electrodes is what allows for the EMPAS to operate at such a high frequency as it mitigates the physical and electrical damage electrodes would cause when higher voltage is applied. Due to the fact that excitation of an electric field is done so remotely in the EMPAS, the delicate quartz is not subjected to harsh physical conditions or erosion. With the aid of an O-ring, the quartz may oscillate almost freely even at high frequencies and the system is able to achieve a higher operating harmonic of frequency. Therefore, the EMPAS is our primary candidate sensing method for detection of binding between the aptamer and HSP10 due to its immensely high sensitivity to molecular interactions and its ability to detect interactions (ie. binding mechanisms) within biological fluids.
Figure 8. Photograph of the EMPAS cell holder (left) which holds the quartz disc approximately 30 µm above the copper coil seen at the center of the disc chamber (adjoining top not shown in photo). Photograph of a quartz disc (right) used for surface modification and measurement on the EMPAS. Discs are 13.6 mm in diameter, 83 µm thick, and are handled with stainless steel metal tweezers.

Figure 9. Representative diagram of the inner workings of the EMPAS during operation. A few factors the EMPAS is sensitive to include mass loading/gravimetric changes, viscoelasticity, and conformational changes, all of which are not mutually exclusive.
1.7 Self-Assembling Monolayers

Since their discovery in the 1980s, self-assembled monolayers (SAMs) have grown significantly in their field and applications in technology.\textsuperscript{63,64} SAMs differ from surfactant monolayers due to the molecules of the former possessing an end group that is capable of reacting to a solid surface (ie. the substrate), thus allowing the formation of a monolayer film.\textsuperscript{65} The most common substrates that exploit this phenomenon are gold and silicon with their respective thiol and alkylsilane molecules.\textsuperscript{63} The chemistry of self-assembly has become the choice method for customizing organic layers on a surface.\textsuperscript{66} In other words, these surfaces can be designed to functionalize specific molecules such as proteins\textsuperscript{67,68}, antibodies\textsuperscript{69} or oligonucleotides\textsuperscript{70,71} to capture target analytes. As such, the ability to functionalize these surfaces is what allows it to be an attractive method in constructing biosensors.\textsuperscript{66}

1.7.1 Silanes and Surface Modification Chemistry

Silane SAMs have garnered a rather appreciative audience in the field of biomolecular sensing. These molecules are capable of forming highly ordered and well-packed monolayers through the cross-linking formation during self-assembly for covalent attachment on a surface.\textsuperscript{63} Although silanes are extremely sensitive to water, pH, and temperature in solution, they have proved to be more robust and stable compared to their thiol counterparts when covalently bound on a surface.\textsuperscript{72} Their increase chemical and physical stability allows for a wider range of chemical modifications in relatively harsher conditions.\textsuperscript{63,65} SAMs are generally made up of three components: a head group, an alkyl chain, and the anchoring tail group (Figure 10).
The head group allows for chemical functionality of the monolayer and can be modified to immobilize specific probes. The alkyl chain provides both length and stability to the overall structure of the monolayer, the latter being a result of van der Waals interactions. Finally, the anchoring tail group is responsible for forming the covalent attachment of the molecule onto the desired substrate. Trichlorosilanes, in particular, are a group of molecules that have an anchoring group with the molecular formula of \(-\text{SiCl}_3\). They are generally chosen for their relative quick reaction with surface silanol groups and are capable of dense packing with a high degree of order. The reactivity of trichlorosilanes to silicon-based substrates, such as quartz, makes the molecules ideal for implementation in biosensor construction.

The mechanism regarding the reaction of organosilane molecules onto solid supports has been a well-defined one. As seen in Scheme 1, the reaction occurs only when the solid surface is hydroxylated. The hydroxylated surface is a critical component in forming covalent bonds with the surface, leading to an overall stronger surface layer and gaining both mechanical and
chemical robustness.\textsuperscript{65} The reaction begins with the physical adsorption of the silanol molecule to the hydroxylated surface via hydrogen bonding forces, and can engage in lateral movement across the surface with the creation and breakage (ie. desorption) of these particular bonds. With an increase in concentration, the silanols begin to aggregate, which is mainly driven by hydrogen bonding but also involves a multitude of van der Waal's interactions such as dipole-dipole interactions. Further aggregation causes a decrease in molecular mobility and induces localization on the surface.\textsuperscript{73} This process subsequently initiates a condensation reaction that causes the covalent linkage of the silanol to the surface, as well as the cross-linking between hydroxyl molecules.\textsuperscript{73,74}

\begin{center}
\includegraphics[width=\textwidth]{scheme1.png}
\end{center}

**Scheme 1. Reaction schematic depicting the mechanism for siloxane self-assembly on a hydroxylated surface (eg.\text{SiO}_2\text{ quartz}).**

In the case of trichlorosilanes, a hydrolysis step is required at the beginning of the reaction schematic in order to convert the molecule into a silanol (R-SiOH\textsubscript{3}). This step requires the presence of water in order for the reaction to occur (\textbf{Scheme 2}). The water molecules may be present in the solvent\textsuperscript{75} with the trichlorosilane molecule or adsorbed on the surface\textsuperscript{72}. The rest of the reaction carries out as seen in \textbf{Scheme 1} when the trichlorosilane has been converted into its silanol counterpart.
Scheme 2. Reaction schematic depicting the extra hydrolysis step in the mechanism when a trichlorosilane molecule is involved in the surface self-assembly process.

When schematically presenting these covalently linked molecules on hydroxylated surfaces, one may believe that the coating of silanes is a uniform process. In reality, however, the case is not as simple. Uniformity of silanes has been historically recorded to be a non-uniform process, in which the molecules commonly arrange themselves on the surface in islands. As such, this molecular phenomenon should be taken into consideration when functionalization of biomolecules onto the surface is to be performed.

### 1.7.2 Biomolecular Immobilization onto Adlayers

As mentioned previously, biosensors are unique in their category as they are comprised of a probe that is capable of recognizing selective biomolecular targets. The attachment of these probes, which usually include biological elements such as enzymes and antibodies, consists of a few different methods."76 One can imagine the importance of orientation when it comes to organizing the probe onto a solid surface. Orientation of the probe to expose its recognition site to allow for optimal target binding is key to a successful biosensor. Nonspecific adsorption of the probe is one approach of immobilization that does not allow for any control over the critical probe orientation, albeit the easiest technique."76 On the other end of the spectrum, there is covalent attachment of the biological probe, which can be achieved either with a direct covalent linkage or with the aid of a crosslinker. Other methods include entrapment of the probe within a membrane, encapsulation of the probe within a porous matrix, and use of the putative biotin-avidin systems."76 However, covalent attachment of probes proves to be the more commonly used and widespread due to their specificity and success towards accurate probe orientation. On a chemical level, a myriad of functionalization chemistries have been designed and explored. These include nucleophilic substitution, click chemistry, and supramolecular functionalization."63
In particular, the covalent attachment of synthetic DNA to allow for enhanced physical and chemical properties began as early as when synthetic aptamers were designed back in the 1990s\textsuperscript{77}, which points towards the notion of how the covalent immobilization of probes on a surface for designing detection techniques remains to be a choice method.

1.8 Surface Characterization Techniques

Characterization of modified surfaces is a crucial step in understanding and studying its physico-chemical properties.\textsuperscript{78} This knowledge gained from characterization gives insight into the behaviours of the surface such that the chemistry can be modified to serve a particular function.\textsuperscript{79,80} To fully understand the properties, one will need to gain information and data regarding factors such as the physical topography, chemical composition, chemical and atomic structures of the surface. Although there is no one technique that can satisfy investigation of all properties, many can be applied to allow for a thorough investigation.\textsuperscript{80} Many different methods have been employed for surface characterization of SAMs. Some techniques aim to investigate structure and growth of the layers while others simply aim to chemically identify the components present.\textsuperscript{81} In this manuscript, focus will be placed on the techniques specifically employed in chemical identification, namely XPS and CAG.

1.8.1 X-Ray Photoelectron Spectroscopy

X-ray Photoelectron Spectroscopy (XPS), also referred to as "Electron Spectroscopy for Chemical Analysis" (ESCA), is technique commonly employed for surface analysis. Its popularity is gained through its ability to acquire of a large amount of quantitative data as well as its adaptability towards a myriad of different sample substrates.\textsuperscript{82} XPS operates under the fundamental photoelectric effect, which was described by Einstein in 1921.\textsuperscript{82–84} This effect illustrated the process of photoelectron emission when a substrate is excited by a light source. The light source, which may be ultraviolet or X-ray in nature, bombards the substrate with a photon that transfers its energy to an electron.\textsuperscript{82,85} With X-rays as a light source, the energy is great enough to exceed the binding energy of core electrons to result in their ejection, while on
the other hand, ultraviolet light is only capable of ejecting valence electrons due to its lower energy.\textsuperscript{82}

\textbf{Figure 11.} Illustration of the photoemission process during XPS acquisition. Example shows elemental carbon irradiated with an X-ray light source to cause the ejection of its core 1s electron, in which its kinetic energy can be measured by a spectrophotometer.

For analysis by XPS, the sample is placed into a vacuum chamber. X-rays of a specified wavelength irradiate the surface with enough energy to eject a core electron, which all have characteristic energies based on the elemental atom it was released from (Figure 11).\textsuperscript{85} This ejected electron, called a \textit{photoelectron}, is detected by the spectrophotometer and its kinetic energy is measured. \textbf{Figure 12} illustrates the energy levels between the spectrometer and sample during XPS analysis.
Figure 12. Diagram illustrating the energy levels during XPS measurements of a conducting material. The spectrometer (left) has a common reference with the sample (right) for electron measurement (\(E_F - \text{Fermi level}\)) in order to ultimately determine the binding energy of the ejected electron. (Image from Ref. 83)

The sample and the spectrometer are placed in electrical contact with one another in order to provide a common reference between the two. This is known as the Fermi level (\(E_F\)), which is the highest occupied energy level\(^{82,83}\). When the sample is irradiated with X-ray photons (\(h\nu\)), a photoelectron is released with a kinetic energy of \(E_k^1\), relative to the sample vacuum level (\(E_v\)), which is the energy level of a free electron at rest outside of the substrate. The kinetic energy of the electron measured inside the spectrometer is given the term \(E_k\) which is used to determine the kinetic energy at the sample surface using the following equation\(^{83}\):

\[
E_k = E_k^1 - (\phi_{\text{spec}} - \phi_s)
\]

where \(\phi_{\text{spec}}\) and \(\phi_s\) are work functions of the spectrometer and sample, respectively. These work functions (ie. energy amount) are required to remove an electron from the solid (Fermi level) to the vacuum (vacuum level). Therefore, the binding energy (\(E_b\)) for the sample can be obtained knowing the photon energy (\(h\nu\)) by using the following equation\(^{83}\):

\[
E_b = h\nu - E_k - \phi_{\text{spec}}
\]
One may notice that $\phi_3$ is not involved in this equation as it is already recognized by the spectrometer. In other words, the measurement of $E_b$ is not dependent on $\phi_3$.  

For chemical composition determination and depth profiling, angle-resolved XPS (ARXPS) is commonly employed. This method allows for variation in the angle between the X-ray and sample by titling the platform (from ~2 to 10 nm) where the sample is situated on. This relationship can be defined with the following equation:

$$d = \lambda \sin \alpha$$

where $d$ is the probing depth of the X-ray light source, $\lambda$ is the inelastic mean free path (the average distance a photoelectron can travel within a solid), and $\alpha$ is the take-off angle between the photoelectron trajectory and the surface.

---

**Figure 13.** Angle-resolved XPS showing probing depth differences. A) Shallower probing depth ($d$) and smaller angle ($\alpha$). B) Deeper probing depth ($d$) and larger angle ($\alpha$).

For a more surface-sensitive technique, the take-off angle should be decreased as seen in Figure 13A, where a shallower probing depth is evident. On the other hand, increased take-off angles will result in greater bulk sensitivity as a result of a deeper probing depth (Figure 13B).
1.8.2 Contact Angle Goniometry

The application of surface wetting techniques towards the knowledge and understanding of surface-liquid interactions has proven to be a critical analysis method.\textsuperscript{86,87} These techniques have been employed in large industrial settings down to nanoscale research and even biological studies.\textsuperscript{86,88} Wettability, or the capacity at which a liquid may spread or adhere to a surface, is commonly determined through the measurement of the contact angle produced by a droplet of liquid. Various techniques are employed to measure contact angle, including goniometry, the Wilhelmy plate, and capillarity.\textsuperscript{86} The focus of this manuscript, however, will be on contact angle goniometry.

In 1805, Thomas Young introduced and described the concept of wettability and contact angle of a liquid on top a solid surface.\textsuperscript{89} When a droplet of liquid is placed onto a surface, three forces are introduced: the solid-liquid ($\gamma_{SL}$), liquid-vapour ($\gamma_{LV}$), and solid-vapour ($\gamma_{SV}$) interface forces. These forces hold a relationship that can be defined by the following equation developed by Young:

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos \theta$$

where $0^\circ \leq \theta \leq 180^\circ$

The Young equation states that the contact angle ($\theta$) of the liquid droplet is defined by the three interfacial forces when at mechanical equilibrium.\textsuperscript{86–88} This equation assumes that at the interface between the liquid and surface, no physical or chemical reactions occur due to the optical nature of contact angle measurements.\textsuperscript{87} However, in practice, the actual theoretical angle calculated from Young’s equation very rarely equates to the experimentally measured angle. Factors such as surface roughness and chemical or physical heterogeneity can all affect the measurement.\textsuperscript{86,87} Figure 14 illustrates the ideal surface for contact angle measurement. When surface-liquid repulsion occurs due to hydrophobic interactions, a larger contact angle is measured. On the other hand, when the surface is hydrophilic, the droplet spreads over a wider area on the surface, creating a smaller contact angle measurement.

A goniometer is an instrument that is capable of measuring precise angles. In contact angle goniometry (the most widely adopted contact angle measurement technique) the instrument is
defined by a horizontal stage located between a CCD camera and a light source, all placed in a linear fashion (Figure 15). When a sessile drop is placed onto the surface sample on the stage, the camera captures the droplet image and its shape is analyzed by computer software.87

Figure 14. Illustration of the interaction between a liquid droplet and a solid surface with a measurable contact angle (θ) at the solid (S), liquid (L), and vapour interface (V) parameters. A) Depiction of a hydrophobic surface with a larger θ value. B) Depiction of a hydrophilic surface with a smaller θ value.
Figure 15. Diagram of contact angle goniometry equipment set-up. Sample is placed on top of horizontal stage between the light source and camera. A syringe is placed perpendicular to the sample to dispense a droplet of liquid.

1.9 Thesis Project

1.9.1 Research Motivation

The foundation for this research project was built upon the dire need for a new testing method for ovarian cancer as the current test is outdated and lacks the specificity and accuracy required for enhancing survival prognosis. With literature coming to a general consensus that early detection is pivotal to the survival of the individual, it is therefore imperative that a technique can be developed to detect early stages of ovarian cancer. This thesis is geared towards the development of a biosensor that can reliably detect for a biomarker released at the early stage of this cancer.
With the promise of a new potential biomarker for ovarian cancer, a new journey towards developing said ovarian cancer biosensor can begin. HSP10 is a promising candidate not only specifically for ovarian cancer, but may be a very general cancer marker. As of now, this project will look into HSP10 as a target for screening detection as it is found to elevated in women with ovarian cancer\textsuperscript{36} and will be the first step towards creating a biosensor to detect for early stage ovarian cancer that can be translated to clinical use in the future.

Previously, our lab has looked into attachment of aptamer probes onto a quartz surface with success for the detection of cocaine in aqueous solutions.\textsuperscript{90} We chose to use aptamers in this project not only for their biotechnical advantages, but also because HSP10 is an immunosuppressant and thus, extracting antibodies against it posed to be difficult. In this project, we have adapted the previous work with DNA aptamers to detect for HSP10. In collaboration with Alexander Romaschin at St. Michael’s Hospital, the Thompson Lab has developed four novel single-stranded DNA aptamers against HSP10: MNA-38, MNB-38, MNC-38, and MND-38, which are the constructs that will be evaluated in this project. In addition, our lab has frequently employed the EMPAS for biomolecular detections.\textsuperscript{61,66,90,91} As it is an extremely sensitive instrument, using this system to detect for probe-ligand interactions will be highly informative for gathering data and information towards the research project.

1.9.2 Research Objectives

The first objective of this project was to determine the binding affinity of the different DNA aptamers towards HSP10. The goal was to select one candidate aptamer that showed the best affinity towards our target protein for immobilization onto quartz surfaces. The second objective was to determine the binding affinity between the aptamers and target protein using the EMPAS. This required the development of novel surface chemistries for His-tag protein immobilization that can be adapted for wider biomolecular applications. The third objective was to choose a candidate aptamer based on the gathered results that would be used for immobilization. The silane surface chemistry used from the previous immobilization step was to be used. All three goals are met in order to move towards advancing research in developing a sensing technique for early-stage ovarian cancer screening and diagnostics.
Chapter 2.
Materials and Methods

Author's note: The experimental work described herein is presented in chronological order. All experiments listed are illustrated in such a way in order to display the thought process in a coherent manner.

2.1 BL21 Rosetta E. Coli overexpression of HSP10

As HSP10 is a protein not readily available for purchase, it is important to outline a protocol that can successfully isolate the protein for biochemical use. First, a pTrcHIS A plasmid\textbf{(Figure 16)} (Invitrogen™ Life Technologies) containing the sequence for HSP10 (\textbf{Figure 17}) at the multiple cloning site (MCS) was obtained from Dr. Alexander Romaschin at St. Michael's Hospital. The plasmid was then transformed, using standard heat shock methods, into Top10 \textit{E. coli} cells. A cell culture of the pTrcHis A/HSP10 plasmid containing Top10 cells was prepared in LB media containing 100µg/ml ampicillin. The culture was incubated at 37°C overnight. The pTrcHis A/HSP10 plasmid was isolated from the Top10 cells using a standard Promega plasmid prep kit. The isolated pTrcHis A/HSP10 plasmid was then transformed into BL21-Rosetta expression \textit{E. coli} cells, using the standard heat shock technique, and grown in a seed culture of LB broth with 100 µg/mL of ampicillin overnight at 37°C. The following day, the optical density (OD) of the overnight culture was investigated using a PerkenElmer Lambda 25 UV/Vis Spectrometer at a wavelength set to 600 nm. Cell stocks were prepared when the OD of the cells had reached over 1 absorbance units (AU). To prepare the cell stocks, a fresh solution of 80% glycerol was made.
Figure 16. The pTrcHisA plasmid. Contains the genetic coding for HSP10 at the multiple clonal site (green). The segment is flagged by the start codon ATG, a 6X Histidine tag, an expression epitope, and an enterokinase recognition sequence. (Adapted from ThermoFisher Scientific Invitrogen)\textsuperscript{92}

Cell stocks of the BL21 Rosetta E. coli cells were plated onto pre-poured agar culture plates containing lysogeny broth (LB) and 100 µg/mL ampicillin (Sigma-Aldrich). A negative control of warm tap water (10 µL) was also plated. All plates were then placed into an incubator at 37°C overnight. The plates were examined the following day for growth. If no growth occurred in the negative control, the cultured cells plates were deemed acceptable for further biochemical use.

The OD of the culture was measured at 600 nm on a PerkenElmer Lambda 25 UV/Vis Spectrometer every half an hour. The cells were allowed to grow until an OD = 0.8 AU was reached, at which point the culture was induced with 1M IPTG (1/1000 of the total volume). The cells were subsequently grown in the induced state for a total of 4 hrs. Samples for SDS-PAGE analysis were taken every hour for a total of 4 hours by taking 150 µL from each of the induced cell culture and a second non-induced cell culture as control. The supernatant was
removed from each sample after centrifuging for 5 min at 45,000 rpm. Then, 30 µL of SDS-PAGE loading buffer was added to the pellet. Gel samples were heated on a hot plate at 100°C for 10 min, loaded into the gel lanes, and run on two 15% SDS-PAGE gels for 45 min at 220V and 500 mA. Gels were stained with Coomassie Blue for 2 min in the microwave at high power.

**HSP10 sequence:**
MAGQAFRKFLPLFDRVLVERSAAETVTKGGIMPEKSQGKVQQATVVAVGSG
SKGGKGGEIQPVSVKVGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD

**Figure 17. Single letter amino acid sequence of HSP10.**

### 2.2 Nickel Affinity Chromatography – purification of HSP10

The BL21 Rosetta *E. coli* cells were pelleted by centrifugation at 20,000 rpm and placed into a -20°C freezer for up to 2 weeks before use. Upon usage, the pellet was re-suspended in cell lysis buffer (50 mM TRIS-HCl, 1 mM EDTA, 100 mM NaCl, 20 mM β-mercaptoethanol, pH = 8.0; 50 mL per litre of cell culture). Following, 10mg/mL of lysozyme (1 mL per litre of cell culture) was added to the suspension and shaken on a bench-top shaker for 30 min at room temperature. Then, 5 mL of 10% Triton-X 100 and 0.5 mL of 0.1 M protease inhibitor phenylmethylsulfonyl fluoride (PMSF) were added to the solution and let to incubate for 15 min at 37°C. The cells were then lysed by a VibraCell sonicator in order to extract the soluble protein from the cells. The sonicator was set to 100% power for 10 cycles of 12 s sonication followed by 1 min incubation on ice. Following sonication, the insoluble cell components were separated by high speed (20,000 rpm) centrifugation for 20 min at 4°C. The resulting supernatant was filtered using a 0.45 µm syringe filter and placed in a 4°C fridge overnight. The following day, the solution was loaded on a HiScreen IMAC FF nickel affinity column (GE Healthcare Life Sciences) connected to a Bio-Rad Econo Low Pressure Purification System (Spectral Medical Inc.) in order to isolate the HSP10 fusion protein from the rest of the transcribed *E. coli* protein. The purification system was set with the running parameter of 1 mL/min, an elution gradient of 0 – 100%, and an absorbance scale of 0.5 AU for loading and 0.1 AU for elution. The protein
fractions were eluted from the column via a linear gradient of imidazole of 0 – 500 mM and the all HSP10 fractions were collected. After collection, the column is washed and stored according to manufacturing instructions.

2.2.1 Buffer Preparation

The running buffer contained 10 mM PBS, 0.5 M NaCl, and 30 mM imidazole at pH = 7.4. The elution buffer contained 10 mM PBS, 0.5 M NaCl, and 500 mM imidazole at pH = 7.4. One powder PBS packet (10 mM phosphate, 0.138 M NaCl, 2.7 mM KCl, pH = 7.4) from Sigma Aldrich was used in making each buffer. After the buffer solutions were prepared, they were sterilized using a 0.22 µM filter.

2.3 Concentrating Purified HSP10

When all fractions from the chromatography session were collected, selected fractions were analyzed using SDS-PAGE to determine which were purely HSP10 and contained no other residual undesired E. coli protein. Two 20% SDS-PAGE gels were prepared using a 15-lane comb. Samples were prepared by taking 20 µL of a fraction mixing with 4 µL of 5X loading dye. Without heating, 5 µL of each sample and 10 µL of the protein ladder were loaded into the gel lanes. As controls, the supernatant from centrifugation after sonication as well as the wash fraction from after loading the supernatant onto the column were also prepared in the same fashion to be loaded in the gel lanes. After loading, the gels were run in 1X Laemmli buffer for 45 min at 220 V at room temperature and then stained with Coomassie Blue in the microwave for 2 min afterwards. The gels were destained with a solution of 40% methanol and 10% glacial acetic acid in deionised water on a spin plate until a light blue background was achieved and then fully destained in distilled water overnight.

After determining the pure HSP10 fractions using SDS-PAGE analysis, the fractions were subsequently pooled together for concentration. Two 3,000 molecular weight cut-off (MWCO) filter tubes were first hydrated with deionised water for 30 min in the centrifuge at 3,500 rpm.
The residual water in the filters was discarded before the pooled HSP10 solution was equally distributed amongst both tubes. In order to keep the solution cool whilst in the centrifuge, the tubes were only centrifuged for 10 min intervals at 4,000 rpm in order to switch out the 15 mL falcon tube holder with a newly chilled one. The protein solution in each tube was concentrated to 1 mL and was pooled into one Eppendorf™ tube for a total of 2 mL to be stored in a 4°C fridge. The following day, all of the concentrated HSP10 solution was exchanged into 0.22 µm filtered PBS buffer via a standard dialysis procedure to remove imidazole and extraneous salts. Thin-layer chromatography was performed (1:1 chloroform:methanol) to visualize removal of imidazole after dialysis.

After obtaining the purified HSP10 solution, concentration was determined using a VWR UV1600PC spectrophotometer to measure its absorbance by running a scan through the wavelength range of 190.0 – 600.0 nm. A spectrum pertaining to the evident protein was obtained. Using the online ExPASy ProtParam tool, the extinction coefficient for HSP10 was estimated and used in conjunction with Beer-Lambert’s Law to determine concentration.

2.4 Enterokinase cleavage of His-tagged HSP10

In a standard dialysis protocol, an aliquotted amount of the purified solution containing the HSP10 fusion protein (usually between 1 – 2 mL) was exchanged into a reaction buffer containing 20 mM Tris-HCl (pH = 8.0 at 25°C), 50 mM NaCl, and 2 mM CaCl₂. The solution was retrieved from the cartridge using a pipette and was incubated with the New England BioLabs® Inc. light chain enterokinase (2.0 µL/mL) for cleavage of the multiple cloning site containing HSP10. Following the reaction conditions suggested on the associated pamphlet, 0.0006% (w/w) of the enterokinase enzyme was added to the HSP10 solution. The solution was then left on a rocking platform to mix overnight (approx. 16 hr) at room temperature. The following day, the enterokinase reaction was inhibited by adding high concentrations of sodium chloride (approximately 2M) to the reaction mixture. The HSP10 solution was then dialysed using the previously described procedure into 0.22 µm filtered PBS for long-term storage. The
concentration of HSP10 was measured once again using the UV/Vis spectrometer and concentrated with centrifugal filters, if deemed necessary.

Aliquots of 50 µL of the solution were taken out at 0, 15, 30, 60, and 120 min after introduction of enterokinase. To stop the enterokinase reaction with the protein, 25 µL of reducing buffer was added to the aliquotted gel samples. In addition, 3 overnight samples of the solution were also obtained in order to determine successful cleavage of the fusion protein using SDS-PAGE. All samples were heated to 90ºC for 5 min and then let to cool to room temperature before loaded onto a 20% SDS-PAGE gel. The gel was run at 220V for 45 min in 1X Laemmlli buffer at room temperature. The gel was stained with Coomassie Blue for 3 min in the microwave and then destained with a solution of 40% methanol and 10% glacial acetic acid in deionised water on a spin plate until a clear background was obtained.

2.5 DNA Aptamers

Using the SELEX method, one aptamer was chosen for its greatest potential in interaction and binding to HSP10. The sequence for this aptamer, named CPN10-38, was provided to us courtesy of Dr. Alexander Romaschin at St. Michael’s Hospital. This aptamer was renamed to MNA-38 when Dr. Miguel Neves designed three more aptamers after the original sequence. These three aptamers were designed to vary in primer region presence and were named MNB-38, MNC-38, and MND-38. The sequences for all aptamers are listed in Table 1 below.

2.5.1 DNA Aptamer Re-annealing

All samples of DNA aptamers are first re-annealed immediately prior to use. The re-annealing process begins with heating the aptamer solution to 90ºC in a water bath for 5 min and then shocked on ice. This process pushes the equilibrium of the aptamer away from its thermodynamic state towards its kinetic state.
Table 1. DNA aptamer sequences against HSP10 written in the 5'→3' direction. Underline indicates primer regions.

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNA-38</td>
<td>GTCTTGACTAGTTAGGCCAACTGGTGCCGGGTGGGATGGATTTGCTTGAGGGGTC TCAATTAGTTAGGCCGTC</td>
</tr>
<tr>
<td>MNB-38</td>
<td>AACTGGTGCCGGGGTGATGGGATTTGCTTGAGGGGTC TCAATTAGTTAGGCCGTC</td>
</tr>
<tr>
<td>MNC-38</td>
<td>GTCTGGACTAGGTTAGGCCAACTGGTGCCGGGGATGGATTTGCTTGAGGGGTC</td>
</tr>
<tr>
<td>MND-38</td>
<td>AACTGGTGCCGGGGTGATGGGATTTGCTTGAGGGGTC</td>
</tr>
</tbody>
</table>

2.6 DNA-Native PAGE for binding confirmation

Using DNA-Native PAGE, binding between the four novel aptamers to HSP10 was assessed and evaluated. 12% DNA-Native PAGE gels were used to run the aptamers of larger size (MNB-38 and MNC-38), while 10% gels were used to run the MND-38 aptamer. Aptamer was first portioned out into an Eppendorf™ tube from its stock solution. Aptamer was re-annealed, and then shocked in an ice bath for 5 min. Gel samples were prepared by combining various ratios (Table 2) of aptamer to HSP10 in 1.5 mL Eppendorf™ tubes for a total volume of 60 µL in order to elicit a binding curve.

After the mixtures of aptamer and protein were prepared, they were then set aside for 1 hour to incubate and allow for binding to occur at room temperature. After this time, 20 µL of 80% glycerol (v/v in deionised water) was added to each gel sample mixture. The tubes were inverted several times to mix and then spun down to collect all liquid at the base of the tube. 5 µL of the samples were loaded into each lane of the DNA-Native PAGE gel. For measurement, 1 µL of blue/green dye (bromophenol blue:xylene cyanol) was loaded into one lane. The gel was run in ½X TBE (TRIS-Borate-EDTA) at 90 V for 1.5 hr in a -10°C chamber. Stains-All™ was used to visualize the gel bands by soaking the gel in a dark chamber for approximately 1 hr. The gel was destained in deionised water while exposed to light.
Table 2. Gel sample preparation for binding affinity. Aptamer concentration held constant as protein concentration varied.

<table>
<thead>
<tr>
<th>Ratio (Aptamer:HSP10)</th>
<th>HSP10 concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>0</td>
</tr>
<tr>
<td>1:0.25</td>
<td>15</td>
</tr>
<tr>
<td>1:0.50</td>
<td>30</td>
</tr>
<tr>
<td>1:0.75</td>
<td>45</td>
</tr>
<tr>
<td>1:1</td>
<td>60</td>
</tr>
<tr>
<td>1:2</td>
<td>125</td>
</tr>
<tr>
<td>1:3</td>
<td>185</td>
</tr>
</tbody>
</table>

2.7 ImageJ and SigmaPlot programs for analysis

Analysis of the different aptamer constructs against HSP10 is performed after each gel has run via electrophoresis and stained accordingly with Stains-All. The analysis was carried out using a program called ImageJ, which is capable of quantifying band colour intensity on the gel. With this program, it is possible to elicit the differences in binding intensity based on the different ratios of aptamer to HSP10 that have run through the gel. The ratios between bound and unbound aptamer are then plotted against HSP10 concentrations in SigmaPlot in which a binding curve using the Langmuir isotherm equation is generated. This program will give an approximate range of the dissociation constant to assist in the selection of which aptamer would be the best candidate for attachment onto a surface.

2.8 Surface modification for HSP10 immobilization

In order to determine the binding affinity between HSP10 and the aptamers more accurately, a scheme was devised for surface modification of quartz (Scheme 3) for future analysis on the EMPAS. AT-cut quartz discs are used in all experiments and are purchased from LapTech Inc.
Scheme 3. Schematic displaying the surface modification of quartz for immobilization of His-tagged HSP10.

2.8.1 Test Tube Silanization

Test tubes must first be silanized prior to any step involving trichlorosilane molecules due to the reactivity of silanes towards glass. For this process, clean and dry test tubes are brought into a glovebox under nitrogen atmosphere. The silane linker, octyldecyltrichlorosilane (ODTS) purchased from Sigma-Aldrich, is used to coat the walls of the test tubes. Anhydrous toluene is first aliquotted into the test tubes such that it fills each approximately half-way. Subsequently,
10 drops of ODTS is added to each toluene-filled test tube. Finally, the test tubes are filled once again with anhydrous toluene and mixed several times by pipetting the solution. A small 1-inch space from the top of the test tube to the end of the solution was left to allow for a rubber stopper to seal. The test tubes are removed from the glovebox and placed onto a spin plate for an overnight reaction (approx. 20 hr).

The following day, the solution inside the test tubes is discarded and each test tube is rinsed 3 times with toluene at normal atmosphere. The test tubes are then placed upside-down in a large beaker and stored in a 160°C oven until needed. This process is only required to be performed once, as long as the test tubes are not subjected to large amounts of water, scrubbing, soap or piranha. Once silanized, the test tubes can be used almost indefinitely.

2.8.2 Quartz Cleaning

Before any modification is performed on quartz, it must be thoroughly cleaned. First, the discs are submerged in a 50:50 solution of Dustbane© liquid soap and distilled water and sonicated for 20 min. During this time, a hot water bath containing empty test tubes is heated to 90°C. While heating, a piranha solution is prepared by mixing 30 mL of 30% hydrogen peroxide with 70 mL of sulphuric acid in a 100 mL graduated cylinder. When the water bath is at 90°C, approximately 5 mL of the piranha solution is pipette into each heated test tube. After filling all test tubes, the discs in the soap-water solution are rinsed with warm tap water to remove all soap suds and then taken out from their respective test tubes. Using a pair of tweezers, the discs are rinsed one last time with tap water and then rinsed with distilled water before dropping them individually into a piranha-filled test tube on the water bath. The discs are allowed to soak in the solution for 45 min.

After this period of time, the test tubes containing the discs are removed from the hot water bath and filled with deionised water to neutralize the piranha solution. The liquid is discarded and the discs are subsequently rinsed 3 times with deionised water, followed by sonication for 2 min. The discs are then rinsed 3 times with spectro-grade methanol and sonicated for another 2 min. Following rinsing, the discs are removed from the tubes, using a small metal spatula and a pair of
tweezers, and dried with a stream of nitrogen gas and placed into scintillation vials for further drying in a 160°C oven for 2 hr. After the allotted time, the discs are moved into a chamber of 70% humidity to sit overnight.

![Molecular structures of reagents used in the scheme for surface modification of quartz.](image)

**Figure 18.** Molecular structures of reagents used in the scheme for surface modification of quartz.

### 2.8.3 Silanization

The discs are taken out of the humidity chamber and individually placed into silanized test tubes that are then brought into a glovebox under nitrogen atmosphere, along with two silane linker molecules, pentfluorophenyl 13-trichlorosilyl-tridecanoic acid 2,2,2-trifluoroethyl (PFP-TTTA) and distilled octyltrichlorosilane (OTS). PFP-TTTA has been previously synthesized and characterized and OTS is purchased from Sigma-Aldrich and distilled in lab ([Figure 18](image)). In the glovebox, diluted solutions of each linker were prepared using anhydrous toluene. In 10 mL of anhydrous toluene, 10 µL of the stock linker is added. After these solutions are prepared, 500 µL of each linker solution is pipetted onto each disc for a 1:1 v/v ratio of PFP-TTTA to OTS at a total of 1 mL per disc. Each test tube is then subsequently sealed with a rubber stopper and taken out of the glovebox to allow for the silanization process to progress for 2 hr on a spin plate.

After two hours have passed, the test tubes were taken off the spin plate and the stopper removed. The solution inside each test tube was discarded and each disc was rinsed 3 times with toluene followed by sonication for 2 min. The discs were then rinsed 3 times with chloroform and sonicated for another 2 min. The discs were removed from the test tubes, dried with a stream of nitrogen gas, and then placed individually into scintillation vials for probe immobilization that would take place a couple of hours later on the same day.
2.8.4 Na,Nα-bis(carboxymethyl)-l-lysine hydrate Functionalization

*Note: The abbreviation “NTA” is a more commonly used for the compound N,N-bis(carboxymethyl)glycine. In this manuscript, the compound used in partner with this abbreviation is Na,Nα-bis(carboxymethyl)-l-lysine hydrate.

Initially, finding a solvent for dissolving Na,Nα-bis(carboxymethyl)-l-lysine hydrate (NTA; Sigma-Aldrich) (Figure 18) but also able to allow for full dissociation of PFP-TTTA head-groups was explored. These solvents included deionised water, 50:50 v/v of methanol/water, dimethyl sulfoxide, and 3:2 ratio of DMF/water.

Each disc was taken out of their respective scintillation vials and placed individually into cooled clean, dry test tubes from the 160°C oven. In a separate test tube, a solution of 10 mM NTA in dimethyl sulfoxide was prepared. The solution was sonicated until all solid particles were dissolved and then mixed thoroughly (5 times) using a pipette. Each disc was introduced to the solution in 500 µL aliquots for a total of 1 mL per disc. The test tubes were then sealed with a rubber stopper and placed on a spin plate for reaction overnight.

The following day, the test tubes were removed from the spin plate and the solution inside was discarded. Each disc was rinsed 3 times with dimethyl sulfoxide and sonicated for 2 min. After sonication, the discs were rinsed 3 times with chloroform and sonicated for another 2 min. Finally, the discs were removed from the test tubes, dried with a stream of nitrogen gas, and then placed individually into scintillation vials for nickel activation later that day.

2.8.5 Activation with Nickel

Discs were taken out of the scintillation vials and placed individually into cooled clean, dry test tubes from the 160°C oven. In a separate test tube, a solution of 100 mM NiCl₂•6H₂O (Sigma-Aldrich) in deionised water was prepared. The solution was sonicated until all solid particles were dissolved and then mixed thoroughly (5 times) using a pipette until an even colour was distributed. Each disc was introduced to the nickel solution in 500 µL aliquots for a total of 1 mL per disc. In the case were an air pocket was trapped underneath the disc, an extra 500 µL of
the solution was added and then removed again to release the bubble. The test tubes were then sealed with a rubber stopper and placed on a spin plate for reaction overnight.

The following day, the test tubes were removed from the spin plate and the solution inside was discarded. Each disc was rinsed 3 times with deionised water and then 3 times with spectro-grade without sonication. After washing, the discs were removed from the test tubes and dried with a stream of nitrogen gas before being placed into individual scintillation vials for HSP10 immobilization to take place later on the same day.

**2.8.6 HSP10 Immobilization**

Each disc was taken out of their respective scintillation vials and placed individually into cooled, clean, dry test tubes from the 160ºC oven. In a separate test tube, a solution of 5 µM His-tagged HSP10 in deionised water was prepared. The protein solution was mixed several times using a pipette. Each disc was introduced to the protein solution in 500 µL aliquots for a total of 1 mL per disc. In the case where an air pocket was trapped underneath the disc, an extra 500 µL of the solution was added and then removed again to release the bubble. The test tubes were then sealed with a rubber stopper and placed on a spin plate for reaction overnight.

The following day, the test tubes were removed from the spin plate and the solution inside was discarded. Each disc was rinsed 6 times with deionised water without sonication and then removed from the test tube to be dried with a stream of nitrogen gas. The discs were all placed into scintillation vials for either short term storage (1 day) or for immediate measurement using the EMPAS.

**2.9 XPS characterization of HSP10 immobilized surfaces**

After modification of the quartz discs, the discs are then sent to Surface Interface (SI) Ontario at the University of Toronto for analysis via X-ray Photoelectron Spectroscopy (XPS). Samples consisted of discs varying in surface modification and were analyzed with the Thermo Scientific K-Alpha instrument operating with an Al Kα X-ray source. Four take-off angles of 27.5°, 42.5°,
57.5°, and 72.5° relative to the normal were used for deep-layer and surface analysis. Analysis for the following elements was also requested for each disc: carbon, chlorine, fluorine, nitrogen, nickel, oxygen, and silicon. Using the ThermoAvantage program provided at SI Ontario, peak fitting and data analysis for each element was performed. All peaks and graphs were calibrated according to the carbon signal at 285.0 eV and background smoothing was set to 0.5 eV.

2.10 Validation of binding affinity using EMPAS

Quartz discs were prepared following the above scheme to immobilize HSP10 on the surface. After the final rinsing and drying of the HSP10-immobilized discs, they were ready for measurement on the EMPAS. The EMPAS is first primed with an unclean disc and an injection of deionised water. This is done by first clamping the disc in the cell holder, while 0.22 µm filtered phosphate buffered saline (PBS) is flown into the cell over the quartz disc at a rate of 50.00 µL/min. During this time, the acoustic resonance envelope of that particular disc is found by scanning the radio frequency generator at 940.000 MHz as well as utilizing the oscilloscope as a visual cue. Using the LabView program, a profile for the resonant wave is outputted where the linear region of the curve is noted to find the amplitude at which the EMPAS will be operating at, which is usually a value within the range of 25 – 40 mV.

Initially, trying to find a correlation for a binding curve between varied aptamer concentrations and frequency shift was attempted. These discs were prepared with the all characterized layers (PFP + OTS + NTA + Ni + HSP10) and subjected to a flow-through of PBS on the EMPAS. Once the baseline stabilized, which is usually around 2000s, an injection of re-annealed aptamer was made. MND-38 was the first aptamer used for this system of evaluation, as it showed little promise from the DNA-Native PAGE gels. Solutions of 10, 25, 50, 100, 250, and 400 µM in deionised water were prepared from a stock solution of 944 µM MND-38 for injection. Each concentration was done in at least one replicate.

Following the main experiments, a series of control experiments were also performed. The first was to investigate the interaction of aptamers with either the silane adlayer or functionalized bulk
layer. A second control was to inject HSP10 to study its interaction with nickel and if this would give a similar binding profile as the aptamer-HSP10 interaction. The third control was to look at the difference between the graphical profiles of HSP10 binding which involves the 6X His-tag, and of imidazole binding, as it is the ring component of histidine involved with chelating with nickel. Serial dilutions of imidazole (from 1M to 5 mM) were prepared to make a final concentration of 5 µM in PBS. As a final control, the difference between deionised water and PBS during injection was also investigated.

It was also important in the experiments to know the amount of time it took for the injected sample to reach the cell chamber. To investigate this, a diluted sample of green dye (Club House®) was injected into the EMPAS. With this, it allowed a visual cue for when the sample would reach the surface of the quartz disc and how long it would remain in the cell before exiting the chamber.

2.10.1 Baseline correction program

The program written for baseline correction is a short segment of code written in Python by Computer Science Master’s student, Liviu-Mihai Calin, of Professor Kyros Kutulakos’ research group at the University of Toronto. The code asks for the user to select a range in data points that is linear. The first and last points, selected by the user, of this range are inputted into the code such that the slope and y-intercept of the linear regression is calculated. Using these values, a new set of y-values are generated by subtracting the regression from the original data. As such, even though a difference between the values is seen on the y-axis, the frequency shift can still be calculated from the newly baseline-corrected graph. These shifts were used to determine if a correlation exists with the varied aptamer concentration.

2.11 Purification of Thiolated Aptamer

A 5’ thiol-modified MNC-38 (tmMNC-38) was ordered from Integrated DNA Technologies® (IDT) as the chosen candidate aptamer for further investigation. The aptamer arrived as a solid
pellet of 376.1 nmol and was taken up in 500 µL of deionised water to make a final stock concentration of 752.2 µM. Purification of tmMNC-38 began with hydrating filter membranes of 3K MWCO Milipore® Centrifugal Filter Units with deionised water. The filter tubes were fully filled with deionised water and centrifuged at 3,500 rpm for 30 min for hydration of the membranes. After this time, all excess water was discarded and tmMNC-38 was added to the tube, and then topped up with 1M NaCl to begin the purification process. The sodium chloride solution was exchanged 3 times into the aptamer at 4,500 rpm in 30 min intervals. Each time, the aptamer solution was concentrated to 500 µL. After the desalting step, the aptamer solution was exchanged into 20 mM borate buffer in preparation for cleavage the following day. The centrifuge tube containing the aptamer solution was topped up with borate buffer and exchanged 3 times at 4500 rpm in 20 min intervals. The solution was concentrated to 1 mL before retrieved from the filter tube and stored in an Eppendorf™ tube at room temperature until needed for disulfide reduction. The aptamer solution should theoretically have a concentration of 100 µM.

2.11.1 Buffer Preparation

1M NaCl: 2.922g of NaCl in 50 mL of deionised water

20 mM Borate Buffer: 0.62 g of boric acid in 500 mL of deionised water

100 mM DTT Buffer: 0.0771 g of DTT in 5 mL of 20 mM borate buffer

2.12 Deprotection and Reduction of Thiolated Aptamer

Fresh 100 mM DTT buffer was prepared prior to reduction of the disulfide bond in the protected thiolated aptamer (Scheme 4). The DTT buffer was combined with the purified aptamer solution in a 1:1 v/v dilution ratio and allowed to incubate for 1 hr on a rocking plate at room temperature. After this allotted time, the reduced solution was inserted into a hydrated 3K MWCO dialysis cassette (Thermo Scientific Slide-A-Lyzer® Dialysis Cassette G2) and dialysed in deionised water to remove the DTT and undesired components. The water was changed once every hour for two hours before allowing it to dialyse overnight. The following day, the water
was changed one last time and let to dialyse for one final hour. After this time, the aptamer solution was removed from the cassette and stored in an Eppendorf™ tube. Using a UV/Vis spectrometer, the final concentration of the dialysed aptamer solution was measured using its absorbance value at $\lambda = 260$ nm. Using the online IDT Oligo Analyzer 3.1 tool, the extinction coefficient ($\varepsilon$) of our particular DNA aptamer was estimated to be 581700 L/(mol•cm). The concentration of the aptamer solution was calculated to be approximately 50 µM.

![Scheme 4](image)

**Scheme 4.** Reduction of disulfide bond in the received thiolated MNC-38 aptamer. Aptamer was received with a dimethoxytrityl protecting group and was reduced with DTT prior to use.

### 2.13 Immobilization of Chosen Candidate Aptamer

Quartz cleaning and silanization with PFP-TTTA and OTS were performed as previously described. On the same day following the silanization step, tmMNC-38 was prepared for immobilization onto the quartz surface (Scheme 5). The 50 µM aptamer solution was first re-
annealed before being diluted to 5 µM in a 50:50 v/v ratio of deionised water to methanol. With the modified quartz discs individually placed in test tubes, 1.2 mL of the 5 µM aptamer solution was pipetted onto the discs in 600 µL aliquots. The discs were then placed onto a spin plate to allow for overnight reaction at room temperature.

The following day, the overnight discs were rinsed 3 times with deionised water and sonicated for 2 min afterwards. This step was repeated once before the discs were removed from the test tubes, dried with a stream of nitrogen gas, and placed into individual scintillation vials for short-term storage. Discs are run on the EMPAS within 1 – 3 days of preparation.

Scheme 5. Schematic for the modification of activated quartz with a mixed PFP-TTTA and OTS adlayer, followed by immobilization of the thiol-modified MNC-38 aptamer.

2.14 Contact Angle Goniometry

Quartz surfaces used for immobilization of tmMNC-38 were cleaned and modified in preparation for measurement using contact angle goniometry. Each layer added onto bare quartz was examined, including after being measured on the EMPAS. The sample disc is first placed onto the platform and the camera is manually adjusted to allow for the platform and sample to be in view. A syringe is then placed perpendicularly above to the sample disc and adjusted
accordingly such that the tip of the needle is in focus of the camera (Figure 19). Using the program, water is selected as the heavy phase and air as the light phase. By applying gentle pressure on the syringe plunger, a water droplet is released from the needle tip and allowed to drop onto the surface at room temperature. After making sure the droplet is in focus by the camera, five frames of the image are taken at 1 second intervals. The baseline for the surface is manually traced before the contact angle program calculates the angle for the droplet. The contact angle is calculated for each frame and then averaged to give the final value. For every layer added to the surface, several samples were prepared for replicates. Measurements were taken once for each disc.

After the discs had been run on the EMPAS with injections of HSP10, the discs were carefully removed from the cell holder, noting the side that was exposed to the protein. These discs were then taken to the goniometer and measured twice – once right after the EMPAS run and then measured a second time after rinsing twice with deionised water. The rinse was a done in order to remove most of the excess and unbound protein on the surface.

Figure 19. Equipment set-up for the contact angle measurement of quartz discs, in which a syringe is placed perpendicular to the sample surface such that the tip is visible to the camera (left). Visual output from the camera showing a live-view of the sample set-up (right).
2.15 Binding of HSP10 onto tmMNC-38-modified Surfaces

Running tmMNC-38-modified surfaces used the previously described EMPAS set-up parameters (940.000 MHz scan, flow rate = 50.00µL/min). Once a baseline for the sample disc was established, an injection of HSP10 is made. HSP10 of the following concentrations (prepared by serial dilution) were injected: 0.5 µM, 5 µM, 100 µM, 500 µM, and 1.3 mM. Each injection was done in replicate or triplicate.

To confirm that the frequency shift seen on the EMPAS corresponds to the occurrence of binding between the immobilized tmMNC-38 aptamer and the injected HSP10, a second set of experiments was set. From previous findings of our lab, it has been seen that non-specific binding and specific binding elicit different responses from the EMPAS. To decipher the difference between specific and non-specific binding of HSP10 to the modified surfaces, it was decided that the thiol-modified aptamer would not be immobilized on the surface (ie. only silane adlayer present). As with the previous parameters set on the EMPAS, injections of 100 µM, 500 µM, and 1.3 mM HSP10 were made. These results are then compared to the results from discs with immobilized aptamer.

2.16 XPS characterization of tmMNC-38-modified Surfaces

With promising results from the contact angle measurements, it was decided that XPS characterization would be the next logical step in order to concretely determine all layers. After modification of the quartz discs with the mixed adlayer followed by tmMNC-38, the discs are then sent to Surface Interface (SI) Ontario for analysis. Samples consisted of discs with subsequent layer modifications and were analyzed with the Thermo Scientific K-Alpha instrument operating with an Al Kα X-ray source. Four take-off angles of 27.5º, 42.5º, 57.5º, and 72.5º relative to the normal were used for deep-layer and surface analysis. Analysis for the following elements was also requested for each disc: carbon, chlorine, fluorine, nitrogen, oxygen, phosphorous, sulphur, and silicon. Using the ThermoAvantage program provided at SI Ontario, peak fitting and data analysis for each element was performed.
Chapter 3.
Results and Discussion

Author's note: The stated results and discussion described herein is presented in chronological order to display the thought process behind each experimental motivation in a coherent manner.

3.1 Over-expression of HSP10 in BL21 Rosetta E. Coli cells

Over-expression of our target protein was achieved through induction of BL21 Rosetta E. coli cells with IPTG. The cell cultures were monitored with UV-Vis spectroscopy to determine the optical density at 600 nm every hour for 4 hours. It was found that cell cultures induced with IPTG kept a similar optical density, while the control counterpart (without IPTG) increased in optical density over time. This observation reveals that the IPTG-induced cell culture is not longer expending energy to replicate, but rather has switched to over-expression of protein. The successful over-expression of HSP10 in BL21 Rosetta E. Coli cells was further confirmed when analyzed with SDS-PAGE (Figure 20).

It is evident in the gel that the samples from the induced cells produced a gradually darkened band over the hours the samples were taken out, which shows successful over-expression of protein. This is especially evident after overnight induction, where the culture was left to grow in the induced state for approximately 20 hours. The band seen from this sample shows the darkest HSP10 band. Comparatively, the non-induced cell cultures remained relatively similar in band intensity when compared to the sample taken before induction (T0). This supports the observation seen when cultures were measured with UV-Vis spectroscopy.
Figure 20. SDS-PAGE analysis of protein overexpression in *E. coli* cells. Running samples taken at time intervals before induction (T0), and after induction for 1 hr (T1), 2 hrs (T2), 3 hrs (T3), and overnight (TON).

3.2 Purification of HSP10 via nickel affinity chromatography

HSP10 was purified using nickel affinity chromatography using the Bio-Rad Econo pump and was visualized using UV absorbance by the attached plotter system. The corresponding graph (Figure 21) approximated which fraction contained purified HSP10, but was further confirmed for purity using SDS-PAGE. Fractions collected from the chromatography column were run on the gel and compared with the solutions collected before the purification process (ie. pellet supernatant, column wash). Using the graph provided from the Bio-Rad pump system, selected fractions were analysed using SDS-PAGE (Figure 22). In both gels, it is clearly evident that in the lanes consisting the supernatant and wash that most of the natural *E. coli* protein is still present, as expected. These proteins lack the His-tag, which prevents them from being retained by the nickel affinity column and therefore, wash out of the column. In the first gel, fractions 5 and 10 depicted no protein, which is explained by the lower gradient of imidazole that is not competitive with protein on the column. Therefore, nothing is eluted at these points, which is also evident in the graph as the absorbance is still at baseline. Interestingly at fraction 13, the gel shows bands of multiple protein sizes and the absorbance increase seen on the graph is indicative
of such. The proteins eluting at this point may have been non-specifically adsorbed on the column and are released upon the increasing concentration of imidazole running through in a competitive manner. It is also important to note that the band corresponding to HSP10 is not evident in the gel, indicating that it is still retained in the column.

As the non-specific proteins are finished eluting, there is a large increase in absorbance seen in the graph, which corresponding to the elution of the target protein, His-tagged HSP10. As the concentration of imidazole running into the column continues to increase, this particular protein is eluted off. When regarding the gel analysis at fraction 16, the band corresponding to HSP10 begins to appear. There is also faint evidence of the non-specific protein as this is the point where a mixture of these proteins eluting off is occurring. As more fractions are analysed, the HSP10 band increases in intensity, indicating increase in concentration as well. At fraction 29, when the absorbance begins to plateau, there is also very little HSP10 left seen in the gel.

![Plotter graph produced by the Bio-Rad Econo System by measurement of protein absorbance (UV) during elution.](image)
Figure 22. SDS-PAGE gels of collected HSP10 fractions. (A) First set of fractions compared to supernatant and wash. (B) Second set of fractions compared to supernatant and wash.

By combining the analyses from both the graph and the gels, the fractions that contained pure HSP10 were selected and combined. To concentrate the solution, the pooled fractions were centrifuged with a 3K MWCO filter. Extraneous salts and imidazole were subsequently removed using standard dialysis protocol. After the purification process, concentration of the final pure HSP10 solution was determined using UV/Vis spectroscopy. The absorbance spectrum produced two distinct peaks characteristic of protein. The larger peak at approximately the 200 nm corresponds to peptide bonds, while the smaller peak around 280 nm is a result of the absorbance of aromatic residues and is the primary absorbance value used for calculation of HSP10 concentration. Using the Beer-Lambert Law, $A = \varepsilon l c$, the concentration ($c$) was calculated, while accounting for the dilution factor used for spectroscopy measurement. $A$ is the absorbance value obtained from the spectrum at 280 nm, $\varepsilon$ is the extinction coefficient (L mol$^{-1}$ cm$^{-1}$), and $l$ is the length of the pathway for light (cm).
Sample calculation:

\[ A = \varepsilon l c \]

\[ 0.1550 = \frac{5960 \text{ L}}{\text{mol} \cdot \text{cm}} \times 1 \text{ cm} \times c \]

\[ c = 2.6066 \times 10^{-5} \text{ M} \]

\[ c = 2.60 \mu\text{M} \]

Accounting for the dilution factor of 50:

\[ c = 2.60 \mu\text{M} \times 50 \]

\[ c = 1300 \mu\text{M} \]

\[ c = 1.3 \text{ mM} \]

The extinction coefficient for our particular protein was generated using an online tool called ExPASy ProtParam. Knowing the HSP10 sequence, it can be inputted into the online tool and a corresponding extinction coefficient will be generated. It is important to know that this value is approximated based on specified parameters such as molecular weight and amino acid composition. The value generated from the online tool for HSP10 was 5960 L/mol\(^{-1}\) cm\(^{-1}\).

### 3.3 Cleavage of His-tagged HSP10

After collecting the purified solution of HSP10 and determining its approximate concentration, the protein was then ready to be cleaved at the enterokinase cleavage site to yield only the HSP10 sequence (Figure 23). This step was only performed when the His-tag was unnecessary, such as during binding interactions in solution or for injection into the EMPAS. Enterokinase is a protein enzyme that cleaves specifically after lysine at the Asp-Asp-Asp-Asp-Lys cleavage site.
Figure 23. Amino acid sequence of expressed HSP10 protein from *E. coli* cells, including His-tag, expression epitope, and enterokinase recognition site. Arrow indicates enterokinase cleavage site.

From the gel, the length of the HSP10 sequence before and after introduction of enterokinase can be compared (Figure 24). Without incubation with enterokinase, there is a dark band corresponding to approximately 17 kDa according to the protein ladder. This is expected as there is extra length in the HSP10 sequence as a result of the His-tag, expression epitope, and enterokinase cleavage site, thereby making the protein seem larger than it truly should be. During the time intervals of enterokinase incubation (0.25 hr – 2 hr), gradual formation of a band is clearly evident. This band, located around 11 kDa, corresponds to the desired and properly cleaved protein, which is known to be 10 kDa in size. Finally, when observing the bands from the overnight reaction, there is full conversion of the His-tagged HSP10 into the putative HSP10.

Figure 24. SDS-PAGE analysis for enterokinase cleavage of HSP10 fusion protein over several time intervals.
3.4 Binding of aptamer and HSP10 using DNA-Native PAGE

With the full cleavage of HSP10, the protein could now be used for binding analyses using DNA-Native PAGE. DNA-Native PAGE is a type of electrophoresis procedure in which the gel does not contain any denaturants or reductants in order to preserve the native structure and charge density of the analytes. This is important for the experiments with aptamer and HSP10 binding where differentiation between the bound and free aptamer state will allow for determination of binding and binding affinity of the two molecules towards each other.

Solutions containing both aptamer and HSP10 were first prepared to allow for binding to occur for approximately 1 hr at room temperature. Different solutions containing a controlled concentration of aptamer, but varied concentration of protein were prepared to determine binding affinity. As more protein is in the solution, less free aptamer will be available and theoretically, the corresponding band intensities will differ and can be analyzed with a program called ImageJ in order to elicit a binding curve and thus the binding affinity for the specific aptamer.

The binding solutions were aliquotted and prepared into gel samples to be analysed by DNA-Native PAGE. Because these biomolecules are new and lack literature experimentation, a long period of time was spent on optimizing gel electrophoresis experiments in order to achieve the best running conditions. As DNA-Native PAGE does not necessarily separate molecules by size only, optimization the percentage of acrylamide in the gel was performed. It was found that our longer aptamer sequences (MNB-38 and MNC-38) required a higher percentage of gel whereas our shortest aptamer sequence (MND-38) required a slightly lower acrylamide percentage in order to achieve optimal separation. Temperature optimization was also performed in order to produce a larger degree of separation between the bands for ease of analysis with ImageJ. It was also decided that the gel experiments would begin with MNB-38 rather than MNA-38 as a surface plasmon resonance (SPR) experiment was already performed for the latter, albeit just one. The binding affinity was deemed to be approximately 500 nM for MNA-38 in the SPR experiment.
From the optimization experiments, it was found that the optimal acrylamide percentage for MNB-38 and MNC-38 was 12%, while MND-38 required a 10% acrylamide gel in order to achieve separation of bands. Separation of the bands was only achieved when the marker band corresponding to xylene cyanol was run off the gel and the band for bromophenol blue was run until it reached the bottom of the gel. All gels were run in a -10ºC fridge in order as this enhanced separation between bands. However, when temperature was decreased below this point, resolution of the bands became very poor and indistinguishable. This was attributed to the gel physically freezing as a result of the lowered temperature. Therefore, it is imperative to keep a constant and exact temperature of -10ºC. The temperature was achieved by loading dry ice in a Styrofoam box large enough to accommodate the electrophoresis equipment. The equipment was elevated with some more Styrofoam to prevent freezing of the buffer at the bottom.

Figure 25. DNA-Native PAGE for aptamer binding. Results from MNB-38, MNC-38, and MND-38 (left to right) binding to HSP10.

After running and staining the gels using the previously described protocol, gels were inspected visually. From Figure 25, it is evident that all aptamers bind to the target protein HSP10 from the appearance of the top band, corresponding to the bound complex. One can also observe that MNB-38 and MNC-38 aptamers produced gels with good resolution and separation of bands. It is also important to note that the band intensity corresponding to the free aptamer diminishes as more bound complex is being formed, which is key for analysis using ImageJ. After generating a band intensity value for each band, the ratio between the bound and unbound bands in each lane is determined. This ratio is plotted against the concentration of HSP10 used in the sample solution to generate a binding curve using SigmaPlot (Figure 26). With the binding curves generated, it was found that the binding affinity for MNB-38 and MNC-38 were 16.0 ± 8.2 µM and 23.3 ± 5.2 µM, respectively.
Figure 26. Binding curves of MNB-38 (left) and MNC-38 (right) generated by SigmaPlot.

The MND-38 aptamer however, produced very poor resolution and separation of bands using the previously described conditions. Further optimization for this particular aptamer was performed but proved to be challenging as the bands became more diffuse with longer and colder electrophoresis conditions. There is also an inexplicable tapering at the higher ratio of aptamer to HSP10 mixture, making it difficult to analyze using ImageJ. This tapering phenomenon seen in one of the MND-38 bands is likely attributed to a phenomenon occurring within the sample mixture, rather than the gel or equipment as this tapering was seen only with that ratio, no matter where or how many times it was loaded in the gel (Figure 27). As a result, a properly visualized gel was not attained and in the end, MND-38 could not be analysed with ImageJ and no binding affinity value was generated for this particular aptamer.

Figure 27. DNA-Native PAGE sample gel of MND-38 aptamer. A "tapering effect" seen at higher concentrations of HSP10 (boxed frames show the same concentration).
3.5 XPS characterization of surfaces

Since it was proving to be difficult to analyse the gels via the proposed method and the dissociation constant could not be established for the MND-38 aptamer, it was decided that it would be best to move away from these experiments. It was then decided that the EMPAS would be employed to elicit binding affinity and associated dissociation constants. This method was chosen as it was previously successfully performed with a cocaine aptamer. However, in order to perform these tests and experiments, it was imperative to develop a new quartz surface chemistry for use with the EMPAS. It was evident that for these tests, HSP10 would have to be immobilized and as a result, would require the His-tag to be retained. As such, enterokinase cleavage of the protein was not performed. In nickel chromatography columns, NTA is commonly used as a moiety for nickel chelation. With this knowledge, it was decided that NTA would be a relevant molecule for incorporation into our surface chemistry. The linker molecule to immobilize all elements onto quartz was decided to be PFP-TTTA as it is known to react with amine functional groups.

The surface chemistry developed here required a bit of optimization at first, as it was relatively novel. The largest hurdle to overcome was immobilizing NTA onto the PFP-TTTA silane adlayer. NTA is a highly hydrophilic molecule, while PFP-TTTA is highly hydrophobic and thus, finding a solvent that can both dissolve NTA and penetrate the adlayer for a proper reaction to occur proved to be difficult at the beginning. We began the experiments by dissolving hydrophilic NTA in a 1:1 v/v ratio of deionised water and methanol, in which the addition of methanol could aid in the hydrophobicity of the solvent overall and allow for the reaction to occur. Three other solvent mixtures were also used: deionised water, dimethyl sulfoxide (DMSO), and 3:2 ratio of dimethylformamide (DMF) to deionised water. All solvents were capable of dissolving NTA, although DMF required slight heating from a heated dryer. Each solvent was used to prepare a 10 mM solution of NTA and let to react with PFP-TTTA silanized quartz discs overnight. These discs were then taken to SI Ontario for XPS analysis to confirm if attachment of NTA was successful. Successful attachment would show a slight increase in nitrogen signal as well as full reduction of the fluorine signal.
The following paragraph will provide a general overview of the prepared surface chemistry on quartz. When NTA is introduced to the surface, a loss of fluorine signal is seen as a result of NTA functionalizing onto the PFP-TTTA and dissociating the head groups. With the introduction of nickel, a trace amount of nickel is present on the surface. Finally, when HSP10 is immobilized, we see a significant increase in carbon and nitrogen, as expected when protein is on a surface. Figure 28 provides an overview of all significant elements from XPS analysis during surface preparation. In another case, the relative percentage of oxygen is seen to decrease due to the increase in surface thickness, thus revealing less of the underlying quartz (chemical formula: SiO₂) for analysis by XPS. The discussion herein will go into detail about each significant element and corresponding peak signal.

![Figure 28. XPS analysis of HSP10 immobilized quartz surfaces at 72.5° relative to the normal. Summary of indicative elements.](image)

When bare quartz is silanized with PFP-TTTA, an increase in fluorine is seen as a result of the fluorinated head groups, which indicates that the quartz was successfully silanized with the linker molecule. From the different solutions of NTA, varied results amongst the different solvents used were seen (Figure 29). Although deionised water was able to readily dissolve NTA, the solvent itself did not provide the hydrophobicity needed to provide a reaction with the
PFP-TTTA molecule and thus, there is no nitrogen signal produced and is in fact, very similar in profile to the blank disc (aka. clean quartz disc without surface modification). The addition of methanol or DMF to water in order to increase hydrophobicity also did not prove successful in either case. However, it was found that dissolving NTA in DMSO gave a slight nitrogen signal (boxed frame in Figure 29), expected from the two nitrogen atoms in NTA. Albeit weak, this signal suggests that NTA has properly attached onto the silane adlayer.

Figure 29. Graphical comparison of the nitrogen signal obtained via XPS at 72.5° relative to the normal. Various solvents were prepared for dissolving NTA and subsequent reaction with adlayer-modified quartz discs. Boxed frame shows a weak nitrogen signal.

In conjunction to the analysis of the nitrogen signal, the fluorine signal is an important factor to analyze as well and provides further supporting evidence for NTA attachment. As suggested from the lack of a nitrogen signal, reduction of the fluorine signal after NTA introduction was not seen for NTA dissolved in deionised water, water:methanol or DMF:water. For NTA dissolved in DMSO, on the other hand, a significant reduction in the fluorine signal is seen and is almost comparable to the blank. However, there is still a slight fluorine signal remaining, which was attributed to the close packing of the PFP-TTTA molecules, in which the head group is rather bulky. This steric hindrance may have affected the ability for NTA to react with all molecules. As a result, a diluent or spacer was suggested to build a mixed adlayer when laying
down trichlorosilane molecules. The spacer suggested was OTS, which is a trichlorosilane molecule with an eight carbon alkane chain. This molecule is shorter than PFP-TTTA overall, thus allowing for the head group of PFP-TTTA to still be readily available for reaction with NTA. OTS is simultaneously deposited onto activated quartz with PFP-TTTA to better distribute the latter over the surface to alleviate the steric associated with close-packing of a single silane molecule. It was decided that a 1:1 ratio would be beneficial for a complete surface reaction.

![Figure 30](image.png)

**Figure 30.** XPS analysis at 72.5° relative to the normal. Graphical representation of the fluorine signal generated by XPS acquisition for 4 samples of NTA attachment to PFP-TTTA using varying solvents. Boxed frame shows a diminished fluorine signal.

With the new mixed adlayer of PFP-TTTA and OTS, another round of XPS analysis was performed to confirm full reaction of the PFP-TTTA linker with NTA. At the same time, this new mixed adlayer was compared with the homogenous PFP-TTTA adlayer previously described. In both cases, introduction of NTA for attachment results in the loss of the fluorine signal, elucidating functionalization onto PFP-TTTA. Without the OTS diluent, the fluorine
peak corresponding to the fluorinated head group of PFP-TTTA is much larger compared to when the OTS diluent is included in the adlayer (Figure 30). This is simply because there is more PFP-TTTA linker present on the surface in the former compared to the latter. The experiment was performed again regarding the mixed adlayer and was able to achieve reproducible results (Figure 31). Fluorine peaks appear when PFP-TTTA is present and disappear when NTA in DMSO is introduced. These results strongly support that NTA has covalently attached onto the PFP-TTTA linker and has properly dissociated the fluorinated head group for the reaction to occur.

Figure 31. XPS analysis at 72.5° relative to the normal. Graphical comparison of the fluorine signal between PFP-TTTA only and PFP-TTTA with diluent OTS covered surfaces.
Figure 32. XPS analysis at 72.5° relative to the normal. XPS scans for fluorine of individual samples. A) bare quartz. B) Quartz with mixed adlayer. C) Quartz with only PFP-TTTA.

Upon individual sample examination of the fluorine signal, evidence of fluorine is still present in the homogenous adlayer (Figure 32C). This is supports the previously mentioned theory of the linker head groups being closely packed together and thus, is more difficult for NTA to penetrate the layer for a reaction to occur. On the other hand, when OTS diluent is mixed into the adlayer, we see full elimination of the fluorine signal (Figure 32B), indicating that NTA must have reacted with all the head groups and have attached onto the surface via the linker. This is comparable to the blank (Figure 32A). The full elimination of the fluorine signal is ideal and puts the mixed adlayer functionalization of quartz in favour over a homogenous adlayer for molecular immobilization. In Figure 33, the full elimination of the fluorine signal is evident.
Figure 33. XPS analysis at 72.5° relative to the normal. Graphical comparison of the fluorine signal before and after NTA introduction.

Figure 34. XPS analysis at 72.5° relative to the normal. Graphical comparison for the nitrogen signal when NTA is introduced on the adlayer-modified surface. Boxed frame shows weak nitrogen-corresponding signal.
Unfortunately, because the nitrogen signal corresponding to the attachment of NTA to the homogenous PFP-TTTA adlayer was already weak to begin with, a signal for nitrogen with the added diluent was not observed comparatively during surface analysis at 72.5° relative to the normal (Figure 34). However, with the evidence provided by the loss in fluorine signal, we can say with confidence that NTA has properly immobilized onto the mixed adlayer. Upon slightly deeper analysis (angle 57.5°) however, the nitrogen signal becomes more apparent (Figure 35). The peak evident at 400 eV corresponds to both the amide and tertiary nitrogen. The reason for the more apparent signal when probing deeper into the layer is because of the physical location of the nitrogen atoms amongst the bulk layer when NTA has attached (Figure 36). The amide nitrogen is buried deep in the layer, as it served to link the molecule onto PFP-TTTA. Although the tertiary amine is closer to the surface, it is shielded by three carboxylic acids. Also to note once again, the nitrogen signal for the mixed adlayer compared to the homogenous adlayer is slightly diminished as a results of less over attachment of NTA. Ultimately, looking deeper into the XPS analysis has provided greater insight and evidence towards the attachment of NTA onto PFP-TTTA.

Figure 35. XPS analysis at 57.5° relative to the normal. Graphical comparison for the nitrogen signal when NTA is introduced on the adlayer-modified surface.
Figure 36. Surface chemistry depicting the NTA attachment onto PFP-TTTA silane molecules.

Figure 37. XPS analysis at 72.5° relative to the normal. Graphical comparison of the nickel signal for when NiCl$_2$·6H$_2$O is introduced onto the modified surface.
NTA is a tetravalent chelating molecule, coordinating to divalent metal atoms (Ni$^{2+}$, Cu$^{2+}$, etc) with an octahedral geometry.$^{95-97}$ With NTA properly functionalized, the surface must be activated with a metal in order for future protein immobilization.$^{96,97}$ As NTA is frequently paired with nickel in affinity chromatography columns, it was decided that this would be the best ion of choice. Activation of NTA involved introduction of nickel chloride hexahydrate (NiCl$_2$$\cdot$6H$_2$O) in deionised water onto the modified surface. From the acquired XPS data, it is evident that there is nickel present on the surface when the binding energy reaches approximately 856 eV (Figure 37). From this data, the metal complex can be validated as it is around this point in energy that is characteristic of Ni(OH)$_2$ and NiO bonds from coordination.$^{98}$ The evidence provided by XPS elicits the presence of nickel and its appropriate geometry, which is imperative for the binding of our His-tagged protein.

![Graphical comparison for the nitrogen signal at different depths of XPS analysis for when HSP10 is immobilized on the modified surface.](image)

With the activation of NTA appropriately confirmed, the addition of our His-tagged protein was next to be analysed. It is evident that with the addition of His-tagged HSP10 greatly increases the nitrogen signal intensity (Figure 38) to approximately 40 cps at surface analysis (72.5°) and 120 cps at deep layer analysis (27.5°). The increase in nitrogen signal is a direct result of the high amount of nitrogen content in protein and indicates that HSP10 is present on the modified
surface. Amide bonds corresponding to the protein backbone give rise to a peak at around 400 eV, but is overlapped by the larger adjacent peaks. These larger peaks seen around 402 eV and 397 eV correspond to the imidazole ring present in histidine. The increase in signal, is mainly due to more nitrogen content overall as a fact of probing deeper into the protein (27.5°). However, this increase in signal may also be a result of probing at the histidine residues, which are comprised of characteristic nitrogen environments, and therefore give rise to the characteristic signals seen in the spectrum. The histidine residues lie deeper in the surface and therefore produce a larger signal only when probed at a deep angle. The evidence from the XPS spectrum suggests that the His-tags are located deep within the surface and are likely coordinated with nickel in a properly oriented fashion.

3.6 EMPAS measurements for binding affinity

The idea behind using the EMPAS to elicit dissociation constant stemmed from work done by Dr. Miguel Neves, where he was able to find the dissociation constant of the binding between cocaine and its corresponding DNA aptamer. Due to the challenges that arose during gel analyses, it was decided that the following experiments would be modelled after the ones performed for cocaine and its aptamer. With the surface characterization confirmed and completed, it was now possible to move towards analysis of aptamer binding onto the HSP10-immobilized quartz discs using the EMPAS. The prepared discs are placed into the chamber of the EMPAS and PBS buffer is allowed to flow over the surface until stabilization is reached. Upon this time, an injection of re-annealed aptamer is made. Once it enters the chamber with the HSP10-immobilized surface, theoretically, a binding event would take place. By injecting different concentrations of aptamers, one would expect differences in frequency shifts corresponding to differences in those concentrations. As in the experiments with cocaine and its aptamer, a lower concentration of cocaine injected onto the aptamer-immobilized quartz resulted in a smaller frequency shift. On the other hand, a higher concentration of cocaine injection results in a larger frequency shift.
The experiments began with the injection of the MND-38 aptamer, due to its specific challenges that arose during PAGE analysis. Table 3 summarizes the frequency shift obtained for all replicates in all concentrations of MND-38 aptamer injected. It is important to note that cocaine is a very small molecule compared to the protein used in this project, HSP10. Protein is also highly dynamic in solution, which may play a role during binding and ultimately, its effect on EMPAS measurements.\textsuperscript{101–103} As a result, a correlation between frequency shifts and injected aptamer concentration was not achieved. In fact, replicates were also difficult to achieve, which may be attributed to slightly differences and variations that occurred during sample preparation. For example, the number of sites available for protein immobilization may be a contributing factor, as well as the differences in composition and distribution of the underlying adlayer during preparation. As a result, these factors have contributed to rather large standard deviation. In addition, the relative standard deviation (RSD) values exhibited rather poor reproducibility (21.22% - 41.32%). Ultimately, a binding curve was not generated from these results.

Table 3. Summary of frequency shifts from injection of varying concentrations of MND-38.

<table>
<thead>
<tr>
<th>Conc. of MND-38 (µM)</th>
<th>Frequency Shift (Hz)</th>
<th>Average (Hz)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Replicate 1</td>
<td>Replicate 2</td>
<td>Replicate 3</td>
</tr>
<tr>
<td>10</td>
<td>2983</td>
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<td>1945</td>
</tr>
<tr>
<td>50</td>
<td>3014</td>
<td>1642</td>
<td>2715</td>
</tr>
<tr>
<td>100</td>
<td>1854</td>
<td>1105</td>
<td>-</td>
</tr>
<tr>
<td>250</td>
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<td>1629</td>
</tr>
<tr>
<td>400</td>
<td>1660</td>
<td>1040</td>
<td>1166</td>
</tr>
</tbody>
</table>
Figure 39. Average frequency shifts recorded from the EMPAS after injection with various concentrations of aptamer.

However, there is a general trend of a smaller frequency shift as the aptamer concentration increases, as there is a significant difference between an injection of 400 µM MND-38 compared to 10, 25, and 50 µM injections (Figure 39). With this observation, control experiments were performed to analyze this particular concentration of aptamer as it gave relatively good reproducibility and the smallest standard deviation. These control surfaces were to be compared to the sample surfaces modified with all layers: PFP + OTS + NTA + Ni + HSP10 (termed "full platform”).

Two alternative disc preparations were prepared as controls: the first being clean, bare quartz modified only with the mixed PFP-TTTA and OTS silane adlayer (termed "adlayer only”), and the second control with HSP10 non-specifically deposited onto clean, bare quartz (termed "HSP10 only"). The first control was prepared to monitor the interaction of injected aptamer with just the adlayer in which a different frequency shift and profile for this control was suspected, since there is no HSP10 on the surface and thus, the aptamer should not bind to the surface in a specific manner. The second control was performed to determine if the binding of
the aptamer would still occur when protein on the substrate surface is not immobilized in an oriented manner.

As predicted, the EMPAS results (Table 4) show a specific interaction between protein and aptamer when protein was on the surface (Figure 40). When aptamer is introduced to the quartz surface modified only with the mixed PFP-TTTA:OTS adlayer, a large frequency shift is seen ($\Delta f = 6.91 \pm 2.74$ kHz) and exhibits a different graphical profile overall compared to the other samples. Alternatively, when protein is present on the surface – either covalently bound or non-specifically adsorbed – the frequency shift is significantly diminished ($\Delta f = 1.29 \pm 0.33$ kHz and $\Delta f = 1.12 \pm 0.55$ kHz, respectively). The profiles of both the surfaces with HSP10 exhibit similar graphical profiles on the EMPAS, whereas the surface modified with only the adlayer is differed (Figure 41).

Table 4 . Summary of frequency shifts from injection of 400 $\mu$M MND-38 onto different surface samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>Averaged Shift (Hz)</th>
<th>RSD (%)</th>
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<tr>
<td>full platform</td>
<td>1660</td>
<td>1040</td>
<td>1166</td>
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<td>adlayer only</td>
<td>4977</td>
<td>8845</td>
<td>-</td>
<td>$6911 \pm 2735$</td>
<td>39.58</td>
</tr>
<tr>
<td>HSP10 only</td>
<td>731</td>
<td>1511</td>
<td>-</td>
<td>$1121 \pm 551$</td>
<td>49.15</td>
</tr>
</tbody>
</table>
Figure 40. EMPAS results from injection of 400 µM MND-38 DNA aptamer over three differently prepared quartz surfaces. "Full Platform" refers to all characterized layers. "Adlayer Only" refers to quartz modified only with the silane adlayer. "HSP10 Only" refers to bare quartz with protein adsorbed.
Figure 41. EMPAS measurements for each sample depicting different graphical profiles after injection with 400 µM MND-38.

The deduction for the phenomenon seen on the EMPAS measurements is based on specific versus non-specific probe-ligand interactions, as well as the many factors that are contributing to the produced signal. When no protein is present on the quartz surface, the overwhelming factor contributing to the signal is that of mass loading. The introduction of the aptamer causes the overall frequency to resonate at a lower value as a result of the gravimetric changes. On the other hand, when operating with quartz surfaces modified with HSP10 protein, a significantly smaller frequency shift is seen when aptamer is injected onto the surface. In a previous study performed, a non-specific interaction gave a smaller frequency shift compared to a specific
The hypothesized reason for this observed phenomenon is due to the rigidification of the bulk layer when the interaction between protein and ligand occurs. During rigidification, the bulk layer is capable of resonating better with the underlying quartz substrate. This allows for the resonant wave to propagate through the layers more effectively, thereby allowing the system to operate at a higher frequency. The result of the interaction of aptamer with protein on quartz surfaces corresponds to a balance between opposing factors. Rigidification of the bulk layer as a result of an interaction theoretically would show a frequency shift upwards, whereas gravimetric changes would result in a downwards shift. As these two contributing factors oppose each other, the ultimate result would be a diminished signal, which is evident in our EMPAS measurements. Looking back at the results regarding the varied aptamer concentration injections (Figure 39), the reason for the larger frequency shifts observed at lower concentrations may be due to the fact that the stiffening effect from a specific binding interaction is playing less of a role. Without this effect, it is expected that perhaps mass loading would play a larger effect and is therefore, what is being seen as the observed signal. However, we must not negate the fact that there are a multitude of factors that contribute to the observed signal. As these factors have not been examined individually, only speculation can be vocalized.

Reproducibility of the quartz discs with non-specifically adsorbed protein is slightly diminished in comparison to discs with covalently immobilized protein. The RSD values were calculated to be 49.15% and 25.40%, respectively. The lowered reproducibility rate (or higher RSD value) for the non-specifically adsorbed protein may be attributed to improperly oriented protein or protein wash-off, both as a result of it being non-specifically adsorbed. However, the protocol we have described to covalently immobilize His-tagged HSP10 remains superior compared to adsorbed protein as the former provides a larger degree of control over specific protein orientation, thereby minimizing the RSD value and enhancing reproducibility of results. In addition, reproducibility for the adlayer only sample was also poor, with an RSD value of 39.58%. This may be a result of a lack of specific binding of the injected aptamer onto the surface, as no protein was present. This would cause the aptamer to "wash-off" the surface over time and become detached. The evidence for this can be seen from the graphical profile as the shift is initially great, but gradually returns to baseline at a higher frequency (Figure 41).
Evidently, all EMPAS results have a limited number of replicates due to reagent and time constraints. Ideally, it would be best to increase the number of replicates to further appreciate and understand the results obtained. However, with the results gathered from this project, it is sufficient to come to the conclusions made, as the graphical profiles from the EMPAS as well as work done previously in our lab provide a satisfactory amount of information.

From these experiments, an interesting result was discovered. When aptamer was introduced onto the surfaces containing HSP10, a characteristic peak was seen after the initial injection. From the previous work done by several members of the Thompson Group, there has been no history of a characteristic peak resulting from the injection of an analyte. The peak corresponded to the time it took for the injected solution to reach the disc chamber (approximately 3 min at a flow rate of 50.00 µL/min), and therefore this peak is significant to the interaction with the surface. From the previous work done by several members of the Thompson Group, there has been no history of a characteristic peak resulting from the injection of an analyte using the EMPAS. Most often, a decrease in frequency was seen as mass loading became the overwhelming factor, which may be followed by a gradual increase in frequency as analyte washed off the surface. The results seen in these experiments were indeed a novel discovery. As such, more experiments were performed to confirm this result was not an artefact or a systematic error of the EMPAS, but rather, was unique to the aptamer-protein interaction. First, possible interaction between the aptamer with only the mix adlayer present on the surface of quartz was explored. In this control experiment, 400 µM injection of MND was performed, which was described previously. Next, the interaction between HSP10 and nickel-bound NTA was observed in order to determine if the characteristic peak seen was simply due to interactions on the surface. Interestingly, when a low concentration of HSP10 (5 µM diluted in deionised water) was injected, the profile given by the EMPAS was almost identical to the profiles seen with samples. However, when a high concentration of HSP10 (1.3 mM in sterilized PBS) was injected, the characteristic peak was not seen, and rather, the profile was very similar to results seen in the past, as the shift in frequency was mainly due to mass deposition of the protein. Due to this difference, it was decided that exploration into how imidazole interacted with nickel-activated surfaces was worthy. Imidazole is the ring structure component of histidine and is also
the component that is involved in the chelating aspect of nickel-bound NTA. Examining the interaction of imidazole with nickel using the EMPAS was an appropriate measure as it was a smaller molecule and may elicit a different response to when a His-tagged protein interacts. However, when 5 µM of imidazole in PBS was injected, no characteristic peak was seen.

The idea for the next experiment sprouted from the difference in solutions that the injected HSP10 was in, as we believed it may have a possible effect on the sensing ability of the EMPAS. Over quartz discs, injections of either deionised water or PBS was compared. The results were noteworthy as it addressed the speculations held in the past. When deionised water was injected, the characteristic peak that had been seen on all sample data was evident once again. In contrast to when PBS was injected, the same solution of the flow-through buffer, no signal was detected. This strongly indicates that the profile seen in the data collected for injection of aptamer may not be characteristic after all, but rather simply the difference in solution as it was detected by the EMPAS (Figure 42).

PBS is a buffer solution containing a variety of salts and ions, which have an effect on the resonant amplitude of the EMPAS. This is due to the effect of the Helmholtz dielectric layer that occurs on the surface when a liquid medium is run over. Previously, the Thompson group has described the effect of high solute concentration on the resonant envelope amplitude of the EMPAS. It was stated that the resonant envelope amplitude decreases as higher solute concentration is present due to ion screening. This phenomenon reduces ion movement over the surface which causes a reduction in the effective magnetic field. Movement of charge is induced by the magnetic field, which leads to a current that is detectable by the system. With a reduction in the effective magnetic field, a smaller current is induced, leading to a reduced signal. On the other hand, inhibited ions as a result of low salt concentrations have a reduced ability to generate a current due to a lack of movement and thus, result in smaller resonant amplitudes.\textsuperscript{104}
Figure 42. EMPAS graphs comparing differences between solvent injections. Deionised water (A) versus phosphate buffered saline (B) compared. Flow-through solvent used in system is phosphate buffered saline. Graphs are not baseline corrected.

What is seen with the experiments performed with the change in buffer conditions is a situation that lies in between the high and low solute concentration phenomenon described previously. PBS is a buffer packed with solutes and ions. As this is our flow-through buffer of choice, it may be creating the ion screening effect, in which the resonant envelope seen is the one used. However when deionised water – a solution containing no solutes or ions – is injected into the system, when it reaches the sensing surface, a displacement of the solutes already present in the chamber causes an increase in current and therefore, an overall increase in signal. As the deionised water portion leaves the chamber, the signal returns back to its original baseline envelope.
3.6.1 Baseline correction

Baseline correction on EMPAS data was required as the raw data showed significant drift during measurement. The correction is performed in Enthought Canopy, a program used for running code such as Python, using a short segment of code written by Liviu-Mihai Calin. Below are two sample graphs generated from the code to show before and after baseline correction (Figure 43).

![Baseline correction sample graph of a 10 µM injection of MND-38 aptamer in PBS. Raw EMPAS data where significant drift is seen (left). Baseline corrected data to allow for obtainable frequency shifts (right).](image)

3.7 Immobilization of tmMNC-38 aptamer on quartz

As the results from EMPAS regarding binding affinity gathered dismal results, we felt it was time to move away from those experiments. Our next decision on choosing the candidate aptamer was based on the entire collection of results from both the PAGE analyses and EMPAS measurement, as well as the structural characteristics of the aptamers. From the PAGE analyses, we were able to determine that all aptamers bind to HSP10, but it was the strength of that binding that was challenging to determine. These analyses also showed that both MNB-38 and MNC-38 result in binding affinities with no significant differences. MND-38 from both PAGE
and EMPAS analyses proved to be difficult to obtain a binding curve and therefore, was not considered in the selection process. MNA-38 was also never formally measured and therefore, was also opted out in the process. Between aptamers MNB-38 and MNC-38, MNC-38 proved to be structurally more beneficially in regards to surface chemistry and immobilization. Since thiol modification would take place on the 5'-end of the DNA aptamer sequence, the extraneous primer region of MNC-38 would prove to be beneficial for future immobilization. This primer region, as we have deduced to be not necessary for binding from the MND-38 analyses, could potentially act as an extension from the silane adlayer and provide optimal distribution and packing on the surface when immobilized.

3.7.1 Contact Angle Goniometry Measurements

With this decision, thiol-modified MNC-38 (tmMNC-38) was ordered and standard procedures for deprotecting and reduction of the disulfide bond were performed. As a quick method for predicting the proper layers were prepared on the surface of quartz, contact angle goniometry measurements were taken. The results from these measurements show the differences in water interaction with the surface as the chemistry is altered. Clean, bare quartz was seen to be the most hydrophilic as seen by its low contact angle at 3.9 ± 0.4º and high degree of wettability. The reason for its hydrophilicity is due to the exposure of hydroxyl groups, as its chemical formula is SiO₂, making it hydrophilic. The contact angle increases drastically to 88.7 ± 2.0º when the mixed silane adlayer is added, a result of the hydrophobic PFP-TTTA and OTS molecules, which repel the droplet of water. When tmMNC-38 is immobilized, the angle decreases slightly to 74.9 ± 2.0º. Albeit slight, this decrease in angle suggests the presence of immobilized DNA aptamer, which is relatively hydrophilic. Contact angle measurements with similar results were found with the cocaine-aptamer project.⁹⁰

It is also important to note the slight disparity between the contact angles regarding each sample of the same category. When samples are prepared, even if the same chemistry is performed, there will always be slight differences between each sample. Factors such as surface roughness (ie. textured aberrations), non-uniform molecular distribution, as well as placement of the droplet can contribute to the disparities in contact angle measurements.¹⁰⁵
Table 5. Contact angle measurements for each additional layer modified on quartz. Corresponding photos regarding each layer are shown.

<table>
<thead>
<tr>
<th>bare quartz</th>
<th>PFP-TTTA + OTS adlayer</th>
<th>tmMNC-38 immobilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9 ± 0.4°</td>
<td>88.7 ± 2.0°</td>
<td>74.9 ± 2.0°</td>
</tr>
</tbody>
</table>

3.8 EMPAS measurements for HSP10 binding

After the quartz surfaces immobilized with tmMNC-38, binding of HSP10 was tested using the EMPAS. At the beginning of these sets of experiments, the working range of protein concentration was 0.5 – 1300 µM. It was found, however, that no shift was found at the lower concentrations (0.5 and 5 µM) and therefore, focus was placed on the higher concentrations of protein (100, 500, and 1300 µM).
Table 6. Summary of the frequency shifts obtained during initial EMPAS measurements for HSP10 injections.

<table>
<thead>
<tr>
<th>Concentration of HSP10 injected (µM)</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Replicate 3</th>
<th>Averaged Shift (Hz)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
<td>248 ± 430</td>
<td>173.21</td>
</tr>
<tr>
<td>5</td>
<td>0*</td>
<td>0*</td>
<td>542</td>
<td>181 ± 313</td>
<td>173.21</td>
</tr>
<tr>
<td>100</td>
<td>2006</td>
<td>2613</td>
<td>2853</td>
<td>2491 ± 437</td>
<td>17.55</td>
</tr>
<tr>
<td>500</td>
<td>3744</td>
<td>2352</td>
<td>-</td>
<td>3048± 984</td>
<td>32.28</td>
</tr>
<tr>
<td>1300</td>
<td>12031</td>
<td>8554</td>
<td>7844</td>
<td>9476 ± 2241</td>
<td>23.65</td>
</tr>
</tbody>
</table>

*no apparent shift

Figure 44. Graphical comparison of the frequency shifts measurement on the EMPAS between surfaces coated with mixed adlayer PFP-TTTA + OTS only (blue) or immobilized with tmMNC-38 aptamer (red).

It was decided that the control for these experiments would be to test the interaction of HSP10 with the bare adlayer (without tmMNC-38 immobilized). It is expected that with a specific interaction, a difference in shift will be evident compared to a non-specific interaction. Results showed that at lower concentrations, interaction of HSP10 with the adlayer surface elicits a lower frequency shift and at higher concentrations, the frequency shift is larger. The same is true for surfaces with tmMNC-38 immobilized, as there was no significant difference between the
two groups (Figure 44). RSD values were poor at 100 µM injections, but improved at higher concentrations (Table 7).

### Table 7. Summary of the frequency shifts obtained with focus on higher concentrations of HSP10 injections. Comparison made between control (adlayer only) and sample (aptamer immobilized).

<table>
<thead>
<tr>
<th>Concentration of HSP10 injected (µM)</th>
<th>Adlayer Only (PFP-TTTA + OTS) (n=3)</th>
<th>Aptamer Immobilized (+ tmMNC-38) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Averaged Frequency Shift (Hz)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>100</td>
<td>1238.33 ± 561.78</td>
<td>45.37</td>
</tr>
<tr>
<td>500</td>
<td>4173.00 ± 1067.22</td>
<td>25.60</td>
</tr>
<tr>
<td>1300</td>
<td>14402.33 ± 1667.73</td>
<td>11.58</td>
</tr>
</tbody>
</table>

*n=3

These results show that there is no specific binding occurring on the surface with the immobilized aptamer. Several hypotheses were conjured for the results we saw, including 1) aggregation of protein on the surface, 2) reaction of free thiol and amino groups of HSP10 with the adlayer or 3) slow binding mechanisms affected by a fast flow rate. The first hypothesis was negated as there is literature stating that HSP10 dissociates from its heptameric formation into its monomeric form at approximately 1 µM. The second hypothesis is unlikely as the time the protein remains within the chamber is too short for a reaction to occur, especially for PFP-TTTA which is notoriously hydrophobic and the running buffer is PBS. The third theory was a likely contender and thus was further explored on the EMPAS by slowing down the flow rate. It was decided that if the third hypothesis was true, then slowing down the flow rate will allow for the binding of HSP10 to the immobilized aptamer and detection of the lower concentrations should therefore be possible. It was therefore decided to use 0.5 µM injections of HSP10. Unfortunately, the results showed no evidence of binding and were overall inconclusive.
3.9 XPS characterization of aptamer-immobilized surface

To make sense of the data gathered from the EMPAS, XPS characterization was the next logical step as it was imperative to concretely confirm that the surface was in fact prepared properly. Analysis for the following elements was performed for each disc: carbon, chlorine, fluorine, nitrogen, oxygen, phosphorous, sulphur, and silicon.

The element fluorine would be the most indicative of proper surface modification in this case. With the introduction of the mixed adlayer containing PFP-TTTA, the relative atomic percentage for fluorine will increase as a direct result of the head group characteristic to the silane molecule. This is evident in Appendix 2, where after introduction of the mixed adlayer, the fluorine signal is increased. This was also seen with the previous surface chemistry, thus surmising this reaction as robust. With the introduction of tmMND-38 however, little to none of the fluorine signal is reduced, indicating that the aptamer was not properly immobilized. This is further deduced by the lack of phosphorous and sulphur signals, which should increase. Phosphorous would increase as a result of the presence of DNA content, which is rich in this element. Sulphur would also increase as a result of the thiol attachment to the PFP-TTTA linker. One can argue that there is minimal attachment as the fluorine percentage does reduce slightly and the phosphorous and sulphur elements increase slightly, but ultimately, this would not be significant enough to achieve a signal when performing measurements on the EMPAS. As the XPS results did not show proper immobilization of the thiolated aptamer, it is only accurate to also assume that the results from the previous EMPAS experiments are invalid.

Preparation of the methanol-water solvent mixture for aptamer immobilization was to increase the solubility of the adlayer into solution. As seen previously with the surface modification for NTA attachment, several tests for optimization were performed in order to find the most suitable solvent for reaction. It was determined DMSO was the best, however, because the aptamer is a biomolecule, harsh solvents were not considered and thus, the choice for a suitable solvent was rather limited. It was seen with a previous experiments performed in our lab that the aptamer was capable of handling being in a mixed solution of water and methanol and therefore, this method was adopted. However, as evidence in the results show, this solvent mixture was not
capable of immobilizing the aptamer. As such, further optimization is required if it is decided that we continue to work with the mixed PFP-TTTA and OTS adlayer. An alternative approach would be to find a more reactive linker molecule, such as TUBTS, which has been used in the past to attach thiolated DNA aptamers.
Chapter 4.
Summary & Conclusions

The work that has been presented in this thesis has demonstrated great potential for application towards early-stage ovarian cancer detection. We have described a procedure that has allowed for successful purification and isolation of HSP10 grown from *E. coli* cells, as the protein is not readily available for commercial purchase. With this isolated protein, it led into the characterization of a novel surface modification of quartz to allow for the immobilization of the His-tagged protein and aptamer analysis. This novel surface chemistry that we have described can be applied in many other scenarios involving His-tagged proteins. As seen with the EMPAS measurements, there was a specific response due to the interaction between protein and aptamer, causing a smaller frequency shift compared to a non-specific interaction. This not only depicted that the chemistry can be applied to binding interactions and aptamer selection, but also provides insight into the complexity of factors that contribute to acoustic wave signals when interactions occur on the surface. These results show that the measurements for my particular experiment are not optimal for quantitative analysis, but rather should be used for qualitative purposes only.

Finally, the conclusion of this project led to the selection of a candidate aptamer for quartz surface immobilization based on results from project as well as its physical structure. The subsequent experiments showed that the PFP-TTTA linker is perhaps not suitable for biomolecular immobilization as harsh solvents are required for a reaction to occur. With the chosen candidate aptamer, we are one step closer to building a biosensor that can aid in ovarian cancer detection at the early stage.
Chapter 5.
Future Work

As this project is still in its infancy, there is still a multitude of experiments that can and should be done that will bring the final goal to light. The first step is to further optimize the surface chemistry regarding immobilization of the aptamer. As mentioned previously, PFP-TTTA is a notoriously hydrophobic molecule and does not react well with reagents dissolved in an aqueous and hydrophilic solution. As such, further optimization of the surface chemistry for thiol-modified aptamer immobilization is needed. One avenue would be to use a previously described protocol stemming from work done by two past lab members, where a different linker called TUBTS was employed. This linker was successful in immobilizing a thiol-modified aptamer as it is more chemically selective towards thiols compared to the PFP-TTTA linker used in these experiments. In addition, no harsh organic solvents were required and therefore the integrity of the aptamer could be retained.

Upon establishing an optimized surface chemistry for immobilizing the candidate aptamer, it would be beneficial to test the modified surface with alternative proteins to observe for any binding or non-specific adsorption. Alternatively, further control experiments by immobilizing an arbitrary aptamer on the surface to observe HSP10 interaction with the surface can also be done.

In addition to the experiments, investigating the actual binding mechanism between the aptamer and protein would be valuable for observing and understanding the signals produced by the EMPAS. To investigate binding mechanism, nuclear magnetic spectroscopy would be employed as it is the most accurate and efficient way to understand molecular interactions. This also leads into another set of experiments to explore, which look into characterization of the aptamer itself and how it may change in conformation during binding. With this knowledge, further optimization of the surface can be explored if needed.
As the binding affinity experiments described in this manuscript produced rather disagreeable results, looking into other methods that can concretely determine the dissociation constant would be best. Isothermal titration calorimetry is a reliable method for deducing binding affinity and should be employed for best results.

With a properly optimized surface, the next steps for the biosensor would be to move into using biological samples. As the ultimate goal is to test real patient samples, this is an important caveat to overcome. Biological samples would include serum and whole blood, but are notorious for surface fouling due to the complexity of components the samples reserve. As such, there must be another stage of optimization as we look into incorporating antifouling materials and chemistries onto our surface. This will help reduce the non-specific adsorption and fouling that inevitable occurs during biological sample testing. Current members of the lab are also tackling this problem.

The work presented in this thesis is the first steps towards creating a biosensor that can detect for early stage ovarian cancer. As this project is still preliminary, many avenues for research discovery and exploration are possible and there is still a myriad of work that can still be performed. With ovarian cancer being the deadliest gynaecological cancer, it is imperative that the goal of this project come to light. We hope that with these first steps, we can move towards building a biosensor that can be used clinically for the screening of ovarian cancer and preventing a majority of the deaths associated.
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### Appendix 1. Summary of XPS data for immobilization of HSP10 on quartz surfaces at four take-off angles relative to the normal. (n=2)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Angle (°)</th>
<th>Relative atomic percentages (%)</th>
<th>C (285.03 eV)</th>
<th>Cl (204.75 eV)</th>
<th>F (696.7 eV)</th>
<th>N (401.25 eV)</th>
<th>Ni (872.84 eV)</th>
<th>O (532.67 eV)</th>
<th>Si (103.41 eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bare quartz</td>
<td>27.50</td>
<td>7.50 ± 0.55*</td>
<td>0.15 ± 0.05</td>
<td>0.06 ± 0.07</td>
<td>0.59 ± 0.32</td>
<td>0.16 ± 0.06</td>
<td>58.59 ± 0.91</td>
<td>32.96 ± 1.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.50</td>
<td>9.58 ± 1.63*</td>
<td>0.17 ± 0.02</td>
<td>0.24 ± 0.10</td>
<td>0.29 ± 0.04</td>
<td>0.15 ± 0.05</td>
<td>55.57 ± 1.59</td>
<td>34.02 ± 3.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.50</td>
<td>15.36 ± 2.53*</td>
<td>0.21 ± 0.08</td>
<td>0.12 ± 0.16</td>
<td>0.61 ± 0.02</td>
<td>0.26 ± 0.11</td>
<td>64.03 ± 0.71</td>
<td>19.42 ± 3.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.50</td>
<td>23.70 ± 3.08*</td>
<td>0.40 ± 0.07</td>
<td>0.49 ± 0.05</td>
<td>0.46 ± 0.65</td>
<td>0.47 ± 0.11</td>
<td>56.11 ± 0.62</td>
<td>18.38 ± 3.22</td>
<td></td>
</tr>
<tr>
<td>+ PFP + OTS</td>
<td>27.50</td>
<td>26.74 ± 2.97</td>
<td>0.23 ± 0.06</td>
<td>2.73 ± 0.12</td>
<td>0.31 ± 0.21</td>
<td>0.31 ± 0.04</td>
<td>46.93 ± 0.39</td>
<td>22.77 ± 2.24</td>
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</tr>
<tr>
<td></td>
<td>42.50</td>
<td>29.44 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>3.25 ± 0.64</td>
<td>0.33 ± 0.04</td>
<td>0.22 ± 0.01</td>
<td>43.55 ± 0.18</td>
<td>22.99 ± 0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.50</td>
<td>36.36 ± 1.27</td>
<td>0.55 ± 0.37</td>
<td>4.83 ± 0.49</td>
<td>0.38 ± 0.04</td>
<td>0.22 ± 0.10</td>
<td>45.77 ± 1.65</td>
<td>11.91 ± 0.42</td>
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<tr>
<td></td>
<td>72.50</td>
<td>35.91 ± 1.77</td>
<td>0.46 ± 0.41</td>
<td>7.95 ± 1.23</td>
<td>0.44 ± 0.04</td>
<td>0.48 ± 0.02</td>
<td>42.76 ± 3.68</td>
<td>12.01 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>+ PFP + OTS + NTA</td>
<td>27.50</td>
<td>31.42 ± 1.66</td>
<td>0.15 ± 0.19</td>
<td>0.40 ± 0.06</td>
<td>1.62 ± 0.18</td>
<td>0.18 ± 0.07</td>
<td>46.38 ± 1.10</td>
<td>19.86 ± 0.55</td>
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<tr>
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<td>57.50</td>
<td>39.22 ± 1.19</td>
<td>0.36 ± 0.06</td>
<td>0.18 ± 0.09</td>
<td>2.11 ± 0.16</td>
<td>0.16 ± 0.14</td>
<td>47.52 ± 1.57</td>
<td>10.48 ± 0.22</td>
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<tr>
<td></td>
<td>72.50</td>
<td>39.73 ± 0.79</td>
<td>0.71 ± 0.35</td>
<td>0.37 ± 0.52</td>
<td>2.96 ± 1.07</td>
<td>0.64 ± 0.22</td>
<td>44.61 ± 0.30</td>
<td>11.00 ± 0.52</td>
<td></td>
</tr>
<tr>
<td>+ PFP + OTS + NTA + Ni</td>
<td>27.50</td>
<td>33.22 ± 2.05</td>
<td>0.13 ± 0.06</td>
<td>0.44 ± 0.08</td>
<td>1.72 ± 0.09</td>
<td>1.07 ± 0.47</td>
<td>45.06 ± 2.00</td>
<td>18.38 ± 0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.50</td>
<td>35.08 ± 1.65</td>
<td>0.23 ± 0.04</td>
<td>0.38 ± 0.02</td>
<td>1.72 ± 0.25</td>
<td>1.12 ± 0.39</td>
<td>42.53 ± 1.69</td>
<td>18.97 ± 0.59</td>
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<td>42.20 ± 2.54</td>
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<td>0.42 ± 0.19</td>
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<td>44.33 ± 2.45</td>
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<td>0.68 ± 0.42</td>
<td>2.64 ± 0.38</td>
<td>1.96 ± 0.59</td>
<td>47.00 ± 0.45</td>
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<tr>
<td>+ PFP + OTS + NTA + Ni + HSP10</td>
<td>27.50</td>
<td>57.16 ± 1.37</td>
<td>0.30 ± 0.02</td>
<td>0.47 ± 0.00</td>
<td>14.76 ± 0.13</td>
<td>0.26 ± 0.06</td>
<td>22.45 ± 0.65</td>
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<td>0.35 ± 0.08</td>
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<td>15.43 ± 1.68</td>
<td>0.16 ± 0.13</td>
<td>20.14 ± 0.95</td>
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<td>0.54 ± 0.40</td>
<td>17.21 ± 0.81</td>
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<td>21.35 ± 0.38</td>
<td>1.00 ± 0.35</td>
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**Appendix 2.** Summary of XPS data for immobilization of tmMNC-38 on quartz surfaces at four take-off angles relative to the normal. (n=2)

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<tr>
<th>Sample</th>
<th>Angle (°)</th>
<th>C (285.03 eV)</th>
<th>Cl (204.75 eV)</th>
<th>F (696.7 eV)</th>
<th>N (401.25 eV)</th>
<th>O (532.67 eV)</th>
<th>P (130.20 eV)</th>
<th>S (163.90 eV)</th>
<th>Si (103.41 eV)</th>
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<td>8.40 ± 5.80*</td>
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<td>0.17 ± 0.03</td>
<td>0.07 ± 0.09</td>
<td>55.18 ± 2.80</td>
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<td>0.02 ± 0.03</td>
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<td>42.50</td>
<td>7.65 ± 4.34*</td>
<td>0.08 ± 0.04</td>
<td>0.16 ± 0.05</td>
<td>0.09 ± 0.03</td>
<td>62.25 ± 1.67</td>
<td>0.12 ± 0.04</td>
<td>0.00 ± 0.00</td>
<td>29.67 ± 2.60</td>
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<tr>
<td></td>
<td>57.50</td>
<td>8.72 ± 4.04*</td>
<td>0.14 ± 0.04</td>
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<td>0.09 ± 0.01</td>
<td>59.57 ± 1.48</td>
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<td>0.08 ± 0.11</td>
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<td>10.23 ± 3.54*</td>
<td>0.15 ± 0.05</td>
<td>0.15 ± 0.03</td>
<td>0.17 ± 0.01</td>
<td>58.08 ± 0.81</td>
<td>0.13 ± 0.01</td>
<td>0.03 ± 0.02</td>
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<td>0.12 ± 0.01</td>
<td>49.49 ± 0.96</td>
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<td>2.13 ± 0.26</td>
<td>0.12 ± 0.01</td>
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<td>0.09 ± 0.02</td>
<td>0.09 ± 0.12</td>
<td>25.98 ± 0.68</td>
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<td>57.50</td>
<td>25.30 ± 0.16</td>
<td>0.05 ± 0.02</td>
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<td>0.14 ± 0.01</td>
<td>3.74 ± 0.20</td>
<td>0.13 ± 0.06</td>
<td>38.65 ± 0.78</td>
<td>0.16 ± 0.01</td>
<td>0.02 ± 0.03</td>
<td>22.47 ± 0.06</td>
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<tr>
<td>+ PFP + OTS + tmMNC-38</td>
<td>27.50</td>
<td>22.72 ± 6.23</td>
<td>0.07 ± 0.00</td>
<td>1.78 ± 0.52</td>
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<td>30.02 ± 1.46</td>
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<td>0.37 ± 0.14</td>
<td>43.20 ± 0.14</td>
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