Development and Utilization of Shear Mode Acoustic Wave
Biosensors for the Detection of Ovarian Cancer

by

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Department of Chemistry
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Abstract

Recent proteome studies have discovered the presence of heat shock protein 10 (HSP-10) as an immunosuppressant in ovarian cancer patients. Due to the severity of ovarian cancer, the development of highly sensitive techniques for the early detection of this cancer is well in demand. In this manuscript, the thickness shear mode (TSM) acoustic wave biosensor will be used for the real-time and label-free detection of HSP-10 in buffer. The TSM sensitivity for HSP-10 is evaluated based on resonance frequency shifts generated by the biosensor. A nucleic acid aptamer, which is specifically engineered by *in vitro* selection to target HSP-10, is employed as the biosensing element of the biosensor. Alkylthiol-based self-assembling monolayers (SAMs), composed of various linker/diluent molar ratios, are used to immobilize the aptamer onto gold-coated piezoelectric quartz substrates. The TSM biosensing properties for avidin-biotin interactions are also evaluated in order to assess the biosensor response to HSP-10 protein-aptamer interaction.
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1. Introduction

1.1. Ovarian Cancer

1.1.1. Overview

Convenient methodologies that can be applied for clinical ailment diagnosis are in high demand. One such ailment is ovarian cancer, which is one of the most serious gynaecologic cancers responsible for death of approximately 1700 Canadians annually.\(^1\) Recent ovarian cancer proteome studies have discovered that Heat Shock Protein 10 (HSP-10) is being present at elevated levels in the circulatory system of ovarian cancer patients. Aside from its primary function as a co-chaperone in protein folding, it is believed that HSP-10 also functions as an immunosuppressant that allows the progression of ovarian cancer in afflicted patients.\(^2\) The ability to diagnose ovarian cancer on the basis of HSP-10 detection would provide the necessary means for an early and reliable screening technique.

1.1.2. Abnormal Function of HSP-10 in Ovarian Cancer

HSP-10 is part of the chaperonin protein family and has a molecular weight of approximately 10 kDa. It is encoded by a nuclear gene (GeneID, 3336; gene map locus, 2q33.1).\(^2\) In normal cells, HSP-10 is first translated inside the cytoplasm and then transported to the mitochondria where it eventually resides in the mitochondrial matrix. HSP-10 functions as a partner of HSP-60 forming a chaperonin complex (Figure 1), which aids in the process of protein folding. This chaperonin complex is believed to not only accelerate the process of polypeptide folding inside the mitochondrial matrix, but also to reactivate denatured proteins and prohibit the aggregation of non-native polypeptides.\(^2\)
Recent proteomic studies have identified the presence of HSP-10 as an early pregnancy factor (EPF) in the sera and ascites of ovarian cancer patients during the 1\textsuperscript{st} and 2\textsuperscript{nd} trimester of pregnancy. In these patients, HSP-10 was found to be produced by ovarian tumour cells and released to the cytoplasm, extracellular ascites and peripheral blood.\textsuperscript{3} Although the process of HSP-10 release from ovarian cancer cells is poorly understood, increased levels of HSP-10 have been correlated with reduced or diminished expression of CD3-zeta molecules. These molecules are T-cell receptor (TCR)-associated signal transducing molecules, which are significant to the normal immune response as they regulate the expression of T-cells and cytokines required to target the antigen. However, the presence of HSP-10 in the cytoplasm of cancer cells inhibits the signalling of the CD3-zeta molecules and thereby reduces the expression of T-cell receptor molecules (\(\zeta\) and \(\epsilon\)).\textsuperscript{3} As a result, the activation and proliferation of T-cells and subsequently the production of cytokines would be suppressed allowing the cancer to evade immune surveillance.

1.1.3. Other Abnormal Functions of HSP-10

Other proteomic and clinical studies have revealed additional abnormal activities of HSP-10 including the abnormal modulation of the cell cycle and apoptosis. Over-expression of HSP-10 was determined to inactivate proapoptotic Raf and extracellular signal-regulated...
kinase (ERK) (Figure 2). This, in turn, resulted in induced cell proliferation, differentiation and survival, all of which are key factors for tumour growth. Moreover, elevated expression of HSP-10 was found to increase the abundance of the anti-apoptotic molecules, Bad and Bcl-2. These molecules are responsible for reducing the levels of pro-apoptotic Bax proteins. Thus, this interference with the process of apoptosis and cell death resulted in uncontrolled cell proliferation. Due to this abnormal activity, HSP-10 was also characterized as a marker for other types of tumours such as colon cancer (Figure 3), adrenal cortex carcinoma and germ cell tumour of the testis.

Figure 2. Schematic representation of HSP-10 role in cell cycle and apoptosis: (A) HSP-10 interaction with Raf signalling cascade inactivating pro-apoptotic Raf and ERK and thus promoting cell survival. (B) HSP-10/HSP-60 interaction with Bax reducing the amount of pro-apoptotic Bax in the mitochondria and thus inhibiting apoptosis.

Figure 3. Electron microscope images illustrating the expression of HSP-10 in normal human colon (a) is lower than in colon cancer (b) and lymph node metastases of colon cancer (c). Red spots represent HSP-10.
1.2. Biosensors

1.2.1. Background and Overview

Over the last three decades, biosensors have gained increased attention as alternative analytical tools in many fields including environmental, clinical and pharmaceutical disciplines. The appeal of biosensors as monitoring tools in these diverse fields is due to the operational advantages that this technology provides. These include speed, ease-of-use, low cost, simplicity, portability, and ease of manufacturing. A biosensor can be defined as an analytical device that converts biochemical interactions, occurring on solid surfaces, into a measureable electrical signal in a real-time and label-free manner ideally. This process generally occurs through the action of three separate components. The first is a biosensing element, the second is a transducer and the third component is an output or data acquisition system (Figure 4).

![Figure 4](image)

**Figure 4.** Schematic representation of a biosensor featuring a biosensing element, a transducer and an output system.

The biosensing element, also known as the bio-receptor, is composed of biological molecules such as antibodies, cell receptors, enzymes or oligonucleotides. A good biosensing element is characterized by two important factors: 1) sensitivity to the analyte, which is the
level of change in state upon interaction with the analyte, and 2) selectivity to the analyte of interest in the sensing environment. During the bio-recognition event, the biosensing element interacts with the target analyte and produces physico-chemical stimuli that are converted into a measurable electrical signal via the transducer. There are various types of transducers to choose from depending on the nature of the stimulus generated during the bio-recognition phase (i.e. thermal, electrochemical, optical or mass-related). Finally, the output system amplifies, stores and displays the electrical signal into an appropriate format.

1.2.2. Piezoelectric Effect

At the end of the 19th century, the discovery of the piezoelectric effect marked the start of a new scientific era in the world of materials science.\textsuperscript{8-10} The piezoelectric effect is the ability of some materials to undergo a charge polarization upon application of a mechanical strain, where a strain is defined as the deformation associated with an applied stress (i.e. force applied per unit area).\textsuperscript{11} The mechanical strain causes an asymmetrical electrical charge separation across the crystal lattice, producing an electrical potential. The charge polarization and electrical potential is proportional to the strain and changing direction with it. The piezoelectric effect occurs only in crystalline materials that lack a center of symmetry.\textsuperscript{12}

1.2.3. Bulk Acoustic Wave Biosensors

In 1959, the piezoelectric effect was utilized in the development of the first bulk acoustic wave (BAW) device when G. Z. Sauerbrey introduced what is now known as the quartz crystal microbalance (QCM).\textsuperscript{13,14} This sensor was based on the correlation of changes in the amount of deposited foreign material to changes in the acoustic resonance frequency of a piezoelectric quartz disc operating in the gas phase. Although it was originally developed to explain frequency shifts by thin-film deposition in the gas phase, Sauerbrey’s model was further
adapted to explain frequency shifts in liquid media by accounting for liquid density and viscosity.\textsuperscript{14} From that point forward, profound development in piezoelectric bulk acoustic wave devices have been made.

An acoustic wave biosensor relies on the mechanical stress induced by a biochemical interaction at the biosensor interface to generate a measureable electrical signal.\textsuperscript{15} The principle behind piezoelectric acoustic wave biosensors involves the generation of an acoustic shear wave by applying an AC potential across the thickness of the piezoelectric material, quartz for example. The application of the AC potential forces the displacement of the electropositive and electronegative atoms resulting in charge separation (\textbf{Figure 5}). The displaced particles return to their original position with the aid of the restoring force from this charge separation.\textsuperscript{15,16} The resulting displacement acoustic wave will propagate vertically until it reflects at a boundary. These acoustic waves are termed transverse waves since particle displacement is perpendicular to the direction of propagation.

\textbf{Figure 5}. Schematic illustration of lateral particle displacement (in the x-direction) and the resulting perpendicular standing shear wave (in the y-direction) through the cross-section of a piezoelectric material.\textsuperscript{17}

At a specific AC potential, the shear wave within the piezoelectric material will give rise to a characteristic resonance frequency. This resonance frequency depends mainly on the thickness and the lattice structure of the piezoelectric material.\textsuperscript{16} The frequency of the wave is
proportional to the reciprocal of the wavelength of the resonance wave and is related to the corresponding thickness of the substrate as shown in Equation 1:

\[ f_s = \frac{v}{\lambda} = \frac{v}{2t_q} \]  

(1)

where \( v \) is the speed of the shear wave, \( \lambda \) is the wavelength and \( t_q \) is the quartz crystal thickness.\(^{18}\)

Material deposition at the biosensor surface will not only exert a mechanical stress on the surface, but also change the thickness of the shear wave propagation path. As a result, this will be transduced by a decrease in the resonance frequency proportional to the amount of the adsorbed material. This property led to the formulation of the Sauerbrey equation (Equation 2):

\[ \Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}} \]  

(2)

where \( \Delta f \) is the shift in resonance frequency, \( f_0 \) is the fundamental frequency of the quartz crystal (9.0 MHz), \( \Delta m \) is the change in adsorbed mass, \( \mu_q \) and \( \rho_q \) are the viscosity and density of quartz respectively, and \( A \) is the surface area of the quartz crystal (0.28 cm\(^2\)).\(^{14}\) Bulk acoustic wave biosensors mainly rely on the piezoelectric AT-cut quartz crystals as transducing platforms since they vibrate at resonance frequencies that are stable over a wide range of temperatures.\(^ {19}\)
1.2.4. **Thickness Shear Mode (TSM) Acoustic Wave Sensor**

The thickness shear mode (TSM) sensor, also known as the quartz crystal microbalance (QCM), is an on-line monitoring device that offers sensitive, label-free and real-time detection of biochemical interactions. It is used in the characterization of bulk and interfacial properties at the sensor-solid and sensor-liquid junctions respectively. It is based on a thin, AT-cut quartz disc transducer sandwiched between two gold metal electrodes (Figure 6). The TSM relies on the exploitation of the piezoelectric effect for sensing and is thus based on the propagation of bulk acoustic shear waves through a quartz substrate (Figure 7). As quartz is a piezoelectric material, a corresponding transverse shear acoustic wave can be generated across the thickness of the disc when an oscillating electrical potential is applied across the surface-attached gold electrodes.

![Figure 6](image1.png)

**Figure 6.** Schematic of the top view of a TSM quartz disc with metal electrodes and electrical contacts. The smaller figure shows the wave motion and the direction of particle displacement.

![Figure 7](image2.png)

**Figure 7.** Cross section through a TSM quartz disc illustrating the thickness shear displacement of the top surface relative to the bottom and the resulting standing transverse shear wave propagating in the y-direction.
The addition of a rigid bio-layer onto the sensor surface increases both the apparent thickness of the sensor surface and the mechanical strain on the resonance vibration. This leads to elongation of the effective wavelength and a decrease in the resonance frequency of the transverse acoustic wave (Figure 8). Thus, the extent of bio-molecular adsorption onto the sensor’s surface can be correlated to changes in the resonance frequency, measured in Hertz, monitored over time.

Figure 8. Schematic representation of the change in acoustic wave wavelength extension without an adsorbed biolayer (a) and with an adsorbed biolayer (b), through a cross section of a TSM quartz disc.

When the TSM is operated with one side of the quartz disc exposed to liquid as opposed to air, a component of the standing wave energy is not reflected at the solid/liquid interface and is instead dissipated into the liquid. This is due to the liquid’s lack of support for shear motion. Under this condition, liquid parameters such as viscosity (\(\eta_L\)) and density (\(\rho_L\)) influence the TSM sensor and are measured as a decrease in resonance frequency and an increase in dissipation. The operation of the TSM in air and liquid are compared schematically in Figure 9. Dissipation of acoustic energy (viscous damping force) is usually measured as a change in motional resistance, measured in Ohms. This variable is related to the energy dissipation in the equivalent electrical circuit of the resonator device (Figure 10).
The TSM response is generated by changes in the impedance of the applied voltage and the current flowing through the crystal. The network analyzer method is employed to fully characterize the sensor response through the computation of the magnitude and phase of impedance for each frequency point. This method stems from an equivalent circuit model (Figure 10), which consists of inductive, capacitive and resistive components.

The TSM instrument consists of three main operational components, (1) a flow through cell holder that holds the 9-MHz piezoelectric quartz disc, (2) a network analyzer that functions as a resonator and collects the changes in resonance frequency and motional resistance, and (3) a computer that collects and monitors all the data. The cell holder consists of two Plexiglas blocks clamped together. Each block consists of an input and an output tube. The input tube is immersed in the sample solution and the output tube is connected to a mechanical syringe.
pump. The gold electrodes on the disc are aligned with the gold leads in the cell holder to complete the electrical circuit. The disc is held in place with the aid of two O-rings. The overall configuration of the TSM is illustrated below in Figure 11.

Figure 11. (A) Schematic of a flow through cell cross-section consisting of the two Plexiglas blocks (1 and 2) clamped together. (B) Picture of a flow through cell consisting of two Plexiglas blocks clamped together.
1.3. Biosensing Element

1.3.1. Overview

When developing a biosensor for the detection of a target of interest, a biosensing element that is specific to the target must be attached to the biosensor surface for selective and sensitive detection. In order to obtain optimal sensitivity and selectivity, the biosensing element must meet several criteria. First, it must have a high binding affinity towards the target of interest. Secondly, the biosensing element should be easily immobilized onto the biosensor surface with a proper orientation and with minimal structural modification to avoid altering its binding affinity for the target. Thirdly, the biosensing element is preferred to be small in size with minimal steric hindering features to produce a densely packed biosensing layer for maximum sensitivity and limit-of-detection. Lastly, the biosensing element should be reversible upon target binding to allow the biosensor to be re-used multiple times so that it is cost-effective. In this study, a specific DNA aptamer produced by an in vitro selection method against HSP-10 was utilized as the biosensing element to target and bind HSP-10.

1.3.2. Nucleic Acid Aptamers as Biosensing Elements

Aptamers are single-stranded DNA or RNA oligonucleotides that are selected from large combinatorial libraries by the process of systematic evolution of ligands exponential enrichment (SELEX).\(^{25,26}\) Aptamers can be selected to bind to a variety of targets including but not limited to proteins, hormones, drugs, ions and organic and inorganic molecules. Nucleic acid aptamers are more attractive than immunological antibodies as bio-receptors in biosensor applications. This is because aptamers have been suggested to bind targets with higher binding affinity through their three-dimensional structure undergoing conformational changes during the binding event.\(^{26}\) Moreover, since aptamers are small than antibodies, this allows them to
form denser bio-receptor layers that results in increased biosensor sensitivity.\textsuperscript{27} Aptamers are also more attractive than antibodies since they can be reversibly regenerated by simple shifts in pH and temperature gradients without loss of function or denaturation.

\textit{In vitro} synthesis of aptamers provides two advantages over the \textit{in vivo} production of antibodies. The first is the ability of aptamers to target toxic and non-immunogenic molecules unlike antibodies, which do not possess this ability.\textsuperscript{27} The other advantage is that aptamers can be easily modified during their synthesis to incorporate reactive groups such as alkyl amines or thiols at their terminal ends. This is important when attempting to anchor the aptamer onto the biosensor surface in a well-ordered and uniform orientation. When compared to aptamers, the immobilization of properly oriented antibodies onto surfaces is very difficult as it requires major structural modifications that may alter function and binding affinity of the antibody. Modification of amino and carboxylic groups has proven to be unreliable for the uniform and oriented immobilization of antibodies onto biosensor surfaces since these groups are present over the entire antibody.\textsuperscript{28} An alternative way to immobilize antibodies in a well-orientated manner is to generate thiol groups at the hinge region of the antibody by cleaving the F\textsubscript{c} region and reducing the sulfur atoms forming the disulfide bridges.\textsuperscript{29,30} This, of course, is a very complicated procedure and may affect the binding affinity of the modified antibody. In light if the advantages of nucleic acid aptamers over immunological antibodies, an anti-HSP-10 aptamer was selected and incorporated at the TSM surface as the biosensing element for the HSP-10 target.
1.4. Self-Assembling Monolayers (SAMs)

1.4.1. Background and Theory

In 1946, W. A. Zisman pioneered the first self-assembling monolayer (SAM) during his research on the formation of hydrophobic monolayer films onto clean metal surfaces.\textsuperscript{31} However, it was not until the 1980s with the introduction of alkylthiol and alkyltrichlorosilane linkers that the interest for SAM chemistry escalated, resulting in greater linker selection and in-depth characterization.\textsuperscript{32-35} Since then, SAM chemistry has been reported on numerous occasions to be one of the most reliable and most rapid chemical techniques for the formation of uniform, stable and functionalizable organic surfaces.\textsuperscript{35-38} In the field of biosensors, SAMs are of great interest for the construction of biosensing elements since they offer the opportunity to immobilize bio-molecules through their reactive and customizable head functions.

SAMs chemistry utilizes bi-functional linkers that are engineered to spontaneously form ordered molecular assemblies onto solid inorganic substrates. These linkers are composed of three distinct parts fundamentally:

1) a highly reactive tail function (Cl\textsubscript{3}Si- or HS-) to covalently anchor the linker onto the underlying inorganic substrate;

2) a functional reactive head group to subsequently immobilize bio-molecules onto the SAM;

3) an organic chain (\textit{i.e.} hydrocarbon or polyethylene glycol chain) to distance the inorganic substrate from the head group function and to provide stability within the assembly through intermolecular interactions between adjacent molecules.

Recent research has been successful in creating densely packed and highly ordered SAMs with functional tail groups that adsorb to a variety of solid surfaces, and functional head groups to
immobilize a variety of bio-molecules.\textsuperscript{39} Furthermore, SAM linker molecules can be prepared by simple synthetic methods at a very low cost and their properties can be easily tuned through linker modification for a variety of applications.

Most SAMs are known to form spontaneously by simple contact between the solution of linker molecules and the inorganic substrate. The packing density, homogeneity and stability of the resulting SAMs are determined by several concerted factors. The primary factor is the interaction between the tail groups of linker molecules and the reactive/exposed atoms of the solid substrate as this defines the distribution of anchoring sites, and thus the density of the SAM.\textsuperscript{40} Substrate surface features, such as surface roughness and morphology, distribution of reactive atoms and surface contamination, also influence interaction between the linker and the substrate. In addition to substrate features, the linker molecules themselves affect the SAM characteristics. For instance, the length of the hydrocarbon chain not only influences the orientation of the linker molecules during the SAM formation, but also the level of intermolecular interactions (i.e. van der Waals and dispersion forces) between neighbouring chains.\textsuperscript{41-44} These intermolecular interactions provide extra stability to the resulting SAM.\textsuperscript{40,45} For example, longer alkyl chains have been shown to form more stable and closely-packed SAMs than shorter chains. Lastly, the steric factors associated with the size of the linker’s head function also influence the packing density of the SAM. Large head functions create steric congestion in the upper region of the assembled SAM. The consequences of steric congestion are reduced packing density and increased angular tilting of the SAM, which infringes on the orientation and functionality of the SAM. Therefore, the packing density and stability of SAMs are determined by both the chemical and physical properties of the molecules that form them, as well as the underlying substrate.
In the field of analytical biosensors, the SAM's packing-density, stability and ability to bind and anchor bio-molecules dictates the quality of the biosensing element, and consequently the performance of the biosensor. As a result, the success of the SAM in immobilizing bio-molecules for biosensor fabrication is dependent on the following two requirements:

1) the SAM must not be altered during the biosensing element immobilization process;
2) the immobilized bio-molecule must retain its biological activity.

In order to avoid SAM alteration during the biosensing element immobilization process, the linker molecules should pack tightly and uniformly during SAM formation reaction so that linker rearrangement, upon introduction and contact with bio-molecules or solvent molecules, is avoided.\(^{42}\) SAM stability depends on the aforementioned factors that control SAM formation. The loss of biological activity upon the immobilization of bio-molecules to SAMs can arise from a variety of factors such as a change in surface or solvent properties,\(^{46-48}\) a change in the molecular conformation upon covalent binding with the SAM’s head function,\(^{49}\) or a drastic change in the molecular micro-environment due to interactions with the underlying surface.
1.4.2. Alkylthiol-based SAMs

Alkylthiol linkers exploit their thiol tail functions to anchor and form SAMs on gold substrates via a gold-thiolate covalent bond.\textsuperscript{50} Formation of alkylthiol SAMs on gold occurs spontaneously when the gold substrate is brought in contact with the alkylthiol linker molecules.\textsuperscript{40} The mechanism of SAM formation starts with the rapid chemisorption of alkylthiol molecules on gold through an oxidative addition of the S-H bond to the gold. This is followed by a reductive elimination of hydrogen and formation of alkylthiolate ions (Equation 3). After chemisorptions, the adsorbed molecules slowly begin re-align and re-organize at a much slower rate lasting several hours to form a well-packed SAM.

\[
\begin{align*}
\text{RSH} + \text{Au (0)} & \rightarrow \text{R} \delta^{+} \text{S} \rightarrow \text{R} \delta^{-} \text{Au} + \frac{1}{2} \text{H}_2 \\
\end{align*}
\]

Attachment of bio-molecules onto the SAM-coated gold substrate occurs through covalent binding between bio-molecules and the SAMs’ head functions. Traditionally, alkylthiol-based SAMs prepared from alkylthiol linkers bear a carboxylic acid head function. This is due to the fact that the thiolated tails of these linkers are very strong nucleophiles. Thus, having a weak electrophile such as carboxylic acid as the head function prevents the premature polymerization of linker molecules in solution. Although, carboxylic acid head functions prevent pre-mature polymerization, they require a pre-activation reaction prior to the immobilization of bio-molecules. The common carboxylic acid pre-activation reaction relies on the well-established \textit{N}-ethyl-\textit{N’}-(3-dimethylamino-propyl)-carbodiimide/\textit{N}-hydroxy-succinimide (EDC/NHS) coupling chemistry.\textsuperscript{51-53} Pre-activation begins with the EDC conversion of carboxylic acid groups (COOH) to unstable \textit{O}-acylisourea intermediates. These intermediates can react with amine- or thiol-terminated bio-molecules to form stable covalent
bonds. However, the O-acylisourea intermediates are highly susceptible to hydrolysis and thus limit the yield of the overall reaction.\textsuperscript{54,55} Subsequent coupling with NHS molecules results in the formation of a hydrolysis-resistant intermediate and a more reactive NHS ester head function.\textsuperscript{52,53} The latter can then directly react with the nucleophilic terminal of bio-molecules and thus form strong and stable covalent bonds.

\subsubsection*{1.4.3. Alkyltrichlorosilane-based SAMs}

This category of SAMs exploits alkyltrichlorosilane linkers for the formation of SAMs on hydroxylated surfaces.\textsuperscript{37,38} Their assembly is based on the covalent binding between the silicon atom of the trichlorosilane tail, and the oxygen atoms on the hydroxylated surface. The formation of trichlorosilane-based SAMs onto hydroxylated surfaces (also called silanization) is commonly carried out in dilute organic solutions. The mechanism of trichlorosilane-based SAM formation is a multi-step mechanism.\textsuperscript{56} It starts with the hydrolysis of the trichlorosilyl tail functions (Si-Cl\textsubscript{3}) into trisilanol species (Si-(OH)\textsubscript{3}) as shown below in Equation 4, where R represents the rest of the linker molecule.\textsuperscript{57} This process is facilitated by the presence of water on the hydroxylated surface, but not in solution to avoid pre-mature polymerization of linker molecules in solution.

\begin{equation}
RSiCl\textsubscript{3} + 3 H\textsubscript{2}O \rightarrow RSi(OH)\textsubscript{3} + 3 HCl
\end{equation}

Once hydrolyzed, the trisilanol groups undergo a reversible adsorption/desorption process at the surface-solution interface during which they condense with the surface hydroxyl groups to form surface-bound silanols.\textsuperscript{56} This condensation process is predicted to be biphasic. The first phase of this condensation process is a rapid nucleation phase that results in the formation of SAM nuclei. The second phase is a much slower nucleation and re-organization phase where linker molecules nucleate and re-organize to achieve a minimal free energy state.
Moreover, cross-linkage between neighbouring silanols is also believed to occur during SAM formation, further increases the stability of the assembly.\textsuperscript{57}

\subsection*{1.4.4. Mixed Linker/Diluent SAMs}

Pure SAMs are known to pack-tightly and form crowded assemblies. These crowded assemblies have been proposed to affect the ability of SAMs to immobilize bio-molecules due to the inherent steric congestion occurring around neighbouring head functions.\textsuperscript{58} This congestion hinders the reactive centre of the head function and prevents it from reacting with the bio-molecule of interest. To overcome this problem, a strategy involving the use of "mixed SAMs" has been proposed.\textsuperscript{39} Mixed SAMs incorporate shorter uni-functional molecules (\textit{diluent molecules}) that space-out adjacent linker molecules within the assembly. This reduces steric congestion around the head functions of SAMs and increases their ability to immobilize larger bio-molecules.
1.5. Surface Characterization Techniques

In the process of biosensor fabrication, the surface properties of the biosensing element are very significant. It is important to analyze the physical and chemical properties of the biosensor substrate before and after SAMs formation, and after the biosensing element immobilization in order to achieve the optimal biosensor design. For this purpose, a combination of different surface characterization techniques can be used to assess the physical and chemical surface properties. The choice of these techniques is determined by several factors including the type of information wanted, the compatibility of the surface with the instrument, the availability of the equipment, and the cost associated with the analysis. In this project, Contact Angle Measurements (CAM) and X-ray Photoelectron Spectroscopy (XPS) were used for surface characterization. These techniques were utilized to quantitatively and qualitatively characterize the biosensor’s surface after the substrate cleaning protocol, coating with different SAMs, and after the biosensing element immobilization.

1.5.1. Contact Angle Measurement (CAM)

Contact angle measurement (CAM) is a simple qualitative and quantitative analysis technique. This technique is based on the measurement of the angle between a horizontal surface and a line tangent to a liquid droplet deposited on the surface. The measured angle provides quantitative and qualitative information about surface wettability and surface hydrophobicity/hydrophilicity. Depending on the polarity characteristics of the surface and the liquid, the degree of droplet spreading on the surface will vary. If the surface and liquid have similar polarity, the droplet will spread forming a flat film over the surface. However, if the surface and liquid have opposite polarity, the droplet will form a sphere with a finite angle (θ) in order to minimize its surface energy (Figure 12).
Figure 12. Schematic representation of the contact angle ($\theta$) at the solid-liquid interface.

When water is used as the test liquid, clean inorganic surfaces such as quartz or gold will have a contact angle close to zero because they are highly hydrophilic. However, the contact angle increases on surfaces that are immobilized with hydrophobic molecules (Figure 13). This is due to the hydrophobic effect of the surface on the water molecules, which forces them to cluster together and form a capped sphere. CAM does not accurately reflect the concentration of materials deposited on the surface. Thus, this technique is only used as a preliminary qualitative tool for assessing the hydrophobicity/hydrophilicity of molecules immobilized on surfaces.

![Figure 13. Schematic representation of contact angle measurements with a droplet of water on a clean surface (a) and increasingly hydrophobic surfaces (b to d).](image_url)
1.5.2. X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS) is a powerful surface characterization technique that provides both qualitative and quantitative information regarding surface elemental composition, down to a depth of 10 nm.\textsuperscript{59-61} It is fundamentally based on the photoelectric effect; the ejection of electrons from a surface upon photon bombardment. In XPS, the surface is irradiated with a monochromatic source of X-ray photons (commonly in the range of 1000-2000 eV) that excite the inner shell electrons of the atoms composing the surface of interest.\textsuperscript{59} As these electrons are excited, they migrate in different directions. Some collide with other atoms within the surface and lose their kinetic energy to give rise to ‘background noise’. Other electrons that are close to the surface (top 3 or 4 layers) escape to the surface exterior and reach the detector with a specific kinetic energy. The detector measures the kinetic energies of the electrons, and then counts the number of electrons associated with a particular kinetic energy. The atomic composition of the material will therefore be obtained by determining the binding energies of the ejected electrons. The binding energy is given by the following equation (Equation 5):

\[ E_{binding} = E_{photon} - (E_{kinetic} + \lambda) \]  

(5)

where \( E_{binding} \) is the binding energy of the atomic orbital from which the electron was ejected, \( E_{photon} \) is the energy of the exciting X-ray photons, \( E_{kinetic} \) is the measured kinetic energy of the electron, and \( \lambda \) is the spectrometer work function. A schematic of an XPS instrument is illustrated in the next page (Figure 14).
In practice, XPS analysis begins by recording a survey spectrum over a region (usually 0-1000 eV) that will provide fairly strong peaks for all the elements present in the sample (except hydrogen and helium). The survey spectrum is then followed by the acquisition of regional spectra around the elemental peaks of interest. Through peak fitting, the conversion of the peak intensities/areas to atomic concentration, the elemental composition of the sample surface can be quantified. For finer analysis of close and/or overlapping peaks and additional information about the chemical environment of the analyzed elements, high-resolution XPS can be performed.
1.6. Research Project

1.6.1. Research Objectives

In light of the need for reliable, real-time, label-free and highly specific diagnostic devices for early cancer screening, the present manuscript describes the preliminary ground work towards the application of acoustic wave biosensors in ovarian cancer detection. The proposed research is intended to utilize the TSM technology as an on-line monitoring tool for the detection of HSP-10 using an anti-HSP-10 DNA aptamer as biosensing element. In this project, the well-known avidin-biotin interaction will be studied in order to characterize the TSM response, and as a reference for the HSP-10 protein-aptamer interaction. Our choice for this system was motivated by the fact that avidin has very high affinity for biotin and that this interaction is actually the strongest known non-covalent biological interaction (Kd ~ 10^{-15} M). Our research objectives are listed below in sequential order:

1) functionalize the TSM gold-coated quartz substrate with alkylthiol-based SAMs;
2) characterize SAMs formation through surface analysis techniques: CAM and XPS;
3) evaluate the immobilization ability of our SAMs using a novel molecular probe;
4) immobilize the biosensing elements; biotinthiol and anti-HSP-10 aptamer;
5) evaluate the TSM biosensing properties for the avidin-biotin interaction in buffer;
6) utilize the TSM to detect the presence of HSP-10 in buffer media.

1.6.2. Research Strategy and Execution

The early stages of our research will focus on the formation of the SAMs onto the TSM biosensor surface. Two commercially available alkylthiol molecules will be utilized for the formation of mixed and pure SAMs. The linker is 11-mercaptoundecanoic acid (11-MUA), and the diluent is 1-butanethiol (Figure 15). SAMs of different linker to diluent ratios will be
investigated in order to achieve the maximum density of immobilized biosensing element, and therefore maximal biosensor response. Once the SAMs are formed onto the TSM gold substrates, the SAM-coated TSM surfaces will be characterized via CAM and XPS. After characterization, we will investigate the immobilization ability of the SAMs’ head functions using a novel Mac F5 test probe (Figure 16).

![11-MUA and 1-Butanethiol](image)

**Figure 15.** Chemical structures of 11-MUA (linker) and 1-butanethiol (diluent).

![Mac F5 probe](image)

**Figure 16.** Chemical structure of the Mac F5 probe.

We will then turn our attention towards immobilizing biotin-thiol and hexylthiol-modified anti-HSP-10 aptamer onto the different SAM-coated TSM surfaces. After that, we will characterize the different surfaces with XPS to determine the success of bio-receptor immobilization, as well as the optimal SAM for maximum bio-receptor density. Next, we will characterize the TSM response for avidin-biotin interaction, in terms of specific and non-specific adsorption of avidin on biotinylated and non-biotinylated SAM-coated surfaces, respectively. Finally, we will utilize the TSM for the detection of HSP-10 in pure buffer over time. Similarly, we will evaluate the TSM response for HSP-10 in terms of its specific and non-specific adsorption on aptamer-functionalized and un-functionalized SAM-coated surfaces, respectively. The HSP-10 protein-aptamer and avidin-biotin interactions will be compared to evaluate the TSM biosensing properties for HSP-10.
2. Experimental

2.1. General Remarks

Anhydrous (toluene and acetone) and spectrograde (MeOH) solvents were systematically used. Freshly distilled (from CaH₂, under high vacuum) anhydrous DMF was used for Mac F5 probe. Avidin (from egg white, lyophilized powder), Dulbecco’s phosphate buffered saline (PBS), EDC, NHS, 11-MUA and 1-butanethiol were purchased from Sigma-Aldrich®. 11-MUA was recrystallized prior to use. Biotin-thiol and the Mac F5 probe, which was used as a test probe, were synthesized in our laboratory by Dr. Christophe Blaszykowski. Hexylthiol-modified DNA aptamer oligonucleotide (nucleic acid sequence: 5'- /5ThioMC6-D/ GTC TTG ACT AGT TAC GCC ACC TTG TGC GGG GTG GTG GGG ATG GAT GTT GCT TGA GGG GTC TCA TTC AGT TGG AGA CTC-3') was synthesized by Integrated DNA Technologies® and modified in Dr. Yingfu Li’s Laboratory at McMasters University. HSP-10 was purchased from Stessgen Bioreagents Corporation®. TSM quartz crystals (AT-cut, with gold electrodes, 13.5 mm in diameter, 9.0 MHz fundamental frequency) were purchased from Lap-Tech Inc.® Formation of SAMs onto the TSM gold surface was carried out in open air, while the immobilization reactions of the probe, biotin-thiol and aptamer were prepared in the glove box maintained under an inert (N₂) and anhydrous (P₂O₅) atmosphere. The crystals were handled with thoroughly pre-cleaned stainless steel tweezers in order to minimize any external contamination. ¹H and ¹³C NMR spectra were recorded on Varian 300 and 400 MHz spectrometers using CDCl₃ as the NMR solvent. ¹H and ¹³C NMR spectra are referenced to the residual solvent peak (CDCl₃: 7.27 ppm and 77.23 ppm, respectively). The abbreviations s, d, t, q, dd and m stand for singlet, doublet, triplet, quadruplet, doublet of doublet and multiplet, respectively. Broad signals are denoted “br”.
2.2. Chemical Synthesis

2.2.1. Biotinthiol Synthesis

The synthesis of biotinthiol 6 was achieved in five steps from commercially available biotin 1 with a 33% overall yield (Scheme 1). Each of the intermediaries has already been reported in the literature in different publications.\textsuperscript{64-66} Our procedure therefore simply consisted of a combination of these previously described syntheses.

![Scheme 1. Biotinthiol synthesis.](image)

**Biotin methyl ester 2.**\textsuperscript{64} To a stirred solution of biotin 1 (900 mg, 3.65 mmol, 1.0 equiv.) in absolute EtOH (30 mL) were added few drops of concentrated H\textsubscript{2}SO\textsubscript{4} at room temperature. After stirring at room temperature overnight, the reaction was submitted to a CH\textsubscript{2}Cl\textsubscript{2}/Na\textsubscript{2}CO\textsubscript{3}-aqueous solution extraction. The combined organic layers were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, filtered then evaporated under reduced pressure to provide 961 mg (97%)
of ester 2 as a white solid. Spectroscopic data were consistent with those reported in the literature.\textsuperscript{64} 1H NMR (400 MHz, CDCl$_3$) δ 5.52 (brs, 1H), 5.15 (brs, 1H), 4.52 (m, 1H), 4.32 (m, 1H), 4.13 (q, $J = 7.23$ Hz, 2H), 3.17 (m, 1H), 2.93 (dd, $J = 12.8$, 4.8 Hz, 1H), 2.75 (d, $J = 12.8$ Hz, 1H), 2.33 (t, $J = 7.6$ Hz, 2H), 1.69 (m, 4H), 1.46 (m, 2H), 1.26 (t, $J = 7.2$ Hz, 3H).

**Biotinol 3.**\textsuperscript{64} To a stirred solution of biotin methyl ester 2 (961 mg, 3.53 mmol, 1.0 equiv.) in CH$_2$Cl$_2$ (10 mL) was added drop-wise DIBAL-H (1.0 M in hexanes, 12.4 mL, 12.4 mmol, 3.5 equiv.) at -78°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2 hours. The reaction was then carefully quenched, at -78°C, by drop-wise addition of MeOH then H$_2$O. After evaporation of the solvents under reduced pressure, the purification was achieved by Soxhlett extraction (EtOH) and provided 796 mg (98%) of biotinol 3 as a white solid; 1H NMR (400 MHz, CD$_3$OD) δ 4.49 (dd, $J = 7.8$, 4.8 Hz, 1H), 4.30 (dd, $J = 7.8$, 4.8 Hz, 1H), 3.55 (t, $J = 6.6$ Hz, 2H), 3.21 (m, 1H), 2.93 (dd, $J = 12.6$, 4.8 Hz, 1H), 2.71 (d, $J = 12.6$ Hz, 1H), 2.16 (s, 1H), 1.74 (m, 1H), 1.57 (m, 3H), 1.45 (m, 4H).

**Biotin tosylate**\textsuperscript{65} \textsuperscript{4} and **biotin thiocetate 5.** To a stirred solution of biotinol 3 (796 mg, 3.46 mmol, 1.0 equiv.) in pyridine (20 mL) was added tosyl chloride (1.75 g, 9.09 mmol, 2.6 equiv.) at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 2 hours. The reaction was then submitted to a CH$_2$Cl$_2$/1 M H$_2$SO$_4$ aqueous solution extraction. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, filtered then evaporated under reduced pressure. The residue (crude 4) was rapidly purified by column chromatography on silica gel (EtOAc/MeOH gradient) to provide 697 mg of an off-white solid. The latter was immediately dissolved in anhydrous MeCN (30 mL) then anhydrous NaI (2.65 g, 17.7 mmol, 5.1 equiv. (relative to 3)) and KSAc (2.06 g, 17.7 mmol, 5.1 equiv.) were successively added at room temperature. The reaction was refluxed overnight then submitted to a CH$_2$Cl$_2$/H$_2$O extraction. The combined organic layers were washed with brine, dried over
anhydrous Na$_2$SO$_4$, filtered then evaporated under reduced pressure. Purification was achieved by column chromatography on silica gel (CH$_2$Cl$_2$/MeOH gradient) and provided 417 mg (42%, 2 steps) of biotin thioacetate 5 as a beige solid. Spectroscopic data were consistent with those reported in the literature.$^{66}$ $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.09 (brs, 1H), 4.84 (brs, 1H), 4.52 (m, 1H), 4.33 (m, 1H), 3.16 (m, 1H), 2.94 (dd, $J = 12.8, 5.2$ Hz, 1H), 2.87 (t, $J = 7.4$ Hz, 2H), 2.75 (d, $J = 12.8$ Hz, 1H), 2.34 (s, 3H), 1.64 (m, 4H), 1.43 (m, 4H).

Biotinthiol 6.$^{66}$ To a stirred solution of biotin thioacetate 5 (410 mg, 1.42 mmol, 1.0 equiv.) in THF (40 mL) was added LAH (95%, 454 mg, 11.36 mmol, 8.0 equiv.) in small portions at 0°C. After addition, the reaction was allowed to warm to room temperature then stirred for 1 hour. The reaction was diluted with EtOAc then carefully quenched with a 1 M HCl aqueous solution. The resulting aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na$_2$SO$_4$, filtered then evaporated under reduced pressure. Purification was achieved by column chromatography on silica gel (EtOAc/MeOH gradient) and provided 291 mg (83%) of biotinthiol 6 as a white solid. Spectroscopic data were consistent with those reported in the literature.$^{66}$ $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 5.15 (brs, 1H), 4.98 (brs, 1H), 4.53 (m, 1H), 4.33 (m, 1H), 3.17 (m, 1H), 2.94 (dd, $J = 12.8, 5.2$ Hz, 1H), 2.75 (d, $J = 12.8$ Hz, 1H), 2.54 (q, $J = 7.3$ Hz, 2H), 1.66 (m, 4H), 1.45 (m, 4H), 1.36 (t, $J = 7.3$ Hz, 1H).
2.2.2. Mac F5 Probe Synthesis

The synthesis of Mac F5 probe 9 was achieved in three steps from commercially available pentafluorophenyl hydrazine 7 with an overall yield of 56% (Scheme 2). This procedure was adapted from a previously described synthesis in the literature.\(^{67}\)

![Scheme 2. Mac F5 probe synthesis.](image)

**N-Pentafluorophenyl-N' -iodoacetyl hydrazide 8 (Iac F5).**\(^ {67}\) To a stirred solution of iodoacetic acid (1.86 g, 10 mmol, 1 equiv.) in dry acetonitrile (20 mL) were added DCC (2.06 g, 10 mmol, 1 equiv.) and pentafluorophenyl hydrazine 7 (1.98 g, 10 mmol, 1 equiv.) at room temperature. After 1 hour, the solution was then filtered using a Buchner funnel to remove the white precipitate, which was rinsed with acetonitrile. The liquid was concentrated using a rotary evaporator, and the residue was filtered through a short column of silica (5 cm height, 3 cm diameter) using 10% MeOH in CH\(_2\)Cl\(_2\). The brown solution was concentrated with the resulting solid being recrystallized twice from chloroform to yield 2.50 g (68%) of 8 as shiny white flaky crystals. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.94 (brs, 1H), 6.24 (brs, 1H), 3.68 (s, 2H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 167.1, 140.3 (m), 139.0 (m), 137.9 (m), 136.5 (m), 135.6 (m), 122.4 (m), -5.4; MS (EI, \(m/z\)) 366 (M\(^+\)).
N-Pentafluorophenyl-N’-mercaptoacetyl hydrazide 9 (Mac F5). To a stirred solution of 8 (1.0 g, 2.7 mmol, 1 equiv.) in MeOH (20 mL) was added potassium thioacetate (0.31 g, 2.7 mmol, 1 equiv.) at room temperature. After refluxing for 1 hour, the solution was cooled to room temperature and 1 M NaOH (3 mL) was added to the flask before stirring for 1 hour. Dilute HCl was then added until the solution was neutral. This was followed by concentration on a rotary evaporator to near dryness. The crude material was transferred to a separatory funnel by alternate rinsing of the flask with water (10 mL) and ether (10 mL) a total of 3 times. After extraction, the organic layer was dried with MgSO₄, filtered and concentrated to yield 610 mg (83%) of 9 as an off-white granular powder (mp = 129-131 °C). No further purification was performed. ¹H NMR (400 MHz, CDCl₃) δ 8.35 (brs, 1H), 6.24 (brs, 1H), 3.25 (d, J = 9.2 Hz, 2H), 2.0 (t, J = 9.2 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 169.0, 140.2 (m), 139.0 (m), 137.8 (m), 136.5 (m), 135.5 (m), 122.7 (m), 26.4; IR: 3293, 1647, 1522 cm⁻¹; HRMS (ESI, m/z) calcd. for C₈H₅F₅N₂OS 273.0115, found 273.0121.
2.3. Surface Engineering

2.3.1. Preparation of Clean TSM Discs

The gold electrodes sandwiching the quartz disc were first wiped with a piece of cotton to remove any rough particles stuck onto the gold surface. The quartz discs were then soaked in 1% solution of sodium dodecyl sulphate (SDS) overnight. The discs were rinsed with a new portion of 1% SDS and sonicated for 5 minutes. Following that, the discs were rinsed three times and sonicated for 5 minutes in separate portions of acetone, ethanol and methanol. The discs were then dried under a gentle stream of nitrogen gas and stored in clean scintillation vials.

2.3.2. Formation of Alkylthiol-based SAMs on Gold

A 10 mM solution of 11-MUA (linker) was prepared by dissolving 218.4 mg of the white crystalline powder (previously recrystallized from hexanes) in 100 mL of absolute ethanol. Similarly, 10 mM solution of 1-butanethiol was prepared via dilution in 100 mL of absolute ethanol. Four different linker/diluent molar ratios were used for SAM formation onto the TSM gold surface (Table 1).

<table>
<thead>
<tr>
<th>11-MUA/ butanethiol Molar Ratios</th>
<th>11-MUA Volume (µL)</th>
<th>butanethiol Volume (µL)</th>
<th>Total Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 3</td>
<td>250</td>
<td>750</td>
<td>1000</td>
</tr>
<tr>
<td>1 : 1</td>
<td>500</td>
<td>500</td>
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<tr>
<td>3 : 1</td>
<td>750</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>1 : 0</td>
<td>1000</td>
<td>0</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 1. Summary of 11-MUA and butanethiol molar ratios and the corresponding volumes used for SAM formation on TSM gold substrate.
Depending on the ratio, the indicated volumetric ratios were added and mixed into separate clean test tubes. The TSM discs were then soaked into the respective alkylthiol solutions for 24 hours. The test tubes were capped with rubber stoppers and placed on a spinning plate. After 24 hours, the discs were rinsed three times with ethanol then sonicated for 5 minutes in a separate portion of ethanol. This step was repeated with methanol. Finally, the discs were dried under a gentle stream of nitrogen gas and stored in clean vials.

2.3.3. EDC/NHS Coupling Reaction

Carboxylic acid activation employed the well-established EDC/NHS coupling reaction, as illustrated below (Scheme 3). NHS and EDC solutions (50 mM each) were prepared by dissolving 45.6 mg of NHS and 60.0 mg of EDC in 10 mL of 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6), respectively. The SAM-coated TSM discs were initially immersed for 60 minutes in clean test tubes containing 1 mL of NHS solution. The test tubes were capped and placed on a spinning plate. After that, the discs were quickly rinsed one time with pure MES buffer, dried and transferred to clean test tubes containing 1 mL of EDC solution. The test tubes were capped and placed on a spinning plate for another 60 minutes. The discs were then rinsed three times with MES buffer and three times with methanol. They were then sonicated for 5 minutes in methanol, dried under a gentle stream of nitrogen gas and finally stored in carefully sealed vials for subsequent Mac F5 probe, biotinthiol or anti-HSP-10 aptamer immobilization.

![Scheme 3](image-url)

Scheme 3. Carboxylic acid activation with EDC then esterification with NHS.
2.4. Immobilization Reactions

2.4.1. Immobilization of Mac F5 Probe

The Mac F5 probe (2 mg) was dissolved in freshly distilled DMF (1 mL) inside the glove box and mixed vigorously for 10 minutes until a clear solution was observed. Equal volumes (1 mL) of the probe solution were portioned into separate clean test tubes in which clean TSM quartz discs were subsequently soaked. The test tubes were sealed with rubber stoppers, removed from the glove box and placed on a spinning plate for 24 hours. The discs were then rinsed three times with DMF followed by three times with methanol and then sonicated with another portion of methanol for 5 minutes. The discs were finally rinsed with another portion of methanol and dried under a gentle stream of nitrogen. These discs were analyzed via XPS to determine the amount of probe that had been immobilized onto the SAMs.

2.4.2. Immobilization of Biotinthiol

Biotinthiol (1 mg) was dissolved in spectrograde methanol (1 mL) inside the glove box and mixed vigorously for 10 minutes until a clear solution was observed. Equal volumes (1 mL) of the biotinthiol solution were portioned into separate clean test tubes in which EDC/NHS activated SAM-coated TSM quartz discs were subsequently immersed. The test tubes were sealed with rubber stoppers, removed from the glove box and placed on a spinning plate for 24 hours. The discs were then rinsed three times with methanol and then sonicated with another portion of methanol for 5 minutes. The discs were rinsed with a final portion of methanol and dried under a gentle stream of nitrogen. These discs were analyzed via the TSM to characterize the biosensor’s response for avidin-biotin interaction.
2.4.3. Immobilization of anti-HSP-10 Aptamer

5 µL of anti-HSP-10 aptamer (5 mg/mL) was diluted into 1 mL of spectrograde methanol. Small portions (1 mL) of the aptamer solution were added to separate clean test tubes in which the EDC/NHS activated SAM-coated discs were subsequently immersed. The test tubes were capped with rubber stoppers, removed from the glove box and placed on a spinning plate for 24 hours. The discs were then rinsed three times with the respective solvent (de-ionized water or methanol) followed by 5 minutes sonication in another portion of the same solvent. The discs were rinsed with a final portion of solvent and dried under a gentle stream of nitrogen. These discs were analyzed via the TSM to characterize the biosensor’s response for HSP-10 protein-aptamer interaction.

Note: The concentrations of the biosensing elements (biotin-thiol and anti-HSP-10 aptamer) required for the functionalization of the TSM crystals were chosen based on theoretical approximation (Appendix A).
2.5. Surface Characterization Analysis

2.5.1. Contact Angle Measurement (CAM)

CAMs were performed in the Department of Chemistry, University of Toronto, Toronto, ON, Canada. The surfaces were analyzed with the KSV contact angle measurement system (KSV Instruments Ltd., CAM101) and ultrapure water as the test liquid. Once the droplets were gently deposited onto the surfaces, they were allowed to settle for 10 seconds then 3 frames were recorded with 1 second intervals. The recorded images were processed automatically into contact angle values measured in angular degrees.

2.5.2. X-ray Photoelectron Spectroscopy (XPS)

XPS analysis was either performed with the Thermo Scientific K-Alpha XPS spectrometer (ThermoFisher, East Grinstead, UK) located at Surface Interface Ontario, University of Toronto, Toronto, ON, Canada. The samples were analyzed with an Al Kα X-ray source (1486.6 eV) at take-off angles of 20° relative to the surface. The samples were mounted onto the holder stage and clipped manually at the edges. The sample area of analysis was set to 300 µm. Peak fitting and data analysis was performed using the Avantage software provided with the instrument. All elemental peaks were shifted (calibrated) to the main C(1s) signal at 285.0 eV and peak identification was performed using the Shirley mode.
2.6. TSM Biosensor Analysis

2.6.1. Analysis of Avidin-Biotin Interaction

Avidin solutions (0.5 mg/mL) were prepared by dissolving 0.5 mg of powdered avidin into 1 mL of PBS buffer. After initializing the TSM components and configuring the system, the biotinylated 11-MUA/butanethiol SAM-coated quartz discs were individually inserted in the flow-through cell. The sample discs were securely sandwiched between the two Plexiglas parts of the flow-through cell. Then, PBS buffer was introduced through the cell at a rate of 40 μL/min, using the syringe pump, until a stable baseline was achieved (20-30 minutes). Next, the pump was stopped momentarily to introduce the avidin solution at the sample inlet at the same rate (40 μL/min). The TSM was allowed to reach a stable baseline response (30-60 minutes). After that, PBS was re-introduced at the same flow rate to remove any loosely or non-specifically bound avidin. Changes to the resonant frequency and motional resistance were observed and recorded on the read-out computer system.

2.6.2. Analysis of HSP-10 Protein-Aptamer Interaction

HSP-10 solutions (92 μM) were prepared by diluting the obtained HSP-10 in PBS buffer. After initializing the TSM components and completing the proper set-up of the system, the aptamer-immobilized 11-MUA/1-butanethiol SAM-coated quartz discs were individually inserted in the flow-through cell. The sample discs were securely sandwiched between the two Plexiglas parts of the flow-through cell. Then, PBS buffer was introduced through the cell at a rate of 40 μL/min, using the syringe pump, until a stable baseline was achieved (20-30 minutes). Next, the pump was stopped momentarily to introduced the HSP-10 solution at the sample inlet at the same rate (40 μL/min). The TSM was allowed to reach a stable baseline response (30-60 minutes). After that, PBS was re-introduced at the same flow rate to remove
any loosely or non-specifically bound HSP-10. Changes to the resonant frequency and motional resistance were observed and recorded on the read-out computer system.
3. Results and Discussion

3.1. Surface Engineering

3.1.1. Formation of 11-MUA/Butanethiol SAMs

The first step towards developing the TSM consisted of functionalizing its surface with SAMs in order to immobilize the biosensing element. The TSM gold substrates were functionalized with 11-MUA/butanethiol SAMs (Scheme 4). Four different molar ratios of 11-MUA/butanethiol (1:3, 1:1, 3:1 and 1:0) were studied in order to examine the effect of the diluent on the SAMs’ packing densities and steric congestion and on the subsequent immobilization of the bio-molecules. The SAM-coated surfaces were prepared after 24 hours of immersion in the corresponding linker/diluent solutions. After SAMs formation, the surfaces were analyzed by CAM with ultrapure water as the test liquid. In addition, these surfaces were also characterized by XPS at an angle of 20° relative to the surface (Table 2). The XPS spectra are attached in Appendix B.

Scheme 4. Formation of 11-MUA/butanethiol (1:1) SAM onto the TSM gold substrate.
Table 2. Contact angle measurements and XPS analysis (20˚ relative to the surface) of SAMs composed of different molar ratios of 11-MUA and butanethiol. *The signal is due to unavoidable surface contamination by adventitious carbon.

According to Table 2, CAM values of the SAM-coated surfaces were significantly higher than the control. In fact, these values are in close agreement with literature values, which range from 45˚ to 55˚ for a pure 11-MUA SAM. This indicated successful SAM formation on the gold. In addition, the CAM values gradually decreased as the 11-MUA/butanethiol molar ratio increased (Figure 17). This behaviour in CAM values indicated that SAMs composed of greater number of butanethiol molecules than 11-MUA molecules were more hydrophobic and exhibited lower surface wettability. The main parameter influencing the contact angle of a water droplet with a SAM would be the polarity of the SAM’s head functions (i.e. surface groups). Since 11-MUA carboxylic acid groups are much more polar and hydrophilic than the butanethiol methane groups, SAMs composed of higher 11-MUA/butanethiol molar ratio would exhibit a more hydrophilic character. Thus, the water molecules would spread out on the surface increasing their interactions with carboxylic acid groups on the underlying surface in order to minimize the surface energy. In addition, the decrease in CAM values with increased 11-MUA/butanethiol molar ratio is also due to the
reduction of the methylene groups exposure in the 11-MUA backbone. In other words, pure 11-MUA molecules of equal lengths would pack tightly and prevent exposure of the hydrophobic sides of the 11-MUA backbone generating a highly hydrophilic SAM with the carboxylic acid groups at the surface. On the other hand, a SAM composed 11-MUA molecules spaced out by shorter butanethiol molecules would be more hydrophobic not only because there would be less hydrophilic carboxylic acid groups at the surface but also because there would be more exposed hydrophobic methylene groups from the linker backbone. Overall, the CAM analysis successfully supported the formation of SAMs composed of different linker/diluent molar ratios based on the surface wettability characterization.

\[ \text{Figure 17. Water contact angle values of a clean control and mixed 11-MUA/butanethiol SAM-coated samples.} \]
The XPS results were based on the relative atomic percentages of elements composing the 11-MUA/butanethiol SAM system (carbon, oxygen and sulfur) and elements contained in the TSM substrate surface (gold). With respect to the clean control crystal, the relative atomic percentages of carbon (\%C) and oxygen (\%O) were significantly higher relative to gold. These signals were unavoidable even with various cleaning methods. They kept arising due to adventitious volatile hydrocarbon compounds in the surrounding environment. For this reason, the trends in the \%C and \%O are considered to be partially indicative of the SAMs’ elemental composition.

**Figure 18.** XPS relative atomic percentages of carbon (\%C), oxygen (\%O), gold (\%Au) and sulfur (\%S) as a function of 11-MUA/butanethiol molar ratio of SAM-coated TSM surfaces.  \%Au and  \%S are multiplied by a factor of 5. The control is a clean crystal.

Regarding the SAM-coated TSM surfaces (samples 1-4), the relative atomic percentages of oxygen (\%O) increased as the 11-MUA/butanethiol molar ratio increased (**Figure 18**). The increase in the \%O is indicative in part of the increase in the number of carboxylic acid groups at the SAM surface; thus, the increase in the number of 11-MUA molecules forming the SAM.
Moreover, the relative percentages of sulfur (\%S) and gold (\%Au) respectively increased and decreased as the 11-MUA/butanethiol molar ratio increased (Figure 18). The increase in \%S signifies increased surface coverage of alkylthiol molecules; in other words, a higher number of alkylthiol molecules adsorb per unit surface area of gold. The higher surface coverage at higher 11-MUA/butanethiol molar ratios is further justified by the decrease in \%Au since increased surface coverage makes it more difficult for the gold photoelectrons to escape the SAM surface due to collisional deactivation.

The reason for this increase in surface coverage with higher 11-MUA/butanethiol molar ratio is hypothesized to be determined by the mechanism controlling the SAM’s formation. As it was introduced previously (section 1.4.1.), one of the main parameters controlling SAM’s formation is the level of intermolecular interactions (i.e. van der Waals forces) between neighbouring molecules.\textsuperscript{41-44} The strength of these intermolecular interactions, and thus packing density of the SAM, has been determined to be in direct correlation with the length of the alkyl chains.\textsuperscript{69} This explains the \%S and \%Au behaviour. In contrast to butanethiol, 11-MUA molecules have a longer alkyl chain, which contribute to stronger intermolecular interactions between adjacent 11-MUA molecules. In addition, the polar carboxylic acid head functions of 11-MUA allow hydrogen bonding between neighbouring chains at the upper part of the SAM. As a result, stronger molecular interactions increase the molecular packing density of the assembly allowing more molecules to adsorb per unit area (i.e. higher surface coverage). Overall, the increase in surface coverage indicated by the \%S and \%Au is due to the increasing level of intermolecular interactions between adjacent molecules with increasing 11-MUA molecules.
3.1.2. Investigating the 11-MUA/Butanethiol SAMs’ Immobilization Ability

Having examined the formation of various 11-MUA/butanethiol SAMs with respect to their surface coverage and packing density, the next step was to evaluate their ability to subsequently immobilize bio-molecules. For this purpose, the novel Mac F5 probe was immobilized onto the SAMs through a coupling reaction involving the EDC/NHS system (Scheme 5). The Mac F5 probe-immobilized surfaces were then characterized by XPS analysis. The XPS signal for fluorine was used to evaluate the amount of the probe immobilized onto the SAMs as fluorine is characteristic of the Mac F5 probe. The relative elemental atomic percentages of the probe-immobilized TSM surfaces generated by surface XPS analysis are summarized in Table 3. Bare SAM-coated surfaces were used as controls for this experiment. The XPS spectra are attached in Appendix C.

Scheme 5. Illustration of Mac F5 probe immobilization onto a mixed 11-MUA/butanethiol (1:1) SAM following EDC/NHS activation of the carboxylic acid head functions.
Table 3. XPS analysis (20° relative to the surface) of Mac F5 probe-immobilized SAM-coated surfaces composed of different molar ratios of 11-MUA and butanethiol. The controls are un-immobilized SAM-coated surfaces.

According to Table 3, the relative atomic percentages of fluorine (F) and nitrogen (N) of the Mac F5 probe-immobilized SAM-coated surfaces (samples) are much higher than those of the un-immobilized SAM-coated surfaces (controls). These results clearly indicate that the EDC/NHS-activated carboxylic acid head functions of all four SAM compositions were capable of anchoring the Mac F5 probe. However, the %F signal varied from one sample to another and followed an interesting trend; the %F decreased as the 11-MUA/butanethiol molar ratio increased (Figure 19).
Since fluorine is characteristic of the Mac F5 probe, the \%F behaviour indicates lower immobilization density of the Mac F5 probe at a higher 11-MUA/butanethiol molar ratio. This behaviour associated with the immobilization of the Mac F5 probe on the 11-MUA/butanethiol SAMs could be attributed to the probe’s bulkiness and steric hindrance. The 11-MUA carboxylic acid head functions are not sterically hindering groups as they are approximately equal in diameter to the rest of the alkyl chain with a diameter of 3.139 Å according to a minimal energy approximation determined by the ChemDraw software. However, EDC and NHS molecules exhibit greater diameter than 11-MUA (9.28 Å and 4.74 Å, respectively). Thus, in a pure 11-MUA SAM, the steric congestion slightly increases as the 11-MUA carboxylic acid head functions undergo EDC/NHS activation. More importantly, the fluorinated benzene and the two amine groups give the Mac F5 probe a very bulky structure, which in turn hinders its proximal reactive thiol group. As a result, it becomes very difficult for
this sterically hindered nucleophilic thiol group to penetrate and react with the NHS ester electrophilic carbonyl group in a pure 11-MUA SAM. In addition, the bulky structure of the Mac F5 probe would also prevent immobilization of more probe molecules on neighbouring immobilization sites. However, SAMs with a lower 11-MUA/butanethiol molar ratio containing higher number of diluent molecules make it easier for the Mac F5 probe’s thiol group to attack the electrophilic carbonyl group and to immobilize to the surface. Besides, these types of SAMs would not prohibit immobilization on neighbouring anchoring sites since the anchoring linker molecules are distanced by the shorter diluent molecules, which ultimately reduce steric congestion and facilitate the immobilization process. Overall, although there are less immobilization sites at lower 11-MUA/butanethiol molar ratio, the %F is higher due to reduced steric hindrance during and after the immobilization of the Mac F5 probe.
3.2. Immobilization of the Biosensing Element

3.2.1. Immobilization of Biotinthiol

After having successfully determined the immobilization ability of our 11-MUA/butanethiol mixed SAMs with the Mac F5 test probe, the next step was to immobilize biotinthiol to subsequently target avidin and evaluate the TSM biosensing properties. For this purpose, the different SAM-coated TSM crystals were immobilized with biotinthiol and analyzed by XPS. Prior to biotinthiol immobilization, the SAM-coated surfaces were first activated via EDC/NHS coupling chemistry followed by immersion in spectrograde methanol solutions of biotinthiol (1 mg/mL) for 24 hours at room temperature (Scheme 6). The relative atomic percentages for the biotinylated TSM surfaces generated by XPS are summarized in Table 4. The controls were non-biotinylated SAM-coated discs. The XPS spectra are attached in the appendix section (Appendix D).

Scheme 6. Illustration of biotinthiol immobilization onto a mixed 11-MUA/butanethiol (1:1) SAM following EDC/NHS activation of the carboxylic acid head functions.
Table 4. XPS analysis (20° relative to the surface) of biotinylated SAM-coated surfaces composed of different molar ratios of 11-MUA and butanethiol. The controls are non-biotinylated SAM-coated surfaces.

<table>
<thead>
<tr>
<th>Samples/Controls</th>
<th>11-MUA/butanethiol Molar Ratio</th>
<th>Relative Atomic Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C (1s)</td>
</tr>
<tr>
<td>Control 1</td>
<td>1 : 3</td>
<td>59.07</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1 : 3</td>
<td>61.06</td>
</tr>
<tr>
<td>Control 2</td>
<td>1 : 1</td>
<td>57.94</td>
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<tr>
<td>Sample 2</td>
<td>1 : 1</td>
<td>56.99</td>
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<tr>
<td>Control 3</td>
<td>3 : 1</td>
<td>53.82</td>
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<tr>
<td>Sample 3</td>
<td>3 : 1</td>
<td>55.09</td>
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<tr>
<td>Control 4</td>
<td>1 : 0</td>
<td>49.85</td>
</tr>
<tr>
<td>Sample 4</td>
<td>1 : 0</td>
<td>56.27</td>
</tr>
</tbody>
</table>

According to Table 4, the relative atomic percentage of nitrogen (\(^\text{\textsuperscript{\textdegree}}\text{N}\)), which is characteristic of biotinthiol, was determined to be much higher for the biotinylated surfaces (samples) than for the non-biotinylated surfaces (controls). This indicates that biotinthiol immobilization was successful on all different 11-MUA/butanethiol SAMs. Besides, the \(^\text{\textdegree}\text{N}\) of the biotinylated surfaces increased as the 11-MUA/butanethiol molar ratio increased (Figure 20). This means, a higher density of biotinthiol was immobilized on SAMs with higher 11-MUA/butanethiol molar ratio. This is opposite to the Mac F5 probe immobilization behaviour where the immobilized probe’s density decreased as a function of increasing 11-MUA/butanethiol molar ratio, which was attributed to the probe’s sterically hindered reactive group.
Figure 20. XPS relative atomic percentage of nitrogen (\(^{14}\)N) as a function of 11-MUA/butanethiol molar ratio of biotinylated SAM-coated TSM surfaces. The controls are non-biotinylated SAM-coated TSM surfaces.

The biotin-thiol immobilization behaviour can be explained by considering its molecular structure. In contrast to the Mac F5 probe, biotin-thiol is smaller in size and not as bulky. Besides, the proximal reactive thiol group of biotin-thiol is distanced from the main ring structure by a 5-carbon alkyl chain, as opposed to the methylene chain of the Mac F5 probe. These two structural criteria of biotin-thiol make its reactive thiol group less hindered and more reactive in comparison to the probe. As a result, it would have the ability to overcome the steric congestion of a pure 11-MUA SAM and more easily attack the electrophilic carbonyl group of the head functions. Hence, biotin-thiol was able to bind to all the anchoring sites of a pure 11-MUA SAM forming a highly dense bio-layer without suffering from steric congestion during and after immobilization.
3.2.2. Immobilization of anti-HSP-10 Aptamer

Having successfully determined the immobilization of biotin-thiol, the next step was to immobilize the anti-HSP-10 aptamer to subsequently target our analyte of interest (HSP-10). For this purpose, the SAM-coated surfaces were first activated by EDC/NHS coupling chemistry followed by immersion in a spectrograde methanol solutions of anti-HSP-10 aptamer (0.025 mg/mL) for 24 hours at room temperature (Scheme 7). The aptamer-functionalized surfaces were analyzed by XPS. The relative elemental atomic percentages of the aptamer-functionalized surfaces generated by XPS are summarized below (Table 5). Un-functionalized SAM-coated surfaces were used as controls. The XPS spectra are attached in the appendix section (Appendix E).

Scheme 7. Illustration of 5’-thio-alkylated DNA HSP-10 aptamer immobilization onto a mixed 11-MUA/butanethiol (1:1) SAM following EDC/NHS activation of the carboxylic acid head functions.
<table>
<thead>
<tr>
<th>Samples/Controls</th>
<th>11-MUA/butanethiol Molar Ratio</th>
<th>Relative Atomic Percentage (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C (1s)</td>
<td>O (1s)</td>
</tr>
<tr>
<td>Control 1</td>
<td>1 : 3</td>
<td>58.07</td>
<td>34.43</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1 : 3</td>
<td>64.80</td>
<td>23.17</td>
</tr>
<tr>
<td>Control 2</td>
<td>1 : 1</td>
<td>56.7</td>
<td>36.24</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1 : 1</td>
<td>66.41</td>
<td>23.42</td>
</tr>
<tr>
<td>Control 3</td>
<td>3 : 1</td>
<td>54.07</td>
<td>38.98</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3 : 1</td>
<td>65.50</td>
<td>19.40</td>
</tr>
<tr>
<td>Control 4</td>
<td>1 : 0</td>
<td>50.85</td>
<td>43.2</td>
</tr>
<tr>
<td>Sample 4</td>
<td>1 : 0</td>
<td>69.58</td>
<td>17.23</td>
</tr>
</tbody>
</table>

Table 5. XPS analysis (20° relative to the surface) of aptamer-functionalized SAM-coated surfaces composed of different molar ratios of 11-MUA and butanethiol. The controls are un-functionalized SAM-coated surfaces.

Phosphorus and nitrogen were both analyzed because they are respectively characteristic of the nitrogenous bases and phosphate backbone of the aptamer. According to Table 5, %N and %P were determined to be much higher for the aptamer-functionalized SAM-coated surfaces (samples) than for the un-functionalized SAM-coated surfaces (controls). This indicated that the aptamer immobilization was successful on all four 11-MUA/butanethiol SAMs. In addition, the %N and %P of the aptamer-functionalized samples generated parallel trends as a function of increasing 11-MUA/butanethiol molar ratio (Figure 21). The %N and %P increased in the beginning reaching a maximum at a 3:1 molar ratio of 11-MUA/butanethiol and then decreased slightly at a 1:0 molar ratio.
Figure 21. XPS relative atomic percentage of phosphorus (A) and nitrogen (B) as a function of 11-MUA/butanethiol molar ratio of aptamer-immobilized SAM-coated TSM surfaces. The controls are un-functionalized SAM-coated surfaces.

This behaviour in the %P and %N suggest that anti-HSP-10 aptamer immobilized more efficiently on the 3:1 as opposed to the 1:0 11-MUA/butanethiol SAM, in the case of biotinithiol. The exact structure and conformation of the anti-HSP-10 aptamer is unknown, but its 78-oligonucleotide sequence suggests that it is bulkier and most likely covers a more extended area than biotinithiol. Although both biotinithiol and anti-HSP-10 aptamer have the same proximal hexylthiol reactive group, the bulkiness of the aptamer could be sterically hindering its reactive group. As a result, it would not be able to penetrate through a pure 11-MUA SAM and reach the electrophilic carbonyl group as the NHS-coupled carboxylic acid head functions would be sterically congested. Overall, the 3:1 11-MUA/butanethiol SAM provided a less sterically congested surface for the immobilization of the aptamer with appreciable number of anchoring sites.
3.3. TSM Biosensor Analysis

3.3.1. Detection of Avidin-Biotin Interaction

In order to characterize the TSM biosensing properties, analysis of the well-known avidin-biotin interaction was performed. For this purpose, two independent sets of experiments were performed using the TSM biosensor. The first set of experiments was dedicated to detect the specific avidin adsorption to biotin. For this set of experiments, the TSM response was monitored upon the on-line introduction of avidin over biotinylated 11-MUA/butanethiol SAM-coated TSM discs (samples) in a continuous flow-through manner. The second set of experiments was dedicated to detect non-specific avidin adsorption. For this set of experiments, the TSM response was monitored upon the on-line introduction of avidin over non-biotinylated 11-MUA/butanethiol SAM-coated TSM discs (controls) in a continuous flow-through manner. Characteristic shifts in resonant frequency (\(F_s\)) and motional resistance (\(R_m\)) generated by the TSM response were calculated from the recorded TSM spectra (Appendix F). A generic spectrum of a TSM response for biotin-avidin interaction is illustrated in Figure 22. Calculated \(\Delta F_s^*\) and \(\Delta R_m^*\) respectively represent the initial shifts in resonance frequency and motional resistance upon avidin introduction. More importantly, calculated \(\Delta F_s\) and \(\Delta R_m\) respectively represent the corrected shifts in resonance frequency and motional resistance after post-washing with PBS buffer and removing any loosely bound analyte. These shifts are reflective of the sensitivity of the biotinylated TSM biosensor for avidin binding and detection.
Figure 22. Generic TSM response for avidin-biotin interaction. $\Delta F_s^*$ and $\Delta R_m^*$ represent the initial shifts in resonance frequency and motional resistance upon avidin introduction, respectively. $\Delta F_s$ and $\Delta R_m$ represent the corrected shifts in resonance frequency and motional resistance after PBS post-wash, respectively.

In general, the TSM response showed a very sudden decrease in resonance frequency upon avidin introduction followed by an instant saturation with both biotinylated and non-biotinylated surfaces. In fact, the decrease in resonance frequency reached saturation limit within an average of 1.5 minutes after avidin introduction onto the biotinylated samples indicating a very rapid avidin adsorption. The decrease in resonance frequency indicates an interfacial coupling (i.e. adsorption) between the introduced avidin and the underlying biosensor surface (i.e. biotinylated and non-biotinylated surfaces). This is because negative frequency shifts are consistent with the mass-loading model of the TSM. Thus, the decrease in resonance frequency would be explained by an increase in surface mass and/or an extension in the effective wavelength of the transverse wave upon avidin interfacial coupling on biotinylated and non-biotinylated surfaces.
The initial and corrected shifts in resonance frequency and motional resistance generated by the samples and controls are summarized below (Table 6). The recorded shifts are average of three independent measurements for each type of sample and control.

<table>
<thead>
<tr>
<th>Samples/ Controls</th>
<th>11-MUA/butanethiol Molar Ratio</th>
<th>Initial Frequency Shift $\Delta F_s^\circ$ (Hz)</th>
<th>Corrected Frequency Shift $\Delta F_s$ (Hz)</th>
<th>Initial Resistance Shift $\Delta R_m^\circ$ (ohm)</th>
<th>Corrected Resistance Shift $\Delta R_m$ (ohm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>1 : 3</td>
<td>-46.98 ± 3.88</td>
<td>-29.86 ± 4.79</td>
<td>+0.98 ± 0.03</td>
<td>+0.23 ± 0.02</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1 : 3</td>
<td>-71.78 ± 9.29</td>
<td>-65.35 ± 9.99</td>
<td>+0.43 ± 0.07</td>
<td>+0.18 ± 0.07</td>
</tr>
<tr>
<td>Control 2</td>
<td>1 : 1</td>
<td>-49.30 ± 3.29</td>
<td>-34.04 ± 4.08</td>
<td>+0.56 ± 0.03</td>
<td>+0.32 ± 0.03</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1 : 1</td>
<td>-76.03 ± 6.84</td>
<td>-72.65 ± 8.86</td>
<td>-1.17 ± 0.09</td>
<td>-1.03 ± 0.07</td>
</tr>
<tr>
<td>Control 3</td>
<td>3 : 1</td>
<td>-47.30 ± 3.96</td>
<td>-27.52 ± 3.27</td>
<td>+0.82 ± 0.03</td>
<td>+1.06 ± 0.03</td>
</tr>
<tr>
<td>Sample 3</td>
<td>3 : 1</td>
<td>-81.81 ± 9.33</td>
<td>-77.27 ± 10.32</td>
<td>+0.37 ± 0.05</td>
<td>-0.21 ± 0.05</td>
</tr>
<tr>
<td>Control 4</td>
<td>1 : 0</td>
<td>-58.97 ± 4.43</td>
<td>-38.80 ± 5.23</td>
<td>+0.88 ± 0.06</td>
<td>+0.73 ± 0.07</td>
</tr>
<tr>
<td>Sample 4</td>
<td>1 : 0</td>
<td>-90.14 ± 7.67</td>
<td>-83.05 ± 9.41</td>
<td>+0.23 ± 0.04</td>
<td>-0.09 ± 0.01</td>
</tr>
</tbody>
</table>

Table 6. Summary of the resonance frequency and motional resistance shifts generated by the TSM in response to avidin specific and non-specific adsorption on biotinylated surfaces (samples) and non-biotinylated surfaces (controls), respectively. The sign indicates the direction of the shift (+ve – increase, -ve – decrease).

According to Table 6, avidin appears to adsorb onto all biotinylated and non-biotinylated surfaces since significant frequency shifts were observed in both cases. $\Delta F_s$ values generated by avidin adsorption on biotinylated surfaces were approximately double those values generated by avidin adsorption on non-biotinylated surfaces for corresponding SAM compositions. In addition, the non-biotinylated controls exhibited more avidin wash-off after re-introduction of PBS buffer than the biotinylated samples as indicated by differences between the initial ($\Delta F_s^\circ$) and corrected ($\Delta F_s$) frequency shifts. Overall, avidin’s specific/non-specific adsorption ratio on biotinylated/non-biotinylated surfaces is 2.3 : 1 on average.
Non-specific avidin adsorption on non-biotinylated surfaces was attributed to charge characteristics, chemical functionalities, and polarity of avidin.\textsuperscript{23} Since avidin is a hydrophilic protein (57\% hydrophilic amino acid residues), it would favour the non-specific binding onto the non-biotinylated SAM-coated surfaces since they are partially hydrophilic as well due to the terminal carboxylic acid groups of 11-MUA. In fact, “Control 4”, which is composed of a 1:0 11-MUA/butanethiol SAM, generated the highest $\Delta F_s^*$ and $\Delta F_s$ values compared to the other controls. This is because it has the most hydrophilic surface due to the maximum number of 11-MUA carboxylic acid head functions. Therefore, non-specific binding of avidin on control surfaces is suggested to be merely determined by the structural chemistry of avidin as well as the polarity character of the underlying surface.

These properties were less influential on biotinylated SAM-coated surfaces since specificity is provided by the surface-bound biotin molecules, which form very strong non-covalent bonds with on-line avidin protein molecules ($K_d \sim 10^{-15}$ M).\textsuperscript{63} Since avidin forms very strong non-covalent bonds with biotin, it is implied that a minimal dissociation of avidin-biotin will occur upon the re-introduction of the buffer. The results were consistent with this notion as minimal wash-off was evident for the samples (i.e. insignificant differences between $\Delta F_s^*$ and $\Delta F_s$). This can be attributed to removal of non-specifically bound, unbound, or excess avidin on the sensor surface. Additionally, the differences in wash-off observed amongst the different SAMs are likely due to the removal of those weakly adhered avidin proteins due to differences in packing efficiency. Therefore, $\Delta F_s$ can be considered as a measure of sensitivity and specificity of the biotinylated SAM-coated TSM surfaces for avidin.
Figure 23. Resonance frequency shifts ($\Delta F_s$) generated by the TSM in response to avidin interaction on different biotinylated and non-biotinylated SAM-coated surfaces composed of different 11-MUA/butanethiol molar ratio specifically and non-specifically, respectively. The frequency shifts are absolute values.

Regarding the biotinylated samples, $\Delta F_s$ values were determined to increase as the 11-MUA/butanethiol molar ratio of the SAM increased, generating a maximum frequency shift at the 1:0 11-MUA/butanethiol SAM (Figure 23). This was consistent with the XPS analysis of the biotinylated surfaces, where the %N of biotinylated surfaces increased with increasing 11-MUA/butanethiol molar ratio. This means, the close-packing of biotinthiol molecules on the 1:0 11-MUA/butanethiol SAM did not affect their sensitivity for avidin. In other words, the steric congestion of 11-MUA and immobilized biotinthiol molecules did not limit the free space required for avidin to complex with biotin nor did it infringe on the conformational changes associated with avidin upon binding afterwards. Therefore, the 1:0 11-MUA/butanethiol SAM-coated surface is not only the most compatible for biotinthiol immobilization, but also most sensitive surface for avidin binding.

Regarding shifts in motional resistance, adsorption of avidin onto the samples and the controls generated insignificant and random positive and negative shifts, as reflected in the
calculated $\Delta R_m^\circ$ and $\Delta R_m$ values. Motional resistance is representative of the acoustic energy dissipation of the TSM sensor into the surrounding environment (i.e. on-line liquid phase). Motional resistance is unaffected by rigid mass loading, and is sensitive to solution viscosity and density. Thus, the minimal changes in $R_m$ observed upon avidin introduction or PBS re-introduction can be attributed to changes in the solution viscosity due to the presence or absence of avidin. Increase in $R_m$ is indicative of a significant amount of energy loss to the environment. Thus, the positive shifts in motional resistance generated by the samples and the controls upon avidin adsorption are indicative of acoustic energy loss from the sensor to the surrounding liquid. However, in some instances the motional resistance failed to stabilize under constant viscosity, but rather gradually declines. This could be due to changes in surface polarity due to conformational changes of the protein.\textsuperscript{70,71} Additionally, often observed increase in motional resistance could be attributed to an increase in the apparent frictional drag experienced by the protein as it is forced to move through the solution with very little acoustic energy.\textsuperscript{15} This is because the acoustic energy is concentrated within the linker, and not within the avidin-biotin region, since the linker is fairly long. Thus, the linker would attempt to drag the protein through the solution causing energy dissipation and thus an increase in motional resistance.
3.3.2. Detection of HSP-10 Protein-Aptamer Interaction

Having characterized the TSM response with the well-known avidin-biotin interaction, the next step was to evaluate the biosensing properties for HSP-10 protein-aptamer interaction. Specific HSP-10 adsorption was assessed by monitoring the TSM response upon HSP-10 introduction over aptamer-functionalized SAM-coated surfaces (samples). Additionally, non-specific HSP-10 adsorption was assessed by monitoring the TSM response upon HSP-10 introduction over un-functionalized SAM-coated surfaces (controls). Characteristic shifts in resonant frequency ($F_s$) and motional resistance ($R_m$) generated by the TSM response were calculated from the recorded TSM spectra (Appendix G). A generic spectrum of a TSM response for HSP-10-aptamer interaction is illustrated in Figure 24. Calculated $\Delta F_s$ and $\Delta R_m$ respectively represent the initial shifts in resonance frequency and motional resistance upon HSP-10 introduction. More importantly, calculated $\Delta F_s^\circ$ and $\Delta R_m^\circ$ respectively represent the corrected shifts in resonance frequency and motional resistance after post-washing with PBS buffer to wash-off any loosely bound analyte. These shifts are reflective of the sensitivity of the aptamer-functionalized TSM biosensor for HSP-10.
Figure 24. A generic TSM response for HSP-10-aptamer interaction. $\Delta F_s$ and $\Delta R_m$ represent the initial shifts in resonance frequency and motional resistance upon HSP-10 introduction, respectively. $\Delta F_s$ and $\Delta R_m$ represent the corrected shifts in resonance frequency and motional resistance after PBS post-wash, respectively.

In general, the TSM response showed a significant *gradual* decrease in resonance frequency upon HSP-10 introduction with a very slow saturation rate mainly with the aptamer-functionalized SAM-coated surfaces. As for the un-functionalized SAM-coated control surfaces, the decrease in resonance frequency was less significant and less gradual reaching saturation more quickly. The decline in resonance frequency indicated an interfacial coupling (*i.e.* adsorption) between HSP-10 and the underlying biosensor surface (*i.e.* immobilized-aptamer or bare SAM). This is because negative frequency shifts are consistent with the mass-loading model of the TSM. Thus, the decrease in resonance frequency would be explained by an increase in surface mass and/or an extension in the effective wavelength of the transverse wave HSP-10 interfacial coupling.
The *gradual* decline in resonance frequency upon HSP-10 introduction over the aptamer-functionalized surfaces was a very unusual but interesting behaviour. In fact, the resonance frequency was decreasing almost uniformly over a period of 35 minutes on average until it reached its saturation limit and stabilized. This gradual frequency decrease means that the interfacial coupling of HSP-10 onto the aptamer-functionalized biosensor surface was taking place at a slow rate. This could be due to slow conformational changes and rearrangements of the HSP-10 over the surface in order to maximize surface binding with the aptamer. A more rational justification would be attributed to slow and complex conformational changes generated by the aptamer in order to bind HSP-10 molecules. Although it is still undetermined for HSP-10, most aptamers are known to undergo conformational changes upon target binding forming specific binding grooves of nucleic acid nucleotides to complex the target with high specificity. Hence, the aptamer molecules onto the surface could be undergoing slow conformational changes individually as they come in contact with the HSP-10 molecules and continue until the HSP-10 are specifically coupled to the aptamer. After all, for a better understanding and more established evidence, structural studies such as Fluorescence Resonance Energy Transfer (FRET) could aid visualizing the conformational changes of both the HSP-10 aptamer and protein upon interaction with one another.

The calculated shifts in resonance frequency and motional resistance generated by the samples and controls are summarized in **Table 7**. These calculated shifts are average of three independent measurements for each type of sample and control.
Table 7. Summary of the resonance frequency and motional resistance shifts generated by the TSM in response to HSP-10 specific and non-specific adsorption on aptamer-functionalized SAM-coated surfaces (samples) and un-functionalized SAM-coated surfaces (controls), respectively. The sign indicates the direction of the shift (+ve – increase, -ve – decrease).

According to Table 7, HSP-10 appears to adsorb onto all sample and control surfaces since significant negative frequency shifts were observed in both cases. $\Delta F_s^*$ and $\Delta F_s$ values generated by HSP-10 adsorption on aptamer-functionalized surfaces were significantly higher than those values generated by HSP-10 adsorption on un-functionalized surfaces for corresponding SAM composition. In fact, the specific/non-specific adsorption ratio of HSP-10 on aptamer-functionalized/un-functionalized surfaces is 2.9 : 1. Thus, it can be said that the selected anti-HSP-10 aptamer has a certain degree of specificity for HSP-10 since its presence as a sensing element was reflected by greater frequency shifts.

Non-specific adsorption of HSP-10 on un-functionalized SAM-coated surfaces of different compositions generated very similar $\Delta F_s^*$ and $\Delta F_s$ values. The re-introduction of PBS...
during the post-wash phase did not seem to cause any further shifts in resonance frequency indicating even less wash-off of HSP-10 molecules from the SAM-coated surfaces, compared to avidin. Non-specific adsorption of HSP-10 on control surfaces could be attributed to the amino acid composition and associated chemical functionalities of HSP-10. Forces such as van der Waals and dispersion forces determined by the polarity character of the protein and SAM molecules could be the reason for this non-specific adsorption.

![Figure 25](image)

**Figure 25.** Resonance frequency shifts ($\Delta F_s$) generated by the TSM in response to HSP-10’s specific and non-specific adsorption on aptamer-functionalized and un-functionalized SAM-coated surfaces composed of different 11-MUA/butanethiol molar ratios. The frequency shifts are absolute values.

On the other hand, the frequency shifts generated by HSP-10 specific adsorption on the aptamer-functionalized sample surfaces followed a parabolic behaviour as the 11-MUA/butanethiol molar ratio increased, peaking for the 1:1 11-MUA/butanethiol SAM (Figure 25). Although the 3:1 11-MUA/butanethiol SAM was the optimal surface for aptamer-immobilization, the 1:1 11-MUA/butanethiol SAM was more compatible for the biosensor’s sensitivity and the detection of HSP-10. This is because the immobilized aptamer molecules on
the 3:1 11-MUA/butanethiol SAM are probably closely-packed and sterically congested due to the high immobilization density on greater number of anchoring sites. As a result, this congestion and close-packing of the aptamer molecules would prevent or at least limit their ability to undergo proper conformational changes in order to specifically capture and complex HSP-10 due to limited free space. However, the greater number of butanethiol molecules in the 1:1 11-MUA/butanethiol SAM assisted in spacing-out the immobilized aptamer molecules, reducing their steric congestion and thus allowing them to undergo unrestricted conformational changes to bind their target.

Adsorption of HSP-10 on the samples and the controls generated positive shifts in motional resistance. As it was previously mentioned, motional resistance is representative of the energy dissipation of the TSM sensor into the surrounding environment (i.e. on-line liquid phase). Motional resistance is affected by changes to viscosity/density of the bulk solution and the degree of shear coupling at the interface. Increase in $R_m$ is indicative of a significant amount of energy loss to the environment. Thus, the positive shifts in motional resistance generated by the samples and the controls upon HSP-10 adsorption are indicative of acoustic energy loss from the sensor to the surrounding liquid.

One way this acoustic energy could be lost to the surrounding would be through apparent frictional drag experienced by deposited materials onto the biosensor surface. Since the aptamer and HSP-10 are both large in size, they would tend to experience frictional drag potentially. This frictional drag would force them to move through the solution with very little acoustic energy. This is because the acoustic energy is concentrated within the linker, and not within the aptamer-protein region, since the linker is fairly long. Thus, linker molecules would attempt to drag the protein through the solution causing energy dissipation and thus an increase in motional resistance.\textsuperscript{15} Furthermore, the rise in $R_m$ could also be due to surface structural
The binding of HSP-10 could induce structural changes in the nucleic acid aptamer. It is known that DNA oligonucleotides tend to stiffen once bound, yielding flexible and rigid regions. These regions of rigidity would tend to move with different velocities and end up experiencing different magnitudes of frictional drag; as a result, generating positive shifts in $R_m$. 
3.3.3. TSM Response for Avidin vs. HSP-10

Avidin-biotin interaction is one of the strongest known bio-molecular interactions with a dissociation constant of $10^{-15}$ M.\textsuperscript{63} For this reason; it was used in this project not only to characterize the TSM response but also as a reference to which the HSP-10 protein-aptamer interaction would be compared. Overall, TSM biosensor was determined to be less sensitive for the HSP-10 protein-aptamer interaction than avidin-biotin interaction. In fact, the sensitivity for the HSP-10 protein-aptamer interaction was less than that for avidin-biotin interaction by a factor of 2.1. Therefore, relative to strong avidin-biotin interaction, the specificity of the selected anti-HSP-10 aptamer for its target (HSP-10) is considered to be significant.

The rate of frequency decline is reflective of the rate at which the bio-molecular interaction at the biosensor interface is taking place. In contrast to the avidin-biotin interaction, which generated an instantaneous frequency decline, the HSP-10 protein-aptamer interaction generated a gradual decline. The rate of the frequency decline was calculated as the ratio between the average initial frequency shift ($\Delta F_{s}$) and the average time required for the frequency to stabilize after initial decline for all samples. On average, the frequency decline rate generated by HSP-10’s specific adsorption was determined to be 5 Hz/min compared to the 54 Hz/min generated by avidin’s specific adsorption. As a result, it can be inferred from the TSM response that the rate of HSP-10 protein-aptamer interaction is 11 times slower than the rate of avidin-biotin interaction. This slow interfacial coupling rate between HSP-10 and its aptamer is attributed to the conformational changes associated with aptamers required for target binding, as opposed to proteins, which already have specific binding sites and undergo minimal conformational changes.
Regarding the controls, non-specific adsorption of HSP-10 was lower than the non-specific adsorption of avidin on bare SAM-coated surfaces. In fact, ΔFₜ values generated by HSP-10’s non-specific adsorption were 2.6 times less than the corresponding values generated by avidin’s non-specific adsorption. This difference in non-specific adsorption could be due to differences in structural properties between HSP-10 and avidin; specifically, amino acid sequence composition and overall molecular weight of the protein. Primarily, HSP-10 is considered to be more hydrophobic than avidin. This is because 60% of HSP-10’s amino acid sequence is composed hydrophobic residues, compared to 43% hydrophobic residues in the case of avidin. Since the SAM-coated surfaces are partially hydrophilic due to the carboxylic acid head functions of 11-MUA, the non-specific adsorption of HSP-10 would be a lot less favoured than the adsorption of avidin due to the hydrophobic effect in play. More importantly, since HSP-10 (10 kDa) is almost 7 times smaller in size than avidin (69 kDa), it is expected to generate a smaller frequency shift than avidin upon surface adsorption according to mass-loading model of the TSM. Even if both proteins have the same capacity to adsorb non-specifically on the surface, avidin would generate a greater frequency shift due to its greater molecular weight. Therefore, structural properties of the analyte do not only influence its specific adsorption, but also play an important role in non-specific adsorption.
3.3.4. Reproducibility of the TSM Biosensor

In order to evaluate the reproducibility of the TSM, the relative standard deviation (RSD) of the corrected frequency shifts ($\Delta F_s$) was calculated for the specific and non-specific adsorption of both avidin and HSP-10 (Table 8).

<table>
<thead>
<tr>
<th>11-MUA/Butanethiol Molar Ratio</th>
<th>Relative Standard Deviation (%)</th>
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<tbody>
<tr>
<td></td>
<td>Avidin Specific Adsorption</td>
<td>Avidin Non-Specific Adsorption</td>
<td>HSP-10 Specific Adsorption</td>
<td>HSP-10 Non-Specific Adsorption</td>
</tr>
<tr>
<td>1 : 3</td>
<td>15</td>
<td>16</td>
<td>21</td>
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<td>13</td>
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Table 8. Relative standard deviation (RSD) of the corrected frequency shifts ($\Delta F_s$) for the specific and non-specific adsorption of avidin and HSP-10 on their respective samples and controls.

Although the TSM was successful in measuring both avidin-biotin and HSP-10 protein-aptamer interaction with very high specificities, it was evident that the overall experimental results exhibited rather low reproducibility, as inferred from the relatively high RSD values (13-21%). Various factors could have affected the reproducibility of the TSM measurements. Primarily, the reproducibility of SAMs formation is a major factor that could have influenced the reproducibility of the biosensor. Although prepared under the same conditions, SAMs formation would have probably been affected by micro-environmental parameters such as surface roughness of the biosensor substrate and heat of reaction solution. As a result, different trials of SAMs formation reactions likely resulted in different morphologies, topologies, packing densities and/or binding affinities. Consequently, this influenced the reproducibility of
the immobilized biosensing layer and thus the biosensor response. In addition, since immobilization of the biosensing elements required pre-activation (esterification) of the 11-MUA carboxylic acid head functions, the discrepancies in this reaction step would add an extra room for error, which would then impact the final biosensor reproducibility. Finally, since the TSM set-up involved electrical contact between the TSM substrate and gold electrodes for its operation, systematic errors in the correct alignment the TSM with these electrodes could have affected the reproducibility of the generated frequency and thus the generated frequency shift.

The RSD values associated with specific adsorption of HSP-10 were on average higher than those of avidin. This could be because the aptamer immobilization was less reproducible than the biotin-thiol immobilization due to complexity and large size of the aptamer. Besides, the RSD values associated with the specific adsorption were relatively higher than the RSD values associated with the non-specific adsorption for both avidin and HSP-10. Since non-specific adsorption took place on bare SAM-coated surfaces, the associated reproducibility would have only been affected by discrepancies in SAMs formation. On the other hand, since specific adsorption took place on functionalized surfaces, the associated reproducibility would have been affected by discrepancies in the SAMs formation, carboxylic acid functions pre-activation and biosensing element immobilization reactions. As result, this explains the observed differences in reproducibility associated with the specific and non-specific adsorptions.
4. Conclusion

This manuscript has presented the development and utilization of the TSM biosensor, functionalized with a selected DNA aptamer as the biosensing element anchored via alkylthiol SAMs, for the detection of HSP-10 in a real-time and label-free manner. The preliminary work consisted in developing the TSM for the detection of avidin-biotin interaction to characterize its biosensing properties. Following that, the biosensor was dedicated to the detection of HSP-10 protein-aptamer interaction in buffer. Developing the TSM for this application required the functionalization of the transducer’s gold substrate with alkylthiol-based SAMs. Thus, different molar ratios of 11-MUA (linker) and butanethiol (diluent) were examined in order to account for steric effects upon immobilization of the biosensing element (i.e. biotinthiol and HSP-10 aptamer) as well as subsequent target binding (i.e. avidin and HSP-10). The conclusions acquired from the work presented within this manuscript are summarized below:

4.1. Surface Engineering of the TSM Biosensing Substrate

- SAM formation of different molar ratios of 11-MUA (linker) and butanethiol (diluent) onto TSM gold substrates resulted in different surface coverage, as inferred by the CAM and XPS analysis;

- 11-MUA carboxylic acid head functions were capable of immobilizing the Mac F5 probe (test probe) in DMF upon activation with EDC/NHS coupling system, as indicated by surface XPS analysis. The optimal SAM for the immobilization of Mac F5 probe was composed of a 1:3 molar ratio of 11-MUA:butanethiol;

- Immobilization of biotinthiol was successfully achieved in methanol with the EDC/NHS coupling system and the maximum surface density of biotinthiol was immobilized by
SAMs composed with a 1:0 molar ratio of 11-MUA:butanethiol, as indicated by surface XPS analysis.

- Immobilization of anti-HSP-10 aptamer was successfully achieved in methanol with the EDC/NHS coupling system and the maximum surface density of anti-HSP-10 aptamer was immobilized by SAMs composed with a 3:1 molar ratio of 11-MUA:butanethiol, as indicated by surface XPS analysis;

- Overall, it can be said that the diluent had an effect not only on the packing density of the SAMs but also on the steric congestion of (bio)-molecules during and after immobilization. In particular, the addition of the diluent reduced steric congestion and helped increase the immobilized density of both the Mac F5 probe and anti-HSP-10 aptamer.

4.2. Evaluation of the TSM Biosensing Properties

- The TSM biosensor, functionalized with the 11-MUA/butanethiol SAM system, was determined to be sensitive for both avidin-biotin and HSP-10 protein-aptamer interaction as inferred by the initial and final corrected TSM resonance frequency shifts;

- The most sensitive and selective surface for HSP-10 protein-aptamer interaction was the 1:1 11-MUA/butanethiol SAM, while the most sensitive and selective surface for avidin-biotin interaction was the 1:0 11-MUA/butanethiol SAM;

- The biosensor’s sensitivity for HSP-10 protein-aptamer interaction was lower than that for avidin-biotin interaction by a factor of 2.1.

- Aptamer-functionalized SAM-coated surfaces bound HSP-10 more specifically than un-functionalized SAM-coated surfaces with a specific/non-specific adsorption ratio of 2.9 : 1 as inferred from the TSM resonance frequency shifts;
- Biotinylated SAM-coated surfaces bound avidin more specifically than non-biotinylated SAM-coated surfaces with a specific/non-specific adsorption ratio of approximately \(2.3:1\) as inferred from the TSM resonance frequency shifts;

- The TSM biosensor exhibited a slightly low reproducibility of resonance frequency shifts with RSD values ranging from 13-21%;

- The TSM response for HSP-10 protein-aptamer interaction was less reproducible than the response for avidin-biotin interaction by a factor of \(1.5\);

- Overall, these preliminary results are clearly indicative of the capacity of a shear mode acoustic wave biosensor in targeting cancer proteins such as HSP-10 with the aid of nucleic acid aptamers as biosensing elements.
5. Work in Progress and Future Objectives

5.1. Overview

The preliminary results disclosed within this manuscript are evident of the immense capacity of shear mode acoustic wave biosensors in the field of protein screening and early-stage cancer diagnosis. The aptamer-functionalized TSM biosensor showed high sensitivity and selectivity for HSP-10 detection in a real-time and label-free manner. This success led us to next test another more sensitive shear mode acoustic wave transducing device: the ElectroMagnetic Piezoelectric Acoustic Sensor (EMPAS).

The EMPAS is an innovative on-line monitoring device that offers the possibility to analyze bio-molecular interactions in a real-time and label-free manner.\textsuperscript{73-75} The EMPAS is very similar to the TSM as it also measures resonant frequency shifts triggered by analyte adsorption. There are three main criteria that differentiate the EMPAS from the TSM and make it more beneficial. First, instead of relying on the application of a potential difference through contacting-gold electrodes to generate acoustic resonance within a quartz disc, the EMPAS operates with an electrode-free environment. It operates using a radiofrequency current to generate an electromagnetic field through a copper coil, which remotely triggers the excitation of acoustic resonance within the piezoelectric quartz disc. In addition, unlike TSM, the EMPAS is frequency tunable and can operate at ultra-high frequencies from 100 MHz to 1 GHz, where it excites the 20 MHz fundamental frequency of the disc to the 53\textsuperscript{rd} harmonic.\textsuperscript{73} This enhances the sensitivity of the biosensor and enables it to operate in liquid media with mixed matrices and a wide range of viscosities and densities as these effects can be tolerated by the increased resonance frequency and thus the acoustic wave energy. Hence, the EMPAS can be applied for bio-molecular analysis in real world samples such as serum, blood and urine.
Furthermore, unlike the TSM gold substrate, the EMPAS quartz substrate can be functionalized with alkyltrichlorosilane-based SAMs. Although alkylthiol-based SAMs are known to exhibit higher order, packing and homogeneity compared to alkyltrichlorosilane-based SAMs, the latter offer superior chemical stability since they involve the formation of covalent bonds with the underlying hydroxylated substrate. In addition, alkyltrichlorosilane linkers can covalently anchor bio-molecules in a single site-specific and coupling-free step.\(^{39}\) This is because alkyltrichlorosilane linkers rely on more reactive ester head functions rather than carboxylic acid head functions as esters are both compatible with the Cl\(_3\)Si moiety and do not require pre-activation with EDC/NHS coupling chemistry. After all, alkyltrichlorosilane-based SAMs offer the possibility of generating more stable and reactive surfaces for subsequent bio-receptor immobilization, which ultimately enhances the biosensing properties of the biosensor.

### 5.2. Work in Progress

Currently, we are studying alkyltrichlorosilane-based SAMs with the EMPAS. Two novel alkyltrichlorosilane-based linker/diluent systems are being investigated for the detection of avidin-biotin interaction: TUBTS/HTS system and PEG-TUBTS/7-PEG system (Figure 26). These molecules are part of a new generation of alkyltrichlorosilane molecules, which were recently synthesized in our laboratory with the exception of the commercially available HTS. The optimal time for the formation of these two linker/diluent SAM systems on quartz substrates were asserted using contact angle goniometry and X-ray photoelectron spectroscopy. Biotinthiol was then successfully immobilized onto these SAMs and the biosensing properties of the resulting assemblies for avidin were evaluated with the EMPAS.
Now, our attention is focused on determining whether these SAMs are suitable for immobilizing anti-HSP-10 aptamer. We are working to determine the optimal ratio of linker/diluent for aptamer immobilization for both TUBTS/HTS and PEG-TUBTS/7-PEG SAM systems. We are relying on CAM and XPS analysis as surface characterization techniques to confirm aptamer immobilization. If we can successfully use these TUBTS/HTS and PEG-TUBTS/7-PEG SAMs for the immobilization of our aptamer, the next step would then be for us to use these aptamer-functionalized SAM-coated quartz substrates for the detection of HSP-10 with the EMPAS. Thus, we can evaluate the biosensing properties of the EMPAS for the detection of HSP-10.

5.3. Future Objectives

Following the current optimization of the TUBTS/HTS and PEG-TUBTS/7-PEG SAM systems for the immobilization of HSP-10 aptamer and the subsequent EMPAS analysis of the HSP-10 protein-aptamer interaction, we intend to pursue five objectives as part of the completion process of this work. These include:

1) conducting structural studies via Fluorescence Resonance Energy Transfer (FRET) in order to determine the extent of the conformational changes induced by both the anti-HSP-10 aptamer and HSP-10 itself upon interaction with one another;
2) engineering monoclonal anti-HSP-10 antibodies to immobilize onto the TSM and EMPAS substrates for the subsequent biosensing of HSP-10 in order to compare the sensitivity of the antibody with the aptamer;

3) incorporating aptamers of similar oligonucleotides sequence selected from the same library pool as biosensing elements for targeting HSP-10 and comparing their specificity and selectivity for HSP-10 through inferred biosensors’ response;

4) utilizing the EMPAS for the detection of HSP-10 in real-world samples such as serum, blood or urine since the EMPAS system can operate at ultra high frequencies and tolerate acoustic energy dissipation in viscous and dense media;

5) finally, examining the reversibility of the biosensing element to regenerate the aptamer-functionalized biosensor substrates from the bound target (i.e. HSP-10) via induced pH or temperature changes taking advantage of the high alkyltrichlorosilane-based SAMs stability in order to develop a reversible biosensor with reduced costs.
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Appendix A: Theoretical approximation of the bio-receptor concentration required to functionalize the TSM surface

- assume 100% 11-MUA coverage and maximum packing density

1) Total area of the gold substrate: \( A_{\text{gold}} = 0.28 \, \text{cm}^2 \)

2) Theoretical radius of 11-MUA: \( R_{11-\text{MUA}} = 3.139 \, \text{Å} = 3.139 \times 10^{-8} \, \text{cm} \)

3) Area covered by a single 11-MUA molecule:

   \[
   A_{11-\text{MUA}} = \pi R^2
   = \pi (3.139 \times 10^{-8} \, \text{cm})^2
   = 3.904 \times 10^{-15} \, \text{cm}^2
   \]

   - assuming area of interstitial spaces is neglectable

4) Area covered by a single bio-receptor molecule:

   \( A_{\text{bio-receptor}} = 3.09 \times 10^{-15} \, \text{cm}^2 \)

   - assuming both biotin-thiol and anti-HSP-10 aptamer bind on all 11-MUA anchoring sites

5) Number of bio-receptor molecules required to functionalize the TSM gold substrate:

   \[
   N_{\text{bio-receptor}} = \frac{A_{\text{gold}}}{A_{\text{bio-receptor}}}
   = \frac{0.28 \, \text{cm}^2}{3.09 \times 10^{-15} \, \text{cm}^2}
   = 9.06 \times 10^{13} \, \text{molecules}
   \]

6) Moles of bio-receptor required to functionalize the TSM gold substrate:

   \[
   n_{\text{bio-receptor}} = \frac{N_{\text{bio-receptor}}}{N_A}
   = \frac{9.06 \times 10^{13}}{6.022 \times 10^{23}}
   = 1.50 \times 10^{-10} \, \text{moles bio-receptor}
   \]

Converting to respective concentrations of avidin and HSP-10 considering each crystal was immersed in 1 mL volume of the bio-receptor solution,

**Concentration of avidin required:**

\[
C_{\text{biotin}} = \frac{(n)(MW_{\text{biotin}})}{(V)}
= (1.50 \times 10^{-10} \, \text{moles})(246 \, \text{g/mol}) / (0.001 \, \text{L})
= 3.69 \times 10^{-5} \, \text{g/L} = 3.69 \times 10^{-5} \, \text{mg/mL}
\]

**Concentration of HSP-10 required:**

\[
C_{\text{aptamer}} = \frac{(n)(MW_{\text{aptamer}})}{(V)}
= (1.50 \times 10^{-10} \, \text{moles})(24500 \, \text{g/mol}) / (0.001 \, \text{L})
= 3.67 \times 10^{-3} \, \text{g/L} = 3.67 \times 10^{-3} \, \text{mg/mL}
\]

Therefore, the required concentrations of biotin-thiol and anti-HSP-10 aptamer were \(2.95 \times 10^{-5}\) mg/mL and \(2.94 \times 10^{-3}\) mg/mL, respectively. However, we used higher concentrations: 1.0 mg/mL of biotin-thiol and \(2.50 \times 10^{-2}\) mg/mL.
Appendix B: XPS survey spectra of bare 11-MUA/butanethiol SAM-coated TSM surfaces

Figure B. 1. XPS survey spectrum of 1:3 11-MUA/butanethiol SAM-coated TSM disc.

Figure B. 2. XPS survey spectrum of 1:1 11-MUA/butanethiol SAM-coated TSM disc.
Figure B. 3. XPS survey spectrum of 3:1 11-MUA/butanethiol SAM-coated TSM disc.

Figure B. 4. XPS survey spectrum of 1:0 11-MUA/butanethiol SAM-coated TSM disc.
Appendix C: XPS survey spectra of the Mac F5 probe-immobilized SAM-coated TSM surfaces

**Figure C. 1.** XPS survey spectrum of 1:3 11-MUA/butanethiol Mac F5 probe-immobilized TSM disc.

**Figure C. 2.** XPS survey spectrum of 1:1 11-MUA/butanethiol Mac F5 probe-immobilized TSM disc.
Figure C. 3. XPS survey spectrum of 3:1 11-MUA/butanethiol Mac F5 probe-immobilized TSM disc.

Figure C. 4. XPS survey spectrum of 1:0 11-MUA/butanethiol Mac F5 probe-immobilized TSM disc.
Appendix D: XPS survey spectra of the biotinylated SAM-coated TSM surfaces

**Figure D. 1.** XPS survey spectrum of 1:3 11-MUA/butanethiol biotinylated TSM disc.

**Figure D. 2.** XPS survey spectrum of 1:1 11-MUA/butanethiol biotinylated TSM disc.
Figure D. 3. XPS survey spectrum of 3:1 11-MUA/butanethiol biotinylated TSM disc.

Figure D. 4. XPS survey spectrum of 1:0 11-MUA/butanethiol biotinylated TSM disc.
Appendix E: XPS survey spectra of the aptamer-functionalized SAM-coated TSM surfaces

Figure E. 1. XPS survey spectrum of 3:1 11-MUA/butanethiol aptamer-functionalized TSM disc.

Figure E. 2. XPS survey spectrum of 1:1 11-MUA/butanethiol aptamer-functionalized TSM disc.
Figure E. 3. XPS survey spectrum of 1:1 11-MUA/butanethiol aptamer-functionalized TSM disc.

Figure E. 4. XPS survey spectrum of 1:0 11-MUA/butanethiol aptamer-functionalized TSM disc.
Appendix F: TSM response spectra for Avidin adsorption on biotinylated and non-biotinylated SAM-coated surfaces

Figure F. 1a. TSM spectrum for avidin adsorption on a non-biotinylated 1:3 11-MUA/butanethiol SAM-coated surface (control).

Figure F. 1b. TSM spectrum for avidin adsorption on a biotinylated 1:3 11-MUA/butanethiol SAM-coated surface (sample).
Figure F. 2a. TSM spectrum for avidin adsorption on a non-biotinylated 1:1 11-MUA/butanethiol SAM-coated surface (control).

Figure F. 2b. TSM spectrum for avidin adsorption on a biotinylated 1:1 11-MUA/butanethiol SAM-coated surface (sample).
**Figure F.3a.** TSM spectrum for avidin adsorption on a non-biotinylated 3:1 11-MUA/butanethiol SAM-coated surface (control).

**Figure F.3b.** TSM spectrum for avidin adsorption on a biotinylated 3:1 11-MUA/butanethiol SAM-coated surface (sample).
Figure F. 4a. TSM spectrum for avidin adsorption on a non-biotinylated 1:0 11-MUA/butanethiol SAM-coated surface (control).

Figure F. 4b. TSM spectrum for avidin adsorption on a biotinylated 1:0 11-MUA/butanethiol SAM-coated surface (sample).
Appendix G: TSM response spectra for HSP-10 adsorption on aptamer-functionalized and un-functionalized SAM-coated surfaces

**Figure G. 1a.** TSM spectrum for HSP-10 adsorption on an un-functionalized 1:3 11-MUA/butanethiol SAM-coated surface (control).

**Figure G. 1b.** TSM spectrum for avidin adsorption on an aptamer-functionalized 1:3 11-MUA/butanethiol SAM-coated surface (sample).
**Figure G. 2a.** TSM spectrum for HSP-10 adsorption on an un-functionalized 1:1 11-MUA/butanethiol SAM-coated surface (control).

**Figure G. 2b.** TSM spectrum for avidin adsorption on an aptamer-functionalized 1:1 11-MUA/butanethiol SAM-coated surface (sample).
Figure G. 3a. TSM spectrum for HSP-10 adsorption on an un-functionalized 3:1 11-MUA/butanethiol SAM-coated surface (control).

Figure G. 3b. TSM spectrum for avidin adsorption on an aptamer-functionalized 3:1 11-MUA/butanethiol SAM-coated surface (sample).
Figure G. 4a. TSM spectrum for HSP-10 adsorption on an un-functionalized 1:0 11-MUA/butanethiol SAM-coated surface (control).

Figure G. 4b. TSM spectrum for avidin adsorption on an aptamer-functionalized 1:0 11-MUA/butanethiol SAM-coated surface (sample).