The development of an aptamer-based surface plasmon resonance (SPR) sensor for the real-time detection of glycated protein

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A Dissertation

entitled

The Development of an Aptamer-based Surface Plasmon Resonance (SPR) Sensor for the
Real-time Detection of Glycated Protein by

Rui Zheng

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biomedical Engineering

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December 2012
An Abstract of

The Development of an Aptamer-based Surface Plasmon Resonance (SPR) Sensor for the Real-time Detection of Glycated Protein

by

Rui Zheng

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Engineering

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December 2012

The direct detection of blood proteins can benefit a number of scientific and clinical applications, one of which is glycated protein monitoring for diabetes diagnosis and treatment. The formation of glycated proteins is proportional to the average glucose concentration over a certain past time period. Thus, the protein glycation level can reflect the long-term glycemic compliance according to a specific protein’s half-life. Albumin has a short half-life of 2-3 weeks and the glycated albumin (GA) level could indicate the average plasma glucose concentration on a monthly basis, which provides valuable complementary information for more complete assessment of glycemic control in comparison to conventional glucose and HbA1c testing. (HbA1c is a common long-term glucose indicator with a half-life of 7-8 weeks).

According to a literature review, no glycated protein monitor, other than HbA1c, is currently on the commercial market for diabetes guidance. A cost-effective, real-time
monitoring approach to assess the HbA1c and other proteins (such as GA) could bring significant advantages for diabetes diagnosis and treatment, whereas the existing monitoring technology does not make this economical.

In this research project, the feasibility of using an aptamer-based surface plasmon resonance (SPR) sensors for the real-time detection of glycated blood proteins (i.e., GA) has been studied. For the purpose of establishment, the well documented thrombin binding aptamers were immobilized onto a gold sensing surface using a two-step amine coupling method. The experimental results show that the aptamer functionalized SPR is a well suited sensing platform for selective blood protein detection applications.

To obtain the specific receptors that enable selective detection, the human serum albumin (HSA) binding aptamer has then been developed using the magnetic beads-based Systematic Evolution of Ligands by EXponential amplification process (MB-SELEX). The experimental protocol of the *in vitro* selection process have been standardized. The developed aptamers have shown a common pattern in sequence and a similarity in secondary structures.

Furthermore, the glycated albumin (GA) binding aptamers have been identified and the sensor performance has been characterized under different binding conditions. The aptamer functionalized sensor has shown a better performance in both sensitivity and selectivity for the detection of GA compared to the well documented phenylboronate monolayer-based sensor, indicating that aptamers are a better receptor choice for GA detection applications.

Finally, a novel multi-channel aptamer functionalization sensor and a feedback data processing model have been developed based on the glycated and non-glycated albumin
binding aptamers. The experimental results demonstrate that the aptamer-based SPR sensor has the ability to detect GA levels within a 25% error range.

This dissertation provides the essential foundation experiments to demonstrate the feasibility of using aptamer-based SPR sensor for the detection of glycated protein. It is expected that the further engineering and improvement of the sensor will eventually lead to a fast, reliable, label-free, and cost-effective glycated protein detection sensor, which may prove useful in the diagnosis and treatment of diabetes.
This dissertation is dedicated to my mom FangQiong Zhu.
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Chapter 1

Introduction

1.1 Background and Significance

The direct detection of blood proteins as the biomarkers could benefit a number of scientific and clinical applications such as drug research and environmental monitoring [1], early disease diagnosis and treatment [2]. A biomarker is a characteristic indicator of a biological phenomena or state (i.e., a high plasma glucose concentration could indicate potential diabetes; thus glucose is a biomarker as an indicator for diabetes) [3]. There are different types of biomarkers ranging from small molecules (i.e., glucose and cholesterol) to complex systems (i.e., organelles and cells), as shown in Figure 1-1. Currently, the biomarker market mainly has two areas, including biomarker discovery and biomarker detection. Biomarker discovery represents the largest area of application for biomarkers. Because of the limited availability of new biomarkers, biomarker discovery is expected to complete its late growth stage and attain maturity in the year of 2015 [4]. Biomarker detection research, on the other hand, is currently in its nascent stage with significant progress in the development of diagnostics and therapeutic guidance [5]. The trend of the biomarker market is shifting from the biomarker discovery to biomarker detection. The market size of the biomarkers is forecasted to nearly double from $21.2 billion in 2012
upwards to ~ $34 billion by 2017 [6]. One of the important applications of biomarker detection, which recently draws increasing attention from researchers and physicians, is the use of glycate proteins for the early diagnosis and treatment guidance of diabetes [7, 8].

Over the past decades, diabetes has become one of the most widespread and serious diseases all over the world. The global market for diabetes drugs and devices accounted for $41.9 billion in 2010 and is expected to attain a market size of $114.3 billion in 2016 with a high annual growth rate of 18.2% [9]. Individuals with diabetes have an inability to control their blood glucose within a normal range (70-130 mg/dL [10]), which might cause serious short-term and long-term complications such as a higher risk for digestive, cardiovascular, nerves, and ocular diseases, bone and joint disorders, and other
physiological problems [7]. The main goal of diabetes care is to prevent these complications through proper glycemic control and early stage diagnosis of the disease. Since diabetes is directly related to a high plasma glucose concentration, presently, the diagnosis of diabetes is dependent on the observation of symptoms of hyperglycemia and the detection of higher casual plasma glucose concentrations. However, hyperglycemia symptoms are usually varying among individuals and the plasma glucose concentration could fluctuate frequently during normal activities (e.g. digestion and exercise) [11]. Usually, a conventional point-of-care invasive type device is used to assess the plasma glucose concentration. This measurement needs to be performed several times a day, which brings considerable physical pain. Therefore, other diagnosis methods which are more reliable and require fewer measurements could result in an increase in patient compliance and healthcare savings.

Over the last decade, the use of glycated proteins has been suggested as a standard laboratory measurement for diabetes diagnosis and control assessment. Among different blood proteins, glycated hemoglobin (HbA1) is the most widely studied glycated protein for diabetes control. HbA1 is made up of HbA1a, HbA1b, and HbA1c, depending on the attachment of different sugars. HbA1c is the primary one used for glycemic control formed by a non-reversible process of D-glucose attaching to a specific amino-terminal site of the β-chain of hemoglobin. This process comprises three stages of non-enzymatic reactions (Figure 1-2), known as the Maillard reactions [12-14]. The first stage of the reaction is the fast but reversible formation of an aldimine base, whereas the second stage is the considerably slower and non-reversible formation of the HbA1c. The final stage of the reaction is the formation of hemoglobin advanced glycation end product Hb-AGE.
The rate of formation of glycated hemoglobin is proportional to the free blood glucose concentration. For example, an increase of HbA1c of 1% (i.e., measured as the percentage of glycated protein over total protein) corresponds to an increase of mean glucose concentration in plasma of ~ 35 mg/dL (1.95 mmol/L) [15]. Protein half-life reflects the overall time that the protein could be exposed to the free glucose. The half-life of HbA1c is ~ 7-8 weeks and it is concluded that the HbA1c level could provide information of average plasma glucose concentration for 1-3 months [16]. If the HbA1c level exceeds certain threshold (e.g., 7% as suggested [17, 18]), a possible diabetes condition could be identified. Also, a lower HbA1c level could indicate good glycemic control over the past 1 to 3 months. Currently, FDA and American Diabetes Association (ADA) accepts the HbA1c as a approved indicator for long-term average glucose concentration measurement [16].

![Diagram of nonenzymatic formation of HbA1c](image-url)

Figure 1-2: Schematic presentation of nonenzymatic formation of HbA1c [19].
Besides HbA1c, recently, glycated albumin (GA) has been reported as a potentially more accurate indicator of shorter term average glucose concentration compared to the current gold standard HbA1c by independent clinical studies [20, 21]. Albumin is a common plasma protein which plays important roles in drug delivery and blood pressure regulation [22]. The formation of GA follows a same mechanism as the formation of HbA1c. Albumin has a shorter half-life of 2-3 weeks. Thus, the GA level could indicate the average plasma glucose concentration on a monthly basis, which provides valuable complementary information for more complete assessment of glycemic control in comparison to just glucose and HbA1c testing.

Currently, common laboratory methods to measure glycated proteins are high performance liquid chromatography (HPLC), immunoassay, and electrophoresis [23]. The chromatography method uses a phenylboronic acid immobilized column to separate the target protein from complex samples and the concentration is then obtained by UV absorption spectroscopy [24]. This is a preferred method because its reusable column. However, it is time consuming which requires ~ 1 week to assess the result for a standard laboratory test. Immunoassay uses fluorescence labeled antibodies to directly detect the glycated proteins. It is relatively faster than HPLC, but not as cost friendly (e.g., one of cheapest single test enzymatic HbA1c assay kits on the market costs ~ $300 [25]). Electrophoresis can either utilize phenylboronic acid or antibodies as the receptors within the electrophoresis gel, but it is not accurate enough for concentration measurement applications [26]. As for portable detection solutions, there are some commercially available point-of-care devices for HbA1c monitoring [27], however, a recent study shows that 6 out of 8 current HbA1c instruments do not meet the general accepted
analytical performance criteria [28]. Therefore, an improved glycated protein detection method with advantages of high accuracy, low cost, and fast response could be very beneficial. According to a recent literature review, no glycated protein monitor, other than HbA1c, is currently on the commercial market for diabetes guidance [23]. At present, the global HbA1c monitoring market is estimated at $272 million, which is low compared to the overall diabetes market size [29], however, there is a great projected growth of HbA1c and other glycated proteins monitoring in the future. A more cost-effective, real-time monitoring approach to assess the HbA1c and other proteins (such as GA) could bring significant advantages for diabetes diagnosis and treatment [20, 21], whereas the existing monitoring technology does not make this economical.

Aptamers are oligonucleotides that mimic the behavior of the antibodies and can be generated though an in vitro selection process called SELEX (systematic evolution of ligands by exponential amplification). Aptamers have the ability to bind specific target molecules with advantages of low cost and high specificity. Surface plasmon resonance (SPR) is an optical sensing technique which is commonly used for concentration measurement with advantages of high sensitivity and fast response. Based on the combined advantages of aptamer and SPR technologies, we proposed an aptamer-based SPR sensor for the real-time detection of glycated proteins. Through specific development of the aptamer and novel sensor design, an aptamer-based GA detecting SPR sensor has been successfully developed and validated. The testing results show that the developed sensor has a great potential for the detection of GA level with low cost (< $1 per test), fast response (< 5 min per test) and a comparable accuracy (~ 5% in standard error), which can be further improved towards the commercialization of this
technology. The same technique could also be directly applied to develop biosensors for the detection of other glycated proteins.

1.2 Research Objectives

The key technical objective of this study is to demonstrate the feasibility of using aptamer-based SPR biosensor to specifically detect glycated proteins in physiological levels in real-time. The following aims were defined in this study to accomplish this key objective:

1) For proof of concept, using existing protein binding aptamers (i.e., thrombin binding aptamers) to functionalize a SPR sensor to demonstrate that aptamer-based SPR sensor has the ability to selectively detect blood protein.

2) Development of target protein (i.e., albumin) specific aptamers with an in vitro selection procedure.

3) Optimization of sensor performance in terms of sensitivity, selectivity, and cost efficiency by optimizing sensor surface and aptamer modifications, binding conditions and the sample loading method.

4) Identification of the advantages and comparison of the performance of the developed sensor to existing sensing methods, such as phenylboronic acid monolayer-based sensors.

5) Further enhancement of the sensor performance by use of multi- or mix-layer surface chemistries and/or multi-channel functionalization to tune the sensor towards practical sensing applications.
1.3 Organization of Dissertation

This dissertation consists of eight chapters. The scope of each chapter is as follows:

Chapter 1 gives an introduction and defines research objectives of this dissertation.

Chapter 2 reviews the SPR technique, including the physics, configurations, applications, instrumentations, and commercialization aspects.

Chapter 3 provides a review of SELEX, including the mechanism and a discussion of common methods used in practice.

Chapter 4 presents the development of a thrombin binding aptamer-based SPR sensor using a two step immobilization method. The results demonstrate that the aptamer-based SPR sensor is a promising platform for blood protein detection.

Chapter 5 provides the detailed experimental procedures and discussion for the identification of human serum albumin (HSA) binding aptamers via a magnetic beads adsorption SELEX (MB-SELEX).

Chapter 6 presents the direct detection of GA using thiol-terminated aptamer-based SPR. The results show that the aptamer functionalization has better specificity and broader working pH range compared to other well documented methods such as phenylboronate monolayer for the detection of GA.

Chapter 7 describes GA level detection using biotin-terminated aptamers. A novel multi-channel functionalization method and a feedback data process model are presented.

Chapter 8 outlines the conclusions of this study. The unsolved problems and the recommended future research directions are discussed.
Chapter 2

An Overview of Surface Plasmon Resonance (SPR)

2.1 Background

Surface plasmon resonance (SPR) is an evanescent wave-based refractive index (RI) sensitive spectroscopic technique which allows for the real-time detection of biomolecular interactions. The first observation of the surface plasmon phenomenon was reported by Wood in 1902 [30]. The excitation of surface plasmons using attenuated total reflection was then demonstrated independently by Kretschmann [31] and Otto [32]. To date, SPR has become a popular core technology integrated into several research-based biosensing devices because of its ultra-sensitivity, fast response and potentially inexpensive instrumentation as compared to many other high performance sensing approaches (i.e., Raman/IR spectroscopy, fluorescence labeling enzyme assay, liquid chromatography, mass spectrometry, and etc.).

The phenomenon of SPR occurs at a metal-dielectric interface. Incident light with a proper wave vector can resonate with free electrons in the surface of a metal, introducing electromagnetic waves known as surface plasmons along the metallic surface. During this condition, the light leaving the surface will become attenuated which results in a change
in the wave vector. By detecting this change, the RI of the dielectric medium can be identified. This forms the basis of a SPR sensor. To generate strong surface plasmons, metals with high free electron density such as gold and silver are preferred. Because of its high resistance to oxidation and ease for surface functionalization, gold is the most widely used metal in SPR sensing. All SPR sensors in this study are based on gold.

One of the most remarkable advantages of SPR sensors is their high sensitivity, which makes SPR well suited for ultra-low concentration detection applications. For example, sugar detection is one of the early and most widely studied biosensing applications. Table 2.1 provides a comparison of the detection range of phenylboronic monolayer-based sugar biosensors (phenylboronic monolayer is a widely used recognition layer for surface binding based sugar sensors). The detection range of the SPR-based sensor is approaching the pM range, in comparison to other sensing systems which only detect within the µM to mM range.

At present, the development of SPR is moving towards the design of compact, low-cost, reusable, and high throughout biosensors. This chapter will provide a fundamental overview of SPR physics, configurations, applications, and instrumentation.
Table 2.1: Comparison of detection range of phenylboronic acid-based sugar biosensors.

<table>
<thead>
<tr>
<th>Sensor system</th>
<th>Sugar</th>
<th>Detection range (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potentiometry [33]</td>
<td>fructose, glucose</td>
<td>3.4-40.8, 3.4-40.8</td>
</tr>
<tr>
<td>Voltammetry [34]</td>
<td>fructose, mannose, glucose</td>
<td>0.0001-1, 0.0001-1, 0.01-1</td>
</tr>
<tr>
<td>Colorimetry [35]</td>
<td>fructose, glucose</td>
<td>1-50, 1-180</td>
</tr>
<tr>
<td>Flurometry [36]</td>
<td>fructose, glucose</td>
<td>1-20, 1-50</td>
</tr>
<tr>
<td>SPR [37]</td>
<td>fructose, mannose, glucose</td>
<td>$10^3$-0.1, $10^7$-0.1, $10^9$-0.001</td>
</tr>
</tbody>
</table>

2.2 SPR Sensing Configurations and Modeling

To successfully develop an optics-based biosensor, two key components should be independently considered. First, an optical platform that provides high sensitivity to changes in sample concentration; Second, an immobilized opto-chemical transducing medium that enables specific detection. This section overviews the first key component (different SPR sensing configurations). The second component key component will then be discussed in section 2.3.

Figure 2-1 shows a typical SPR sensor setup that converts light wave properties to analyte concentration changes. The input of this SPR sensor, which is sample concentration, interacts with the recognition receptors (which specific binds to the target analyte), resulting in a RI change that directly relates to the analyte concentration and/or binding kinetics [38]. Since a SPR signal is usually generated in real-time, this also enables ultra fast biosensing.
2.2.1 Propagating SPR (PSPR) Sensing with Angular Modulation

Among all the different SPR modulations, the angular modulation-based Kretschmann configuration originally presented by Liedberg et al. in early 1980s [39] is the most common technique used in both laboratory built and commercially available SPR systems. Figure 2-2 shows a typical Kretschmann SPR sensor configuration with angular modulation using a prism as an optical coupler. This SPR setup is based on a propagating surface plasmon (also known as the planar surface plasmon), so it is also abbreviated as PSPR. A monochromatic incident light is totally reflected at the metal-dielectric interface. An electromagnetic field wave component commonly referred to as an “evanescent wave” excites a surface plasmon. The strength of coupling between the incident wave ($K_i$) and the surface plasmon wave ($K_p$) is observed at multiple angles of incidence. The angle which yields the strongest coupling (i.e., where the maximal absorbance of the reflected light is observed) is known as the SPR angle.
For more specific analysis, the incident wave vector $K_i$ can be given by the following equation:

$$K_i = \left(\frac{2\pi}{\lambda}\right)n \sin \theta_i , \quad (2.1)$$

where $K_i$ is the vertical component of the incident light wave vector, $\theta_i$ is the incident light angle, $\lambda$ is the wavelength of the incident light and $n$ is the RI value of the prism.

The wave vector of the surface plasmon ($K_p$) can be described by the following equation:

$$K_p = \left(\frac{2\pi}{\lambda}\right)\sqrt{\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2}} , \quad (2.2)$$

where $\varepsilon_1$, $\varepsilon_2$ are the dielectric permittivity constants of the metal and the dielectric exit medium (sample loaded on top of the metal surface). Surface plasmon resonance occurs when
\[ K_i = K_p \ , \ (2.3) \]

In above equations, since both the RI value of the prism and the dielectric constant of the metal will be fixed for monochromatic incident light (\( \lambda \) is a constant). The resonance condition (Equation 2.3) is mainly dependent on the incident angle \( \theta_i \) and the dielectric constant of the loaded sample. For a functionalized surface as shown in Figure 2-2, the dielectric constant \( \varepsilon_2 \) is determined by the specific bindings of the receptors to their targets. When the binding happens, the SPR angle will shift accordingly (as shown in Figure 2-3). Thus \( \varepsilon_2 \) which contains sample concentration information can be determined from above equations. More accurate modeling of the PSPR system will be discussed in section 2.2.2.

![SPR shift diagram](image)

Figure 2-3: A typical Kretschmann SPR response profile: a change of sample RI value results in a SPR angle shift from solid line to dotted line.
By detecting the real-time SPR angle shift (as shown in Figure 2-4, the SPR response is the SPR angle shift), binding interaction models can be fitted to determine factors such as kinetic or affinity constants. One of the most widely used 1:1 Langmuir binding models is listed as follows:

\[
\frac{dR}{dt} = -K_d R, \quad (2.4)
\]

\[
R = \frac{K_a[A]R_{\text{max}}}{([A]K_a + K_d)} \left[1 - e^{-([A]K_a + K_d)t}\right], \quad (2.5)
\]

\[
K_{eq} = \frac{K_a}{K_d}, \quad (2.6)
\]

Assuming the reaction is \( A + B = AB \), where \( A \) is the injected analyte, \( B \) is the immobilized ligand, and \( AB \) is the analyte–ligand complex. In a SPR system, the response \( R \) is proportional to the amount of \([AB]\) and the \( R_{\text{max}} \) is proportional to the initial \([A]\). The terms \( k_a \) (the kinetics parameter of the association phase), \( k_d \) (the kinetics
parameter of the disassociation phase), and $K_{eq}$ (the kinetics parameter of the equilibrium state) are the parameters of interest, where the experimental curves are recorded during the association, equilibrium association, and dissociation phases as shown in Figure 2-4. The factor $k_d$ can be obtained from the dissociation phase by equation 2.4. Then the term $k_d$ can be substituted in equation 2.5 to obtain $k_a$ from the association phase. Finally, the parameter $K_{eq}$ is calculated as the ratio of association and dissociation rate constants as equation 2.6.

It should be noted that the penetration depth of the evanescent field for planer gold is $\sim 200$ nm. Therefore, without other enhancement approaches, the detectable range of a typical gold layer and prism coupled SPR sensor is within $200$ nm above the gold surface, which should cover most biological binding effects.

The PSPR response is usually defined in response unit (RU), where 1 RU = $10^{-6}$ refractive index unit, which corresponds to approximately 1 pg of protein / mm$^2$ adsorbed by a Kretschmann configuration SPR sensor [40]. For reference, a typical resolution of a commercial Kretschmann configuration SPR sensor is $\sim 0.1$ to 1 RU [41]. In most applications, the contribution of either the rate of change of the SPR signal (i.e., the slope of the binding kinetics curve in Figure 2-4) or the response difference before and after sample injection (i.e., amount bound in Figure 2-4) can used as the sensing signal. The latter is commonly used for concentration measurement applications. In Figure 2-4, the SPR response can be either RU, mdegree, or nm, depending on different SPR configurations.
2.2.2 Angular Modulation PSPR Modeling Using Fresnel Multilayer Reflection

Theory

The interaction between the incident light wave vector and a surface plasmon in the most commonly applied Kretschmann SPR configuration can be accurately modeled using Fresnel multilayer reflection theory [42]. Three layers \( p, m, \) and \( d \) have been added together as the key components for the Kretschmann SPR configuration, where \( p \) stands for the prism layer, \( m \) stands for the metal layer and \( d \) stands for the dielectric layer (sample layer). The amplitude of reflected light \( A_R \) can be given as:

\[
A_R = r_{pmd} A_I = \left| r_{pmd} \right| e^{i\theta} A_I , \quad (2.7)
\]

where \( A_I \) is the amplitude of the incident light wave, \( r_{pmd} \) is the amplitude reflection coefficient, and \( \theta \) is the phase shift. Furthermore, the amplitude reflection coefficient is given by:

\[
r_{pmd} = \frac{r_p + r_{md} e^{2ik_{mx}q}}{1 + r_p r_{md} e^{2ik_{mx}q}} , \quad (2.8)
\]

where

\[
k_{ix} = \sqrt{\left(\frac{2\pi}{\lambda}\right)^2 \varepsilon_i - k_z^2} , \quad (2.9)
\]

\[
r_{ij} = \frac{\varepsilon_i k_{ix} - \varepsilon_j k_{jx}}{\varepsilon_i k_{ix} + \varepsilon_j k_{jx}} , \quad (2.10)
\]

for the common TM polarization.
The i, j stands for p, m and d accordingly and q is the metal film thickness, ε is the dielectric constant of different layers. The total reflectivity of the incident light $R$ (intensity of the reflected light) is then given as:

$$R = |r_{pmd}|^2. \quad (2.11)$$

The modeling was performed by MATLAB and the code is listed in Appendix A. It is assumed that the prism is the commonly used BK7 glass ($n_p = 1.51$); the metal layer is gold ($\varepsilon_m = -25 + 1.44i$ at incident wavelength $\lambda = 800$ nm); and the sample layer is water ($n_d = 1.329$). Figure 2-5 shows the metal film thickness affecting the total reflectance. For a film thickness equal to 50 nm, the metal layer has the maximal theoretical absorbance of 100% at the SPR angle. Therefore, for angular modulated SPR, 50 nm is the optimal thickness of the gold layer to obtain the best sensitivity. In real world, since the exact monochromatic light source does not exist, a 100% absorption rate of the incident light will not be possible. However, using high quality monochromatic light sources such as lasers, better absorbance can be achieved to enhance sensor performance compared to lower quality light sources such as LEDs.
Figure 2-5: Reflectivity as a function of the angle of incidence.

Figure 2-6a shows the SPR angle shift as the function of the different sample RI values. As the sample RI value increases (or concentration of the sample increases), the SPR angle also increases accordingly. Their relationship is perfect linear in ideal situation as shown in Figure 2-6b. Using a highly precise rotation control or a high resolution linear detector (i.e., the SensiQ SPR system as described in section 2.6), a detection resolution of ~ 1 RU or less can be achieved.
Figure 2-6: (a) Reflectivity as a function of the angle of incidence with different RIs (b) SPR angle shift as the function of sample RI (n values as shown).
2.2.3 Other SPR Configurations

SPR imaging (SPRi) is a recently developed highly integrated SPR system which allows for the simultaneous monitoring of multiple biomolecular interactions in real-time. The basic physics of the SPRi is the same as the Kretschmann SPR setup. As shown in Figure 2-7, instead of a single sensing spot, for SPRi the entire metal surface is illuminated with a broad beam light and the reflected light is captured by a CCD camera. The CCD camera continuously monitors the changes occurring in different sensing spots and provides the real-time SPR response data. Since every spot can be a specific receptor, with the injection of a single sample, the interaction between multiple receptors and the sample can be analyzed all at once. Therefore, SPRi combines the advantages of the conventional SPR (high sensitivity) with high throughput capabilities for label-free measurement. Recently, SPRi has been successfully applied to the study of biomolecular interactions [43], detection of adsorption and desorption of multiple proteins [44], real-time study of antigen–antibody reactions [45], and the detection of cancer biomarkers [46].

Figure 2-7: The scheme of a SPRi system.
The Kretschmann configuration-based SPR requires a precise optics setup and relatively expensive instrumentations (i.e., prisms, lasers and detectors). Recently, another type of SPR sensor is taking advantage of the state of art nano-fabrication techniques and enables the development of miniaturized prism-less sensors. This type of sensor is based on the principle of localized SPR (LSPR). LSPR is not dependent on the planar surface plasmons, but the localized plasmons within the nano-sized metals. As has been reported, noble metal nanoparticles exhibit a strong UV-visible absorption band which causes color change of the nanoparticles upon interacting with different samples [47, 48], as shown in Figure 2-8a and 2-8b. This absorption band is presented when the incident photon frequency resonates with the localized surface plasmons and can be detected by spectrometers and sometimes is also detectable with the human eye. Therefore, a majority of LSPR phenomenon-based sensors can be categorized as colorimetric sensors. Normally, white light sources and UV-Vis spectrometers instead of lasers and high resolution detectors are used for LSPR sensors. Furthermore, nano-sized sensors only require small sample volumes for measurements. The LSPR technique enables the development of a possible miniaturized, low cost, and low sample consumption biosensor. To date, there have been numerous approaches demonstrated for developing nanoparticle arrays, including top-down lithography using electron beam (EB) [49, 50] and focused ion beam (FIB) [51, 52]; nanosphere lithography formation of nano triangular silver array [11, 53-55]; colloidal lithography formation of nanoholes [56-58], nanodisks [59-61], and nanorings [62-64]; and soft lithography for stamp imprinting periodic nano structures [65-73]. A typical gold nanoparticle-based LSPR biosensor and its response curve are shown in Figure 2-8a and 2-8b. For demonstration, a uniformly
distributed nano-gold layer (~ 20 nm in diameter, Figure 2-8c) was formed by physical vapor coating a 7 nm thickness of gold onto a pre-cleaned glass microscope cover slide, followed by proper heat treatments [74]. The nanogold layer can be further functionalized to form a nanoscale biosensor. It should be noted that the enhanced local electromagnetic fields near the surface of the nanoparticles are also responsible for the intense signals observed in all surface-enhanced spectroscopies, such as surface-enhanced Raman scattering (SERS) [75], as well as, nanoparticle enhanced PSPR sensors [76].

Figure 2-8: Schematic representation of an AuNP-based LSPR biosensor. (a): Biosensing scheme on an AuNP, onto which a chemical layer is deposited to selectively capture a molecule in the presence of interfering agents; (b): The extinction spectrum red shifts with binding of molecules to the AuNP; (c): SEM imaging of a uniform distributed AuNP layer that is capable of LSPR sensing.

One unique and pioneering method to develop reproducible LSPR biosensors was reported by Van Duyne’s group. A uniform triangular silver nanoparticle array was formed on a glass surface utilizing a technique called nanosphere lithography (NSL) [11, 53-55]. A single layer of polystyrene (PS) nanospheres was spin coated onto a flat glass surface and then a thin layer of silver is physically vapor deposited on top of the nanospheres. The triangular geometrical gap between the nanospheres was filled with
silver during the deposition process. After removing the layer of nanospheres, a uniformly distributed triangular silver nano-array was left on the surface. Figure 2-9 provides SEM images showing a reproduction of the nano triangular array using the NSL approach performed by our group in the University of Toledo Center for Materials and Sensor Characterization (CMSC) and the University of Michigan Lurie Nanofabrication Facility (LNF). This array can be functionalized to form an extremely sensitive nanoscale affinity biosensor [77].

![SEM images for the demonstration of nanosphere lithography: Left: a single PS nanospheres; Right: after the physical gold thin layer deposition and removal of PS nanospheres.](image)

Besides LSPR, fiber optics-based SPR sensors use single or multi-mode fiber with polished and metal-coated tip [78, 79] have also been reported for miniaturized sensor development. Kuriharal et al. demonstrated that the fiber tip can be optimized using micro-fabrication technology to etch a micro-cone into the polished end of the fiber to form a micro-prism to achieve a resolution of 800 RU [80]. This is still a much lower resolution compared to the conventional PSPR sensors with reported resolutions on the order of 1 RU. Therefore, currently, because of their maturity and high sensitivity, Kretschmann configuration-based SPR sensors still dominate the SPR sensing market.
2.3 Opto-chemical Transducing Mediums for SPR: Sensor Functionalization

Although SPR is very sensitive to the change in sample concentration, by itself, SPR is not a selective technique. Therefore, the second key component of a SPR sensor will be introduced in this section: an opto-chemical transducing medium that enables specific detection. This is normally achieved through various types of chemical modification at the metal-dielectric media interface. The surface modification serves multiple purposes. The most obvious of which is that it allows the surface to selectively capture the desired analyte in the region for which the SPR interaction occurs. Capturing or adsorbing the target also enhances the relative RI change at the surface and thereby further improves the sensitivity. This is especially important for applications involving small molecules with low concentrations. In addition, through optimizing the surface chemistry, it is possible to avoid other nonspecific interactions to further increase the specificity of the sensor.

2.3.1 Molecular Imprinting Polymers (MIPs)

Molecularly imprinted polymers (MIPs), also known as plastic antibodies, are a type of polymer configuration that mimics the function of antibodies (for reference, an antibody is a physiological target binding protein, which will be discussed in section 2.3.3). MIPs have been widely applied in sensing techniques, bio-separation and chromatography in the last 30 years [81-86]. Figure 2-10 shows the MIP creation steps. First, the functional monomers are allowed to link through a self-assembly process to the template, followed by polymerization with cross-linkers to form a key-lock structure. After the removal of the template, highly specific recognition cavities are created for specific target binding.
For SPR sensors, usually MIPs are coated onto the metal surface directly. Upon absorbing the target molecules, the physical properties (i.e., density, amount of solvent adsorbed, polymer matrix swell or shrink) of the MIP will change which results in a change of the SPR signal. Thus, the absorbed sample amount can be calibrated and determined. To date, different types of MIP-based SPR sensors have been reported, such as micro-beads [87], peptide nanoparticles [88], thin films [89-91] and hydrogels [92]. Figure 2-11 shows a hydrogel thin film surface coating method developed by our group. The thin film Au slide coating procedure was performed-based on a reverse slide immersion approach [93, 94]. Briefly, a 50 nm Au-coated glass slide was first pre-functionalized with vinyl groups. Then a proper amount of pre-polymerization solution was poured between the Au slide and a base glass slide. The slide was transferred to a container with continuous dry nitrogen gas flow and the mixture was allowed to polymerize under UV radiation.
Figure 2-11: The schematic representation of a hydrogel thin film surface coating
A: bare SPR chip; B: pre-polymerization solution; C: glass slide as support.

A cross sectional SEM image of the developed hydrogel MIP thin film with theophylline as template is shown in Figure 2-12 (theophylline is a small organic molecule for drug development/test and environmental monitoring.). The thickness of the layer is ~ 10 µM, which is beyond the ~ 200 nm detection range. However, the osmosis effect will gradually bring the target into the detectable range, and the specific cavities will then lock the target in place. Figure 2-13 shows the SPR detection results for different concentrations of caffeine and theophylline, which are very similar in chemical structure (Figure 2-13, inset plots). The sensor was functionalized using the method described in Figure 2-11 with theophylline as the template. The functionalized SPR sensor shows an enhanced response for theophylline, but a minimal response to the non-target (i.e., caffeine). All the response data was collected in real-time. This result demonstrates the advantages of SPR (i.e., real-time detection and high specificity)
compared to other theophylline detection techniques such as solid phase extraction and liquid chromatography/mass spectrometry [95], which are more time consuming and not as cost effective.

Figure 2-12: SEM image of a thin hydrogel MIP layer coated gold slide.

Figure 2-13: Hydrogel thin film coated SPR sensor for specific theophylline detection.
2.3.2 Self-assembled Monolayers (SAMs)

A self-assembled monolayer (SAM) is an organized layer of immobilized molecules in which one end of the molecule, the “tail group” specifically binds to a target, and another end of the molecule, the “head group” attaches to a certain surface. The most common sensing surface for SPR is gold, while the most common “head group” that binds to gold is thiol (-SH). The majority of SAMs-based SPR surface modifications are based on thiol, such as peptide SAMs for SPR sensing [96, 97], double layer SAMs for supporting DNA [98], and mixed layer SAMs for protein detection [99]. Although SAMs can enable selective binding effects, for complex samples, the selectivity of SAMs is not comparable to other receptors like antibodies and aptamers, as will be discussed in section 2.3.3 and 2.3.4. In most SPR systems, SAMs only function as linkage layers for supporting the receptors and reduce non-specific bindings. The most common SAMs used for SPR linkage layers are 3-mercaptopropionic acid (MPA) and 11-mercaptoundecanoic acid (MUA). These two linear molecules have one thiol head and one carboxyl head. Through the use of a 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) coupling method, protein or any amine containing molecules can be immobilized onto the carboxyl modified gold to enable label free detection. This method will be discussed in detail in Chapter 4.

As mentioned in section 2.2.3, AuNP can be used to enhance the sensitivity of the PSPR sensor. A recent example is to use a double layer SAM containing AuNP and Au/Ag alloy for the enhanced detection of human IgG [100]. For a given sample concentration, the sensor sensitivity had a ~ 80% enhancement by introducing AuNP and a ~ 120% enhancement by introducing Au/Ag alloy.
2.3.3 Antibodies

An antibody is a physiological protein that recognizes and specifically binds unique parts of target. It is the most widely used receptor for biosensors including SPR. Examples using antibodies-based SPR sensor for protein detection include: C-reactive protein [101]; bovine troponin [102]; myoglobin and cardiac troponin [103]; tumor antigen [104]; human hepatitis B virus [105] and many other more have been reported. The most critical part of the sensor development is to immobilize the antibody onto the sensing surface without losing its recognition function. A common approach is to modify the gold surface with a thiol containing SAM with carboxyl groups and then apply the EDC/NHS surface coupling method for the antibody immobilization. This approach can also be applied for the amine-terminated single strand DNA immobilization, which will be discussed in detail in Chapter 4.

2.3.4 Aptamers

Aptamers are specifically developed oligonucleotides that can mimic the function of antibodies. Custom aptamers can be identified from random oligonucleotide libraries for the target by an in vitro selection process called SELEX (Systematic Evolution of Ligands by Exponential Amplification) [106, 107]. A detailed review of the aptamer development by the SELEX method is provided in Chapter 3. Currently, while antibodies are still the most widely used receptors for biosensing applications, aptamers are becoming more popular due to their high affinity and selectivity for various target compounds ranging from small molecules, such as drugs and dyes, to complex biological molecules such as enzymes, peptides, and proteins [38, 108]. Aptamers also have a
number of advantages over antibodies such as a tolerance to wide ranges of pH and salt concentrations, heat stability, ease of synthesis, and cost efficiency, as summarized in Table 2.2.

<table>
<thead>
<tr>
<th></th>
<th>Antibody</th>
<th>Aptamer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possibility to tune kinetic parameters</td>
<td>Not possible</td>
<td>Possible</td>
</tr>
<tr>
<td>Product variation</td>
<td>No variation</td>
<td>High variation</td>
</tr>
<tr>
<td>Stability</td>
<td>Expire quickly if not stored properly</td>
<td>Stable even in room temperature</td>
</tr>
<tr>
<td>Binding condition</td>
<td>physiological condition only</td>
<td>Wide pH and ion strength range</td>
</tr>
<tr>
<td>Ease of production</td>
<td>Difficult and time consuming</td>
<td>Relatively simple using SELEX</td>
</tr>
<tr>
<td>Cost of production</td>
<td>High</td>
<td>Very low comparing to antibody</td>
</tr>
<tr>
<td>Post modification</td>
<td>Limited</td>
<td>Can be performed in any place within the sequence</td>
</tr>
</tbody>
</table>

To date, aptamer-based SPR sensors for the detection of proteins have been reported [109, 110]. A thrombin-binding aptamer functionalized SPR sensor is illustrated in Figure 2-14. Taking advantage of a micro-fluidics system, only a small amount of the sample is needed for highly specific real-time blood protein sensing. The detailed description of this method will be provided in Chapter 4.
Figure 2-14: A portable design of thrombin detection SPR sensor chip.

2.4 SPR Sensing Applications

SPR sensors are exceptionally sensitive to environmental RI changes. Since chemical concentration is directly related to the RI, SPR was first used to detect ethanol concentration in water by Minami et al. in the late 1980s using a basic Kretschmann configuration [111]. Since then, SPR sensors have been used to measure analyte concentrations in various media including gas [112, 113], liquid [114, 115], and solid [116, 117] phases. The RI value is also directly related to the other physical quantities (i.e., temperature, surface flow, film thickness and density). Recently, SPR has also been reported for highly sensitive temperature measurement [118], real-time thin film
thickness monitoring [117, 119], and flow condition analysis [120, 121]. As discussed in section 2.2.1, SPR is also a well-known label-free biosensing method for monitoring surface binding events. From antibodies and aptamers, to small organic molecules and SAMs, different kinds of receptors have been immobilized onto the sensing surface to enable the determination of kinetic data for a wide variety of molecular binding events [122, 123]. Currently, biosensing (i.e., to detect binding events) is the main application area for SPR sensors [124]. Due to their high sensitivity and instant response, SPR sensors usually have better performance than conventional measurement methods [125].

2.5 Commercialization and Future Trends of SPR Sensor Development

SPR technology holds potential for applications in areas including biological studies, health science research, drug discovery, clinical diagnosis, and environmental and agricultural monitoring. However, current SPR sensors only cover a very limited area of the biochemical / biomedical sensing market aimed primarily at research and analytical laboratories [126]. In order to gain a larger share of the biochemical / biomedical sensing market, SPR sensors have to compete with existing technologies on the basis of factors such as cost, ease of use, robustness, sensitivity, and stability. Therefore, future trends of SPR sensor development will move towards the following directions:

1. **New sensing platform development** for improved, sensitivity, portability and cost-efficiency. Current SPR biosensors are limited to a resolution of ~ 0.1 RU [127], which is not sufficient for detecting ultra-low concentrations of low molecular weight analytes. New technologies such as nano-material signal enhancement [100] could be introduced to address this problem. Furthermore, highly integrated systems such as fiber
optics [78, 79] based configurations will reduce the cost and make such systems more portable.

2. Multi-analyte capability. SPRi with multi-receptors is becoming more popular since it enables high throughout biosensing. However, it is only suitable for single analyte measurement with each sample loading. Currently, some reported SPR sensors have integrated multi-channel microfluidic sample delivery systems capable of handling picoliter volumes [121, 128, 129]. In such configurations, it is possible to perform automatic serial dilutions through integrated microvalves. The binding curves for different sample concentrations can be obtained with a single injection. This significantly reduces the time and labor needed. Another innovative fluid delivery strategy using the electro-osmotic flow method (which is a pumpless technique) has also been reported recently [130]. These configurations with their capability of ultralow sample volumes have enabled a new level of high throughput sensing development.

3. Development of advanced recognition elements. For sensing applications involve complex real samples (i.e., blood), advanced receptors which allow for specific and stable binding are needed. The integration of novel recognition elements (i.e., SAMs, aptamers, and nano materials) is the future trend. Graphene is a new class of single atom thick and two-dimensional carbon nanostructure which recently has been successfully integrated into SPR sensors for the real-time sensing of H₂O₂ [131] and blood protein [132]. In those SPR sensors, graphene serves as a reliable stabilizer for immobilizing the receptors, making it a good alternative of SAMs in certain applications.

4. Combination of SPR with another sensing technique. Other sensing techniques combined with SPR have been reported such as Raman spectroscopy [133], mass
spectroscopy (MS), and HPLC [134]. These techniques have capacities that conventional SPR sensors lack of (i.e., a built-in bio-separation function and chemical fingerprint identification). A combination of those techniques could further expand the capability of SPR.

The research and application area of the SPR sensors are so broad and still expanding. This section only provides a brief introduction. With so many possible designs and great potentials, SPR sensors are expected to become a more and more popular sensing platform.

2.6 Core Instruments: SensiQ Discovery and BIAcore 3000

Two commercially available SPR systems are involved in this study: the SensiQ® Discovery (ICx Technologies, Arlington, VA) and the BIAcore® 3000 (GE Lifescience, Pittsburgh, PA). For protocol determination, the configurations and the advantages of the two systems are provided in this section.

The SensiQ® Discovery is a highly integrated SPR system with two independent detection channels. The flow cell is made of steel and it is accessible for maintenance. All optics are integrated into a portable sensor (Figure 2-15a shows the size comparison and 2-15b shows the sensor chip configuration). This sensor is based on the Kretschmann configuration, in which the light from a light-emitting diode (LED) is polarized and then internally reflected from the gold surface. The diverged reflected light is measured with a photodiode array to give the SPR angle. Since a LED is not a high quality monochromatic light source, this system has a relatively low resolution of 1 RU, in comparison to the BIAcore® 3000 which has a resolution of ~ 0.01 RU using a laser as
the light source. In the situation that the receptor layer is too thick to fit the flow cell, a simple sample holder can be attached to the sensing surface for direct sample loading (as shown in Figure 2-15a). However, the sample loading and surface regeneration need manual operation. It also lacks temperature control which makes system normalization more difficult. On software aspect, the data processing program Qdat is limited to process sequenced data generated by SensiQ® system only, which needs a specific experimental design and it is difficult to calibrate abnormal response curves. There is no option for choosing data fitting models, so this system is not suitable for multiple binding model data processing. In terms of cost, the price for each disposable sensor is ~ $80 (2 sample loading).

![Figure 2-15](image)

**Figure 2-15:** (a) SensiQ SPR sensor size comparison (b) SensiQ SPR sensor chip configuration [135].
The BIAcore® 3000 is a commonly used commercialized SPR system which is also based on the Kretschmann configuration. Figure 2-16a shows the flow path of the system; 2-16b shows the sensor chip structure; 2-16c shows the surface of a sensor chip. It has an autosampler pump which allows for programmable sample loading. It also has four detection channels with internal switches which enables multi receptor sensing. Compared to the SensiQ® Discovery system, BIAcore® system has a conservative flow cell design, making the system maintenance more time-consuming (see Appendix B for details). Using sensor chips with thick receptor layers is also not possible. On software aspect, the control software allows for user programmable functions such as sample mixing and conditional injection, which makes the BIAcore® 3000 better suited for complex experimental design. The data processing software BIAevaluation can process multiple data formats including the commonly used ASCII text format (i.e., *.TXT). Therefore, data not generated from BIAcore® 3000 can also been processed. It has comprehensive data fitting models for more practical binding analysis. These advantages make the BIAcore® a more powerful sensing platform compared to the SensiQ®. In terms of cost, the price for the newer introduced BIAcore® SIA kit is ~ $80 per slide (4 sample loadings), which is comparable to the SensiQ® system.

Both systems are well suited for developing reusable sensors. The gold surface can be replaced with lab-made gold slides (see Appendix C for SPR gold slides manufacturing protocol). The lab fabricated slides are not only more cost efficient (e.g., ~ $0.5 per slide), but also easier to functionalize using immersion methods (see Chapter 4 for details). Briefly, for the SensiQ® SPR sensor, the original gold surface can be removed by aqua regia (HNO₃:HCl 1:3 v/v). A proper sized gold slide can then be coupled to the stripped
sensor using optical index matching oil. For the BIAcore® SPR system with the SIA kit, the gold slides can be mounted onto the holder using a double-side adhesive tape. For both systems, the lab-made slides mounted sensors perform the same as the original sensors. This allows for a more cost effective development of new surface modification methods.

Figure 2-16: (a) the flow path of the BIAcore 3000 system; (b) the sensor chip structure; (c) a sensor chip surface [136].
Chapter 3

An Overview of SELEX (Systematic Evolution of Ligands by EXponential amplification)

3.1 Background

SELEX (Systematic Evolution of Ligands by EXponential amplification) is an in vitro selection process for aptamer development (aptamers are oligonucleotides that specifically bind to target molecules). The method was first described in 1990 independently by Ellington and Tuerk’s group using unmodified RNAs [137, 138]. The single strand (ss) DNA aptamers were also developed by Ellington’s group later in 1992 [139]. Since then, aptamers have drawn increasing attention as antibody substitutes and have been widely applied for clinical study and biosensing applications as described in section 2.3.4. This chapter will provide an overview of SELEX, including the basic concepts, method variations, and the protocol adopted in this study (i.e., magnetic beads-based SELEX).
The basic concept of SELEX is illustrated in Figure 3-1. The process begins with the incubation of the target with a very large oligonucleotide library to let free binding occur. The oligonucleotides share same forward and reverse primer sequences with randomly generated sequences in between the primers. This oligonucleotides library is also known as the random DNA or RNA pool, depending on which oligonucleotide is used. Assuming ssDNA is used as the pool, for a randomly generated region of length $n$, the number of possible sequences in the library is $4^n$ (4 nucleotides A,T,C,G, with $n$ possibilities). Therefore, the possible 3D conformation of the oligonucleotides is at least
4, considering the buffer type, salt concentration, pH, and temperature can all affect the 3D conformation and charge distribution. Generally, a larger random region will generate aptamers with higher affinity [140]. In practice, it is recommended to use a comparable molecular weight of oligonucleotides similar to the target for the best chance of binding [141]. During the incubation, oligonucleotides having higher binding affinities to the target will primarily bind. After a sufficient time of incubation, when the binding process is completed, unbound ssDNAs are removed. Bound ssDNAs are then separated from the pool and amplified by PCR to form a new ssDNA pool. Since the ssDNAs with lower affinities to the target have been removed, the new ssDNA pool will contain fewer types of sequences. The new ssDNA pool is then incubated with the target again as the beginning of the next SELEX round. A typical SELEX involves 8-16 rounds. After the final round of the SELEX, the end products with the highest affinity toward the target are now selected. These are collectively called aptamers and can then be sequenced and mass produced. This can be accomplished often at a significantly lower cost compared to the conventional receptors, such as, antibodies discussed in section 2.3.4.

Both ss and double strand (ds) oligonucleotides can be used for the oligonucleotide pool. For developing aptamers without the need of high affinities, ds oligonucleotide can be used [142, 143]. Due to a higher variety of 3D conformations, currently ss oligonucleotides are preferred for the development of aptamer. However, this does involve an extra PCR run to transfer the ds oligonucleotide back to ss oligonucleotide (see chapter 5 for detailed discussion).
Since the invention of SELEX, different types of targets have been used to generate their corresponding aptamers. These targets include inorganic ions (e.g., Zn$^{2+}$ [144] and Ni$^{2+}$ [145]), small organic molecules (e.g., theophylline [146] and cocaine [147]), amino acids (e.g., arginine [148] and histidine [149]), proteins (e.g., thrombin [106] and streptavidin [150]), and complex systems (e.g., whole cells [151] and organisms [152]). According to a recently published book [153], a majority of the aptamers were developed for protein binding. Proteins can form complex 3D structures, which makes them ideal targets for aptamer development. Among different proteins, thrombin is a common blood protein that was the first one used for protein binding aptamer development. To date, thrombin binding aptamers are one of the best documented protein binding aptamers and have been widely used for verification and modeling applications (i.e., to test newly developed aptamer-based biosensing platforms [106], and evaluate new immobilization methods [132]). Chapter 4 provides a feasibility study of using thrombin binding aptamers to develop a SPR sensor for real-time selective blood protein detection. Using well documented aptamers and a novel immobilization method, the developed SPR sensor has been proven as a promising platform for blood protein detection applications.

### 3.2 SELEX Method Variations

Since the introduction of SELEX, the standard protocol has been modified in each step to shorten the reaction time, increase the binding affinity, and lower the cost [38, 154]. This section will introduce the SELEX method variations as part of the standard process.
To start a SELEX process, a randomized oligonucleotide pool needs to be generated. Normally, a standard oligonucleotide pool is suitable for most applications. However, in certain situations, the oligonucleotides are not stable enough in the required binding conditions (e.g., developing aptamers in a high pH environment [108]). A modification of the oligonucleotide could improve the stability which is usually performed by a specific attachment to certain positions within the oligonucleotide. Since DNA is generally more stable than RNA, usually the modifications are for RNA aptamer development. Recently, a 5’ end attachment of Br, Cl, NH₃, and a 2’ position attachment of NH₂, F, and OCH₃ have been reported for improved oligonucleotides stability [155]. Modification of the phosphodiester bond with thiol has also been performed for the stability enhancement and meanwhile a higher affinity has also been achieved [156]. These modification methods have advantages for improved aptamer stability and binding efficiency. However, the modification of the oligonucleotide is costly and time consuming. To use the developed aptamers for binding applications, the modifications need to be kept, which will result in a higher cost.

After obtaining a proper oligonucleotide pool, the next step is to incubate the pool with target and then let free binding occur. This step is straightforward and usually performed in an aqueous buffer. To monitor the successful binding between the oligonucleotide and the target, radioactive labeling (radioactive-SELEX) [157] and fluorescence labeling (fluorescence-SELEX) [158] are commonly used methods which require specific laboratory equipment. These modifications can help to confirm the binding result but they are not required since a successful binding can also be confirmed in the amplification step by PCR. Real-time PCR (RT-PCR) uses fluorescence dye that binds to
dsDNA which enables the real-time monitoring of the DNA amplification. A successful amplification indicates a positive binding in the previous incubation step. This method was used in this study and will be discussed in detail in chapter 5. One unique modification for the incubation step is called photo-SELEX using oligonucleotides carrying photon reactive groups [159]. The binding was allowed to react under a photon radiation. The oligonucleotides that still bind to target without photon radiation were eliminated, so the developed aptamers would only bind to target under photon radiation (i.e., aptamers can be “triggered” with light to bind to target).

An aptamer with good performance should be able to specifically bind to its target. However, for real samples, there might be non-targets that have very similar chemical structures and 3D conformations as the target (i.e., caffeine vs. theophylline; glycated hemoglobin vs. normal hemoglobin). To develop aptamers that can distinguish between these targets and non-targets, simply running more SELEX rounds will not guarantee the high specificity of the aptamers [141]. A modified procedure called counter-SELEX or subtractive-SELEX can be introduced to address this problem [160]. Briefly, after obtaining the bound oligonucleotides, an extra “counter selection” step to eliminate non-target binding oligonucleotides is performed using a non-target as the template. This is an important process to generate aptamers with high specificity to their target, as discussed in detail in section 7.2.4.

After the complete binding of oligonucleotides and target, an elution (or a separation) step to remove the unbound oligonucleotides is followed. This is the most crucial step in SELEX to guarantee the successful development of aptamers [153]. If the unbound
oligonucleotides are not completely eluted or the oligonucleotides are denatured during the elution process, the developed aptamers will have low affinities to their target. Commonly, the elution process is performed by affinity chromatography (LC-SELEX) [161] or gel-electrophoresis (CE-SELEX) [162]. Both methods are time consuming and could potentially release the bound ssDNA from the target molecule due to they require specific running buffers which are different from the binding buffer used in the SELEX incubation step. Recently, other separation methods have been developed to increase the separation efficiency, and avoid the hassle of losing aptamer functionality. Beads-based SELEX is one of the recently applied methods for convenient and effective elution [155]. In beads-based SELEX, the target molecules are immobilized onto the beads and then incubated with the oligonucleotide pool. The beads can be easily pulled out from the solution using a simple centrifugation (for common micro-beads such as agarose beads) or a magnet (for magnetic beads). This makes the separation much easier without introducing other materials. Common beads used for SELEX includes agarose beads [163], magnetic beads [150], and nano metals [164, 165]. Automated SELEX systems have also been reported using beads-based separation methods [166, 167]. Utilizing a programmable auto-sampler, a precise temperature control, and a high level clean environment, the whole SELEX procedure can be “automatically” performed by robots. This method enables high throughput development of aptamer (i.e., a typical 96 well plate can generate aptamers for up to 96 different targets simultaneously); significantly reduces the intense labor required for SELEX; and avoids the potential contamination involved by “manual” operations.
The final step of the SELEX is cloning and sequencing, which is a standard service provided by a number of companies for a reasonable cost (a standard 96 well plate sequencing which gives 96 aptamer sequences costs ~ $200 and needs ~3 days of turnaround time). After obtaining the aptamer sequences, a secondary structure analysis can identify the common regions and structures within the sequences as the potential binding sites. Currently, there are free offline programs (i.e., the widely used mfold [168]) and free online programs (i.e., UNAfold [169]) available for the DNA/RNA secondary structure analysis. After the analysis, aptamers containing the most shared region and structures can be mass produced for affinity test. This could be performed by SPR sensors and the experimental details are discussed in detail in Chapter 4, 6 and 7.

3.3 Magnetic Beads-based SELEX

As discussed, beads-based SELEX is one of the recently applied methods for convenient and effective elution which can be carried out using only standard laboratory equipment (i.e., pipette, magnet, centrifuge) and a PCR machine. We identified Strataclean® agarose beads and magnetic beads (MBs) and performed a preliminary binding test. For a same concentration of the beads and protein, it is found that MBs have a 10 times better binding rate then the agarose beads. Using a magnet rather than centrifugation, separation of MBs from solution is also easier. This method also avoids the potential structural damage and non-specific bindings (i.e., unbound DNA aggregating to the beads during the centrifugation process) involved by centrifugation. Therefore, MBs-based SELEX (MB-SELEX) protocol is pursued in this study.
Figure 3-2 shows the flow chart of the MB-SELEX protocol involved in this study. To start with, the target protein was coupled onto the MB in a proper binding buffer. Then the protein immobilized MBs were mixed and incubated with the random ssDNA pool in the binding buffer, during which the ssDNAs with higher binding affinity to the target were bound onto the surface of the MB. Using a magnet to lock the MBs in place, the supernatant containing the unbound ssDNAs could be easily removed by pipetting. This process was repeated several times to “wash” the MBs to make sure a complete removal of the unbound ssDNAs. The bound ssDNA was then eluted from the MBs via a proper treatment (e.g., heat, ethanol or high ion strength buffer). Again, using a magnet to lock the MBs in place, the supernatant now containing the ssDNAs with higher affinities to
the target could be isolated. Followed by the PCR amplification of ssDNAs, a new ssDNA pool was ready for the next SELEX round. A proper SELEX process usually involves 8-15 rounds [170]. After the last round, the final ssDNA pool could then be cloned and sequenced. The detailed experimental protocol of this MB-SELEX process is provided in Chapter 5.
Chapter 4

A Feasibility Study: Detection and Quantification of Protein using Aptamer-based SPR

4.1 Introduction

To ensure the successful development of the proposed GA detection sensor, a feasibility study was performed to establish the proof of concept using well documented thrombin binding aptamers as the sensor receptors. The combination of a self-assembled monolayer (SAM) as a linker and modified aptamers as receptors to form an aptamer-SAM matrix on a gold surface has been demonstrated as a potentially reliable and easy approach for biosensing [171]. In this chapter, the sensor was prepared by immobilizing amine-terminated aptamers onto a modified gold surface via a two-step immobilization technique. The physical detection was then performed by SPR. The developed sensor has an optimal detectable range of 5-1000 nM and the results show the sensor has good reversibility, sensitivity, and selectivity. Electrochemical Impedance Spectroscopy (EIS) and Atomic Force Microscopy (AFM) were utilized to monitor the formation of the SAMs on the gold surfaces. Aptamer binding capacity was determined by a magnetic beads (MBs) coupling method. The developed sensor shows potential for improvement.
and standardization in the direct detection of other blood proteins (i.e., hemoglobin and albumin) for clinical applications.

4.2 Materials and Experimental

4.2.1 Materials and Instrumentation

The identified aptamers were synthesized by Integrated DNA Technologies (Coralville, IA), including a 15bp aptamer (APT1): 5’-NH$_2$-(CH$_2$)$_6$-GGTTGGTGGTTGG-3’, and a 34bp aptamer (APT2): 5’-NH$_2$-(CH$_2$)$_6$-CTATCAGTCCGTGGTAGGCAGGTTGG-GGTGACT-3’. The 6 carbon bone between the actual DNA sequence and the amine termination helps to distance them so they do not interact with each other to interfere the immobilization process.

Tosylactivated MBs were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma Aldrich (Carlsbad, CA) at the highest purity available. Aptamer solutions were prepared with 1M pH 8 phosphate buffer and 3-mercaptopropionic acid (MPA) solution was prepared in ethanol. Protein sample solutions were prepared using a 0.1 M pH 7.2 PBS buffer solution with 5 mM KCl and 1 mM MgCl$_2$. The phosphoric acid (PPA) used in this study was 100 mM. All other solutions were prepared in deionized (DI) water.

AFM images were captured using the Veeco Nanoscope system (Plainview, NY). SPR measurements were performed using a commercial grade SensiQ Discovery system (ICx Technologies, Arlington, VA) at 25 °C. In this study, the SPR response profile was recorded by the SensiQ control software and then processed within MATLAB®.
Note: In case the SensiQ system fails to provide real-time SPR responses (RU values), the SPR dip profiles need to be saved and curve fitted to find the dip position shift and then calculated to obtain the RU values. The MATLAB code for the curve fitting and dip position calculation is provided in Appendix D.

4.2.2 Experimental

Electrochemical impedance spectroscopy (EIS) measurements were carried out using a Gamry Reference 600 potentiostat (Warminster, PA) in 5 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ solution with KCl as a supporting electrolyte. All the experiments were carried out at room temperature with the solutions purged with nitrogen gas for 15 minutes and the nitrogen blanket was maintained during the experiments. The experiments were performed at 25 °C. Impedance spectra were collected in the frequency range from 0.1 Hz to 100 kHz with a potential amplitude of 5 mVrms at 10 points per decade. EIS results were analyzed by fitting the experimental impedance data to electrical equivalent circuit models. Parameters of the electrical-equivalent circuits were obtained by fitting the impedance function to the measured Bode and Nyquist plots with a complex nonlinear least square (CNLS) program built into the Gamry EIS 300 electrochemical impedance spectroscope.

Aptamer coupling to MBs was performed as followed: 10 nmol of amine modified aptamer was coupled to 10 mg washed MBs in a shaker incubator at 37 °C for 18 hours. The unoccupied binding sites were blocked by Bovine Serum Albumin (BSA). The MBs were washed thoroughly and then 10 nmol of thrombin was mixed with the aptamer-coupled MBs for 2 hours in a shaker at room temperature. The control group was
prepared by exactly the same method except for the absence of aptamers. The total and unbounded proteins were measured with a carboxyl functionalized SPR sensor provided by SensiQ.

To use an aptamer-based SPR sensor for detecting blood proteins, thrombin and antithrombin aptamer were chosen for demonstration purposes. Gold slides were prepared by physical vapor deposition (PVD) forming a 1 nm layer of titanium and a 50 nm layer of gold onto pre-cleaned microscope cover slides (see Appendix C for detailed protocol). They were dried in nitrogen gas before usage. To functionalize the gold slides, they were immersed in the 10 mM MPA solution for 30 min and then washed with ethanol and DI water. After the slides were dried, they were immersed in a water solution of N-hydroxysuccinimide (NHS) and N-(3-dimethylammonopropyl)-N-ethylcarboxidiimide hydrochloride (EDC) (NHS 0.05 M, EDC 0.2 M) for 30 min. The slides were then washed with DI water and then immersed in the 5 μM aptamer solution. Finally, the slides were rinsed with the PBS buffer to flush off non-specifically adsorbed proteins. Then the slides were ready for measurement. This two-step surface functionalization procedure is illustrated in Figure 4-1.

Figure 4-1: Schematic diagram of the sensing surface functionalization procedure.
Non-coated (i.e., no gold) SensiQ base sensors were used and then custom modified with the developed gold-based SPR sensing surfaces. Specifically, the fresh aptamer immobilized gold substrates were coupled to the stripped sensors with index matching optical oil. Followed by the load of 100μL 1 M ethanolamine (EA) at a flow rate of 20 μL/min to block the non-occupied MPA activated by the EDC/NHS, followed by an injection of 100 μL of 100 mM phosphoric acid (PPA) at 50 μL/min to remove the non-specific binding. The running buffer was 0.1 M pH 7.2 PBS. The sensor was first normalized with the buffer for 10 min, then the thrombin sample (25 μL) at concentrations of 5 nM, 25 nM, 50 nM, 250 nM 500 nM, 1000 nM, 2000 nM were loaded at 5 μL/min. Samples with BSA were all prepared with 400 nM BSA. All data was recorded at 290s, 300s, and 310s after the sample injection and averaged. Sensor regeneration was performed by the injection of 100 μL PPA at 50 μL/min followed by washing with the running buffer.

4.3 Results and Discussion

4.3.1 EIS Measurement

Electrochemical impedance spectroscopy (EIS) has been proven as a powerful tool for surface characterization. The successful immobilization of each functionalized layer was confirmed through EIS measurements. Figure 4-2 shows the Nyquist plots of impedance spectra at different electrodes. The bare gold electrode represented a very small circle at high frequencies, suggesting a very low electron transfer resistance to the redox probe dissolved in the electrolyte solution (curve A). When the MPA was immobilized on the
electrode and treated with EA and PPA, the electron transfer resistance ($R_{et}$) increased to 125 $\Omega$, (curve B). Then, when 5 $\mu$M of the APT1 aptamer was added and bound with the SAM, $R_{et}$ increased to 600 $\Omega$ (curve C). Note that the reactive sites on the gold electrode were blocked by EA (ethanolamine) to prevent non-specific adsorption of aptamers onto the gold surface, thus making sure that the aptamers were attached only to the SAM. The $R_{et}$ increase is attributed to the electrostatic repulsion between the immobilized aptamer and the redox probe, causing a barrier for the interfacial electron transfer. These results indicate successful immobilization of the SAM layer onto the gold surface and stable bonding of the aptamer to the SAM.

Figure 4-2: Nyquist plots of impedance spectra obtained in 100 mM PB solution (pH 7.2) containing 5 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: (A) Bare Au; (B) Au/MPA/EDC-NHS/EA/PPA; (C) Au/MPA/EDC-NHS/EA/PPA/APT1. The right plot shows the ($R_{et}$) of each layers. Impedance spectra were collected in the frequency range from 0.1 Hz to 100 kHz with a potential amplitude of 5 mV rms at 10 points per decade.

An atomic force microscopy (AFM) measurement has also been conducted to verify the immobilization as shown in Figure 4-3a for the bare gold surface and 4-3b for the
aptamer immobilized surface. After the aptamer immobilization, the surface roughness has increased, indicating a successful immobilization has been achieved.

Figure 4-3: AFM images for (a) bare gold surface and (b) aptamer immobilized surface.
4.3.2 MB-based Maximum Binding Capacity Study

It is assumed that all aptamers were coupled to the MBs completely since twice the amount of the MBs, as instructed by the MB manufacturer’s instruction sheet, were used along with the longest allowed reaction time. After the modified MBs were thoroughly washed, thrombin was added and the concentration change was measured using a carboxyl modified SPR sensor. It is also assumed that the refractive index is controlled only by the concentration change of the added thrombin.

Other experimental variables such as protein degeneration and temperature had minor influences on SPR results and thus were not considered. As shown in Figure 4-4, the concentration change of thrombin was insignificant for the control group (less than 3%) which was not functionalized by aptamer. This indicates that the concentration change in the two experimental groups was mainly due to the binding between the aptamer and thrombin. For APT1 and APT2 groups, the mixture of aptamer functionalized MBs and thrombin solution was allowed to react for 18 hours and the reaction was considered to be completed based on the MB manufacturer’s specifications. Thus, the final concentration reflected the maximum mol/mol binding capacity of aptamer to thrombin. The results showed the binding ratio of APT1 (57.1%) has a slightly better capacity than APT2 (55.2%). Both aptamers had more than 50% mol/mol binding ratio to thrombin, indicating that they are good receptor candidates for thrombin sensing applications. In practice, not all the aptamers may bind to the MBs, and therefore the actual binding capacity of two thrombin binding aptamers toward thrombin may be slightly greater. It should be mentioned that MBs binding to aptamers could form a MB-based biosensor by involving fluorescence labeling [32]. However, the sample
preparation procedure of this method is complicated. Here we only utilized the MBs to determine the maximum binding capacity of the aptamers used in this study. This could facilitate choosing the best aptamer from multiple candidates in future optimization studies.

![Aptamer/thrombin binding ratio in mol by MBs coupling method.](image)

The control group consists MBs without functionalized by aptamers and all binding sites blocked by BSA; The APT1 group consists MBs functionalized by APT1 and unoccupied binding sited blocked by BSA; The APT2 group consists MBs functionalized by APT2 and unoccupied binding sited blocked by BSA. The error bars represent the standard deviation of the values determined from three samples.
4.3.3 SPR Results

A typical real-time SPR sensorgram is shown in Figure 4-5. After the injection of the mix of the EDC/NHS and the aptamer, a baseline shift ~ RU is observed indicating a successful immobilization of the aptamer. Upon the injection of thrombin, a larger baseline shift is obtained which is related to the sample concentration. For more specific analysis, two different aptamers were immobilized on gold surfaces and the binding performance of each one was compared. For reference, samples of different thrombin concentrations (5 nM, 25 nM, 50 nM, 250 nM, 1000 nM, 2000 nM) were individually loaded onto a bare Au, an APT1, and APT2 sensor, respectively. A secondary experiment was then performed using the same thrombin concentrations, however, with a 400nM BSA confounding component added to each thrombin sample for comparison. As shown in Figure 4-6, thrombin only group, for the thrombin-only experiment, the SPR shifts were very low for the bare Au sensor surface even for the relatively high thrombin concentrations; which was expected. In contrast, for the aptamer modified sensors the SPR shifts were significantly enhanced and the optimal detection range was 5 nM to 1000 nM (linear range). As compared to the thrombin only group, the responses are nearly identical indicating the developed APT1 and APT2 sensors are highly specific to only thrombin; which was expected. This is further illustrated in Figure 4-6, which shows the SPR shift for the 500 nM thrombin concentration with and without 400 nM of BSA. Adding BSA to the sample had minimal effect on the SPR response for the aptamer modified sensors, indicating a good selectivity of the sensor toward thrombin. This is in contrast to the bare Au sensor, which experienced a significant change between the thrombin samples with and without BSA. The APT1 modified sensor did have a slightly
higher shift than the APT2 sensor for all the thrombin concentrations. The slope of the fitting line for APT1 is also slightly larger than APT2 in the linear response range (Figure 4-8), again demonstrating a slightly better sensitivity. These two aptamers bind to different sites of thrombin, thus the affinity to the target should be different in both the interfacial binding environment and in solution [172]. In the MBs binding tests, we demonstrated that APT1 had a slightly higher binding capacity than APT2, which corresponds to the SPR results in terms of sensitivity of the functionalized sensor. A potential cause of this could be due to the smaller aptamer having a greater probability to access the binding sites of the target protein. Another possibility is that larger aptamers are thought to have more complicated secondary structures that require an extra spatial flexibility to form bonding with target molecules [173].

![Figure 4-5: A typical SPR sensorgram.](image-url)
Figure 4-6: SPR response of bare Au and aptamers-modified sensor. All data points were averaged from 3 experimental data readings. Samples were thrombin only and thrombin with 400 nM BSA. Inside plots are same data plotted in logarithmic scale for better visualizing the points in lower concentration range.

The MPA layer is known to have excellent coverage rate on gold and is widely used for antibody immunization for biosensing purposes [28]. MPA is cost effective and the related SAMs are easy to prepare. Our results show that the amine-modified aptamer can be easily immobilized onto the MPA layer and the sensor performance was comparable to antibody-based sensors. Imprecision guidelines for blood proteins quantification has been proposed by several reports and the common finding is that an imprecision of 3% for quantification methods is adequate for clinical purposes [174-176]. In our tests, three
sensing slides were prepared for each aptamer and also the control group. The sensor to
sensor performance was consistent when using the freshly prepared samples, yielding
relatively small errors for each measurement and averaging less than 2.5% standard
deviation of the total signal (error bar showed in Figure 4-7). Adding BSA did introduce
a slightly larger error and by lowering the flow rate and increasing the sample loading
time, the error can be reduced although deemed not significant enough to be considered.
The majority of the error is thought to be caused by temperature variance; thereby placing
the sensor in a temperature controlled environment should help increase the accuracy.
Recently, aptamer immobilization has been done on a variety of surfaces for sensing
purposes such as magnetic nanoparticles [177], gold nanoparticles [178], and carbon
nanotubes [179]. Our sensing surface in this study had an optimal dynamic range from 5
nM to 1000 nM, which is comparable to or greater than the largest reported dynamic
ranges for thrombin aptamer-based sensing techniques. The thrombin concentration range
in the human blood is reported to be within the low nanomolar to low micromolar range
[180], suggesting that the reported method could be well suited for potential in vivo
thrombin quantitative detection.
To test the reversibility of the sensor, fixed sample concentrations were repeatedly loaded to the sensor 10 times. The sensor regeneration was done by PPA, as previously described. The average SPR response with error bars for standard deviation using thrombin concentrations of 50 nM, 250 nM and 500 nM are shown in Figure 4-8. All data were obtained from freshly prepared sensing slides. According to our experience, the SPR response would decrease about 0.5% for each loading for a same sample concentration. All the sensing slides maintained more than 95% of the original SPR shift response after the 10th loading. It was also noticed that the second sample loading usually had the greatest response change as compared to the following loadings. We expect that with longer PPA injection time, the sensor recovery rate can be even higher.
This depends on the experimental requirements. The appearance of BSA did lower the sensitivity of the sensor (e.g. in Figure 4-8, the appearance of BSA did reduce the slope slightly in the response curve), although it did not affect the reversibility of the sensor. Figure 4-8 demonstrates that sensor maintained a linear response with and without the appearance of BSA in the 50 nM to 500 nM sample range.

![Figure 4-8: SPR responses of different sensing surfaces for 50 nM, 250 nM, 500 nM thrombin with and without 400 nM BSA, upper axis (APT1), lower axis (APT2); The zero position of lower axis has been shifted intentionally to better distinguish between data points that would be overlapping.](image)
4.3.4 Further Optimization and Development of the Sensor

Recently, some groups have successfully developed aptamer-based biosensors for the detection of proteins [147, 181-187] and related SPR-based aptamer sensors have also been reported [109, 110]. For the detection of different blood proteins, the key factor is to find the aptamer that specifically and directly binds to the target protein of interest [188]. The developed aptamer can then be amine-terminated and immobilized onto the gold surface-based on the same methods described in this chapter to form a target specific sensor for almost any protein.

Better performance of the sensor may be possibly achieved by using a mixed length spacer layer [189], such as 11-mercaptoundecanoic acid (MUA) combined with MPA to increase the sensitivity and specificity [190]. This may due to such a configuration can potentially help to form and maintain the specific shape of the immobilized aptamers. Furthermore, inserting a hydrophilic group such as ethylene oxide onto the 5’-end of the aptamer may reduce non-specific protein binding [171]. However, using our two-step immobilization method, in which a SAM was first immobilized followed by the aptamers, this should be a more cost effective and controllable method compared to adding all of the modifications to the aptamer monomer at once. Consequently, by specifically designing the SAM, e.g. optimizing the hydrocarbon length and introducing co-adsorbents [191], we can still use the simple amine-modified aptamer as the receptor to improve the sensitivity and selectivity.

Since the surface density of immobilized aptamer affects the efficiency of target capture, and the surface coverage was mainly determined by the aptamer immobilization concentrations [172], in this study we used relatively high aptamer concentrations (i.e., 5
μM) to guarantee the density and the uniformity of the surface. However, closely packed aptamers might prevent the target molecules from approaching their respective binding sites efficiently, and at meantime, they may inhibit themselves from folding into secondary structures. Therefore, by optimizing the aptamer immobilization concentrations, the performance of the sensor may be improved. For the two step immobilization method, spacing the aptamers could be also done by adjusting the MPA SAM density, or by co-incubating ethanolamine and aptamer at various molar ratios.

4.4 Conclusions

This chapter demonstrated the feasibility of using aptamer functionalized SPR for the detection and quantification of a specific protein via a two-step immobilization procedure. The advantages of the SPR sensor (i.e., low sample consumption, lack of labeling requirement, high sensitivity, and fast response) have demonstrated SPR to be a promising platform for blood protein sensing applications. The developed immobilization method has demonstrable cost efficiency, good reversibility, and uniform density. To accomplish the main goal of this dissertation, specific albumin and glycated albumin binding aptamers need to be identified, which can be performed through an in vitro selection process. The next chapter will provide a detailed experimental protocol for the development of human serum albumin binding aptamers, as the initial step for the development of a glycated albumin level sensor.
Chapter 5

Aptamer Development: Magnetic Beads-based

Systematic Evolution of ligands by EXponential amplification (MB-SELEX)

5.1 Introduction

Chapter 3 provided an overview of the SELEX process and identified MB-SELEX as the method for aptamer development. Briefly, MB-SELEX is a recently applied method that allows for convenient and effective aptamer development. It can be carried out using only standard laboratory equipment (i.e., pipette, magnet, centrifuge) and a PCR machine and it also avoids the potential structural damage and non-specific bindings. This chapter defines the experimental protocol of the MB-SELEX process for generating human serum albumin (HSA) binding aptamers. To develop a standard protocol, two book chapters [142, 143] were initially referenced. Although these provided detailed experimental steps, they focused on the development of dsDNA aptamers, which only need one PCR run for each respective SELEX round. The wide range of possible 3D
conformations of ssDNA makes it the preferred aptamer candidate for most applications [38]. This study used ssDNA for the aptamer development.

Radioactive labeling and gel analysis can be used for binding conformation. These two methods are reliable but not efficient and convenient. In this chapter, several other journal papers [192, 193] involving dsDNA to ssDNA transformation were referenced with modifications to fit our current experimental capabilities. Real-time (RT) PCR was used to monitor the DNA formation. Blue and white screen was used for the DNA cloning. The blue-white white screen allows for the direct monitoring of successful ligations in vector-based gene cloning. DNA of interest is ligated into a vector. The vector is then transformed into competent cells. The competent cells are grown in the presence of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (commonly known as X-gal). If the ligation is successful, the bacterial colonies will be white; if not, the colonies will be blue. This technique enables the quick and easy detection of successful ligation. The final aptamers sequences were obtained using a commercialized sequencing service.

5.2 Materials and Experimental

5.2.1 Materials and Instrumentation

Buffers:

The protein-magnetic bead (MB) binding buffer (buffer A: 0.1 M Na-phosphate buffer pH 7.4) was prepared according to the manufacturer’s instruction [194]. Briefly, 0.262 g NaH₂PO₄·H₂O (MW 137.99) and 1.442 g Na₂HPO₄·2H₂O (MW 177.99) were dissolved
in distilled water and then the total volume was adjusted to 100 mL. The binding buffer
detailed in Chapter 4 was used for protein and ssDNA binding (0.1 M pH 7.2 PBS buffer
with 5 mM KCl and 1 mM MgCl$_2$). All buffers were filtered by 0.45 µM syringe filters
before use. All dilutions and solutions were prepared with deionized (DI) water.

**Oligonucleotides and other chemicals:**

The random **DNA pool**: 5'-GGATTTGCTGGTGCAGTACAGTGGATCC -N$_{44}$-GGATCCTTAGGAGCTTGAAATCGAGCAG-3' (N$_{44}$ are 44 random DNA bases), the **forward primer**: 5'-GGATTTGCTGGTGCAGTACA-3', and the **reverse primer**: 5'-CTGCTCGATTTCAGCTCCT-3' were synthesized by Integrated DNA Technologies (Coralville, IA). The magnetic beads (Dynabeads™ M-280) and the cloning kit (TOPO™
TA cloning kit) were purchased from Invitrogen (Carlsbad, CA). Chemicals and proteins
were obtained from Sigma Aldrich (Carlsbad, CA). Pipette tips and tubes were
DNase/RNase free from Fisher Scientific (USA). The LB medium plates (1% Tryptone;
0.5% Yeast Extract; 1% NaCl; 1.5% Agar; pH 7.0) used in the cloning process were
prepared following the protocol listed in Appendix E.3.

**Instrumentation:**

The PCR was performed using the Bio-rad iQ5 real-time PCR system and the iQ
SYBR™ PCR kit obtained from Bio-rad (Hercules, CA). The UV absorption
measurements were performed using the Molecular Device Spectra Max 384 Plus 96 UV-
Vis well plate reader (Sunnyvale, CA). The MATLAB® codes for processing the raw data
from this device are provided in Appendix F.
5.2.2 Experimental

As discussed in Chapter 3, SELEX is time consuming and requires extreme attention to detail in the experimental process. Due to the number of steps involved, it would be very difficult to perform trouble-shooting for an unsuccessful experiment. Therefore, strict adherence to the developed protocol and the use of verification process are important to guarantee the successful development of aptamers. Appendix G provided an agarose gel electrophoresis protocol for PCR end product verification and purification. All potential contamination should be avoided. A generally applicable MB-SELEX protocol for the development of protein binding aptamers has been standardized in this study.

5.2.2.1 Protein to MB coupling

The Invitrogen Dynabeads™ M-280 Tosylactivated MBs were chosen as the protein carrier for its reliable coupling results as proven in Chapter 4. MBs can bind to any free amine containing molecule (usually proteins) in proper buffers, as shown in Figure 5-1. The protein coupling to MBs was performed following the manufacturer’s instructions with minor modifications (detailed protocol listed in Appendix E). Briefly, the washed MBs were incubated with target proteins at 37 °C for coupling and then stored in 2-8 °C for future use.
5.2.2.2 Binding and Amplification

After the target protein was coupled to the MBs, the random ssDNA pool was added for selective binding (detailed protocol listed in Appendix E.2). The ssDNAs with higher affinity to target protein will stay bound with the MBs. The unbound ssDNAs were removed by pipetting with a magnet to stabilize the MBs on the bottom of the tube. The mix was then re-suspended in water and heated to 95 °C for 3 min to release the bound ssDNAs for PCR amplification. The first PCR run was the exponential phase using both forward and reverse primers. The temperature cycle is listed in Appendix Table E.2. The second PCR run was the linear phase using forward primer only to amplify the ssDNAs using a same temperature cycle. After 8 to 12 SELEX rounds, for the last SELEX round, only the first PCR run was performed, and the “final extension” time was increased from
5 min to 15 min to ensure 3’ adenylation of the PCR generated DNA. This is important for the transformation of the DNA to the TOPO™ vector in the cloning step. It should be noted that the binding buffer will affect the final aptamer product since the ssDNA’s 3D conformation is mainly controlled by the environmental parameters (i.e., buffer strength, type, pH, and temperature). Therefore, to maintain the environmental parameters, the binding buffer should be consistent.

### 5.2.2.3 Cloning and Sequencing

Before cloning, it should be ensured that the last run PCR was using both forward and reverse primers with *Taq* polymerase and a final extension step of 15 min to ensure 3’ adenylation. The PCR product can be verified using agarose gel electrophoresis (see Appendix G for experimental details). Prepare LB (Luria-Bertani) medium plates for culturing cells (see Appendix E.3 for experimental details). The TOPO™ colony assay is based on the blue and white screening technique that allows for the direct monitoring of successful ligations in vector-based gene cloning. A TOPO™ vector contains genes that make a cell resistant to antibiotics (i.e., ampicillin) and show blue with appearance of X-gal. The DNA insertion site for the TOPO™ vector is designed to separate the X-gal gene. Thus, a DNA inserted (or ligated) vector on longer carries the X-gal gene and will show a normal white color with the appearance of X-gal. This allows for the visual determination of the ligation results. For the DNA transformation to the TOPO™ vector, the fresh PCR product was mixed with the vector for 5 min in room temperature. The vectors were then added to the competent cells from the TOPO™ kit, and incubated on ice for 5 min,
followed by heat-shock for 30-90 seconds at 42 °C without shaking to transfer the vectors into competent cells. The cells were then plated in the LB medium plates and incubated at 37 °C for growing. If the vectors were successfully transferred into the competent cells, the cells will contain antibiotics resistant gene that ensures them to survive in the ampicillin culture plates. After ~ 12 hours, the white colonies are indentified and then cultured in new LB medium plates for sequencing service (see Appendix E.4 for details).

5.3 Results and discussion

5.3.1 Real-time PCR Results and DNA Concentration Measurement

To guarantee sufficient bindings, a high DNA concentration (10 µg total for 4 µL beads) was used for the initial protein binding. The RT-PCR curves are shown in Figure 5-2. The upper 4 curves are the bound DNA eluted from the beads for PCR; the lower 4 curves are samples with known initial concentrations for standard curve generation. The initial relative fluorescence unit (RFU) values represent the DNA concentrations. From Figure 5-2, the bound DNA from 4 repetitive samples were all above the 100 ng standard curve, indicating the binding ratios for the 1st round SELEX to be above 1 %, which is too high if all the bindings were specific. One possible reason is that the high initial concentration of DNA caused an aggregating effect and washing with DI water could not sufficiently remove these non-specific bindings. Therefore, although a high DNA pool concentration is needed for complete binding (especially for the initial SELEX rounds), the concentration should still be within a reasonable range to avoid non-specific bindings.
Figure 5-2: RT-PCR curves for high initial random DNA pool concentration samples. Lower 4 curves correspond to 5 ng, 25 ng, 50 ng, and 100 ng standard samples (from low to high); Upper 4 curves correspond to the bound DNA eluted from the beads from 4 independent samples.

To determine an optimal range of the starting DNA pool, different concentrations were tested using RT-PCR. Figure 5-3 shows the RT-PCR curves for an optimized starting DNA pool concentration (1 µL of 12 µg/mL ssDNA, 12 ng total ssDNA for 4 µL beads). There was no significant dsDNA formed until the cycle 3-5, indicating relatively low starting DNA template concentrations. Cycle 5-15 was the exponential amplification phase, indicating a successful amplification of the bound DNA. A lower initial DNA concentration (10 pg total DNA for 4 µL beads) was also investigated. No significant amplification was shown within 30 cycles, indicating no sufficient binding to the MBs. Therefore ~10 ng to 100 ng DNA for 4 µL MBs is the optimal initial binding condition for this study.
Figure 5-3: RT-PCR curves for 12 ng initial DNA pool concentration; 16 samples in parallel.

Table 5.1: PCR end product concentration calculation using UV absorption data.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>A_{260nm}</th>
<th>Concentration (µg/mL)</th>
<th>A_{260nm}/A_{280nm}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.355</td>
<td>273.6</td>
<td>1.65</td>
</tr>
<tr>
<td>2</td>
<td>0.345</td>
<td>265.0</td>
<td>1.63</td>
</tr>
<tr>
<td>3</td>
<td>0.344</td>
<td>264.1</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>0.376</td>
<td>291.6</td>
<td>1.74</td>
</tr>
<tr>
<td>5</td>
<td>0.303</td>
<td>228.9</td>
<td>1.56</td>
</tr>
<tr>
<td>6</td>
<td>0.258</td>
<td>190.3</td>
<td>1.71</td>
</tr>
<tr>
<td>7</td>
<td>0.325</td>
<td>247.8</td>
<td>1.64</td>
</tr>
<tr>
<td>8</td>
<td>0.337</td>
<td>258.1</td>
<td>1.74</td>
</tr>
<tr>
<td>9</td>
<td>0.221</td>
<td>158.5</td>
<td>1.51</td>
</tr>
<tr>
<td>10</td>
<td>0.421</td>
<td>330.3</td>
<td>1.64</td>
</tr>
<tr>
<td>11</td>
<td>0.395</td>
<td>308.0</td>
<td>1.67</td>
</tr>
<tr>
<td>12</td>
<td>0.361</td>
<td>278.8</td>
<td>1.74</td>
</tr>
<tr>
<td>13</td>
<td>0.357</td>
<td>275.3</td>
<td>1.77</td>
</tr>
<tr>
<td>14</td>
<td>0.381</td>
<td>295.9</td>
<td>1.63</td>
</tr>
<tr>
<td>15</td>
<td>0.376</td>
<td>291.6</td>
<td>1.70</td>
</tr>
<tr>
<td>16</td>
<td>0.371</td>
<td>265.0</td>
<td>1.59</td>
</tr>
</tbody>
</table>
After the exponential amplification phase, a linear amplification phase using PCR with forward primers only was carried out to generate enough ssDNA for the next SELEX round. Figure 5-4 shows the typical linear amplification phase RT-PCR curves. No significant amount dsDNA was formed and therefore all the curves are relatively flat. A quick and convenient method for determining the PCR end product concentration is using UV absorption at 260 nm. A standard calibration curve using the known DNA concentrations for the PCR end products concentration determination is provided in Figure 5-5. Furthermore, the purity of the DNA can be determined by the ratio of $A_{260\text{nm}}/A_{280\text{nm}}$. A ratio above 1.5 indicates high purity of the DNA samples. The corresponding PCR end product concentration measurements in Figure 5-4 are provided in Table 5.1. If the ratio is lower than 1.5, a quick DNA purification process such as ethanol participation needs to be applied before using the PCR end product for next steps.
A 5-10 fold increase of absorption at 260 nm should be achieved after the PCR run, indicating a successful linear amplification of the ssDNA.

![Graph](image)

Figure 5-5: The standard curve for DNA concentration measurement using 100 μL sample volume and O.D. 260 nm.

### 5.3.2 Blue-white Screen for Cloning

As discussed, the TOPO™ cloning kit is a blue-white screen based technique that allows for the detection of successful ligations in vector-based gene cloning in a quick and easy manner. The TOPO™ vector size is 39K bp, and the forward primer is M13 (-20). To ensure a sufficient cloning, the optimal molar concentration of the PCR product should be 2 times of the vector molar concentrations. Therefore, for 1 μL of vector, 26 pg of 100 bp of the PCR end product is needed.
Figure 5-6 shows the blue-white screen for visual cloning confirmation. Sample #1 are the original cells from the TOPO™ kit grown on a normal LB plate. This confirms the cells are alive with good vitality. Sample #2 are vector inserted cells grow on a LB plate containing ampicillin. Since the original cells do not carry the gene for resistance to the antibiotics, the growth of the cells indicates a successful vector transfer to the cells. Sample #3, #4, and #5 are the vector transformed cells growing on the ampicillin and X-gal containing LB plates using different plating methods. Sample #3 has non-uniform distributed colonies, which is due to an improper spreading process. Sample #4 has equally distributed colonies using a glass bead plating method which can help ensure the uniform spreading. Sample #5 has a low colony density, which is due to a very low vector transformation rate. The TOPO™ cells used in this study were stored in -20 °C instead of recommended -80 °C, which could possibly cause a lower cell viability after thawing. Increasing the heat shock time from 30 seconds to up to 90 seconds could increase the vector transformation rate. However, longer heat shock time would potentially damage the cells, causing cell death or lose of functionality. Therefore, the heat shock time needs to be carefully adjusted according to the final transformation rate observed from the blue-white colony plates. If most colonies are blue (sample #4), increasing the ice incubation time before heat-shock for up to 1 hour could increase the target DNA insertion rate to the TOPO vector, yielding more positive white colonies. Sample #6 shows the final colonies plated on the ampicillin and X-gal LB plates in numbered lines ready for sequencing. No plasmid extraction process is involved here since it is not needed for the commercialized sequencing service.
Figure 5-6: The culture plates for cloning and sequencing (red circles in #5 sample showing blue and white colonies).
5.3.3 Aptamer Sequence and Second Structure Analysis

Using a commercial sequencing service, the vector sequences can be obtained in two business days with a reasonable cost (~ $200 for 96 sequences; see Appendix E.4 for details). The aptamer sequences can be identified by searching the forward and reverse primer sequences in the sequencing results. If the sequences between the two primers are 44 base pairs, these 44 sequences is an aptamer candidate. For the first SELEX run, 5 culture samples (cloned from the 8th round SELEX end product) were sequenced and 4 out of 5 samples showed positive insertion. The random regions of the sequenced ssDNAs are listed in Table 5.2.

<table>
<thead>
<tr>
<th>#</th>
<th>Sequences of random region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CTCTCCGGCCGCTGACCCAGTTTGGAGGGGGAGGAGGAGGCCGCGG</td>
</tr>
<tr>
<td>2</td>
<td>ACGGCCACTGGTTTCATCCGCGATGAGATGTGTGTCAACTTTAT</td>
</tr>
<tr>
<td>3</td>
<td>CAATAACCGATTGTATCAGAAACGTTTAACTTTGGATCTCTTT</td>
</tr>
<tr>
<td>4</td>
<td>TAGCGACACACGTGGCCGCTGGTTCGCCGGGAGCCACGGATCTTT</td>
</tr>
</tbody>
</table>

In Table 5.2, the common regions within the aptamers have been highlighted. The most common region is “GGA”, which appears 6 times in all 4 aptamers except aptamer 2. To better understand the potential binding sites within the aptamer, a secondary structure analysis was conducted and the results are shown in Figure 5-7. It clearly shows that all the aptamers form at least 1 loop structure which contains > 10 bp in the mid-region and an overall bulge–loop structure, which might be essential for exclusive recognition of and binding to HSA. One of the glycated HSA (GA) binding aptamers identified by Higashimoto’s group is shown in Figure 5-10 [195]. This aptamer also has a bulge–loop structure. Higashimoto’s group indicated the ACC or CCA region or so called “motif”
might be the potential binding sites for the GA. An additional SELEX run involving a lightly glycated HSA for counter-selection (see Section 5.4 for detailed description) was also conducted and the aptamer sequences are provided in Appendix H.

Figure 5-7: Predicted secondary structures of the HSA binding aptamers corresponding to sequences in table 5.2.
(Temperature: 25 °C; Na concentration: 100 mM, Mg concentration: 1 mM)
5.4 Future Research and Conclusion

An albumin molecule has multiple sites available for glycation [196]. Different from the glycation level which usually refers to the percentage of the albumin that has been glycated with regard to the total albumin, glycation rate refers to how many sites within a single albumin molecule have bound to glucose. It is expected that the 3D conformation and the charge distribution are significantly different between full GA and HSA, but very similar between the lightly glycated GA (single GA) and HSA. Therefore, as mentioned in Section 3.2, the development of a specific single GA binding aptamer that has low affinity to HSA could be challenging. The SELEX protocol needs to include a counter-selection process as shown in Figure 5-9 using MB-SELEX as demonstration. Similar to a normal MB-SELEX process, a large random DNA pool is initially mixed with the target immobilized MBs to select the DNAs with higher binding affinities. After releasing the bound aptamers from the target protein (i.e., single GA), the control protein (i.e., HSA) is used to remove the non-target protein bound aptamer. Upon the removal of the HSA binding DNAs, only single GA binding aptamers will be selected. This method

Figure 5-8: Predicted secondary structure of a GA binding aptamer [195].
allows for the development of a single GA specific aptamer that has a low affinity to HSA. For future research, this counter-selection SELEX method is essential to generate aptamers that have ability to distinguish analytes with similar chemical structures.

Figure 5-9: A demonstration of counter-selection involved MB-SELEX process for single GA binding aptamer development.

In this chapter, the HSA binding aptamers have been successfully developed via a MB-SELEX process. In the next chapter, the glycated albumin binding aptamers will be identified and the binding performance will be compared with the existing sensing methods. To develop a GA level sensor, both the GA and HSA binding aptamers will be used to enable specific detection, which will be discussed in the chapter 7.
Chapter 6

A Comparison Study of Phenylboronate Monolayer and Aptamer-based SPR Biosensor for the Specific Detection of Glycated Albumin

6.1 Introduction

Human Serum Albumin (HSA) binding aptamers have been developed according to the experimental details provided in Chapter 5. In this chapter, Glycated Albumin (GA) binding aptamers have been identified and the binding performance has been compared to the existing well documented phenylboronate monolayer. Phenylboronate monolayer-based biosensors have been reported for the real-time direct detection of the glycated hemoglobin by SPR [197], quartz crystal microbalance [198] and electrochemical [199], which are based on the mechanism that the boronic acid can form covalent bonds to the surface sugar sites of a glycated protein, as shown in Figure 6-1. This reaction occurs significantly faster in a basic aqueous environment compared to neutral or acid aqueous environments. Therefore, most phenylboronate monolayer-based sensors require a basic
environment for the optimal binding. One of the most commonly used boronate molecules for monolayer formation on gold is 4-mercaptophenylboronic acid (MPBA) and it was used to form the phenylboronate monolayer in this study. To our knowledge, all documented phenylboronate-based sensors are for the detection of pure sugars or glycated hemoglobin. No such sensor for glycated albumin (GA) or complex samples has been reported yet. In this study, both a phenylboronate and glycated albumin binding aptamer monolayer have been immobilized onto gold and the minimal immobilization time, optimal binding condition, sensor sensitivity and specificity have been investigated to provide an insightful view of applying different receptors for the GA sensing. The developed sensor has shown a better specificity and a broader working pH range over the well documented phenylboronate layer-based sensors for the detection of GA.

Figure 6-1: The molecular structure of MPBA and the binding between sugar (glucose as shown) and boronic acid group.
6.2 Materials and Experimental

6.2.1 Materials and Instrumentation

The sequences of the glycated albumin binding aptamers were adopted from the Yamagishi et al.’s work [195] and synthesized by Integrated DNA Technologies (Coralville, IA): **APT1**: 5’-CH₅(CH₂)₅-(SH)₂-(CH₂)₆-CGGAAACCAGACCACCCCA-ACCAAGGCCACTCAGTCGAACCAGCCAACACTCACCCCA-3’; **APT2**: 5’-CH₅(CH₂)₅-(SH)₂-TAACTCAGTCATACTTGACTGATTGTGCAAACAACACACCCCTAAACAGTC-3’. The thiol termination of the aptamer allows for direct binding of the aptamer to the gold surface. These aptamers contain cytosine-rich sequences and show typical loop structures. According to Yamagishi et al.’s conclusion, the cytosine-rich bulge-loop structure and the ACC(C) or (C)CCA motif may be essential for exclusive recognition of the glycation sites within the protein [195]. The Reductacryl™ resin was obtained from Calbiochem (Darmstadt, Germany) to reduce the thiolated aptamers for better immobilization. Glycated hemoglobin was purchased from US biological (Swampscott, MA). All protein samples were prepared fresh before testing. All other chemicals and proteins were purchased from Sigma Aldrich (Carlsbad, CA) at the highest purity available.

SPR measurements were performed with a commercial grade BIAcore® 3000 system (GE Lifescience, Pittsburgh, PA) at 25 °C with SIA kit sensor chips. Extra gold sensing slides were manufactured via a physical deposition of 1 nm of thickness of titanium and 50 nm thickness of gold onto pre-cleaned microscope glass cover slides.
6.2.2 Experimental

The phenylboronate modified sensing surface was prepared by immersing the pre-cleaned gold slides in 1 mM MPBA in ethanol for 12 hours. For the thiol-aptamer modified sensing surface, the aptamer was reduced by the Reductacryl\textsuperscript{TM} resin with 1 mM pH 7.5 PBS buffer at a ratio of 1 mg aptamer to 50 mg resin and the mixture was stirred at room temperature for 15 min. Then the aptamer modified sensing surface was prepared through immersing the pre-cleaned gold slides in 1 µM reduced aptamers in 1 M pH 8 phosphate buffer containing Reductacryl\textsuperscript{TM} resin at 37 °C and shaking at 100 rpm for 12 hours. After the incubation, the MPBA treated gold slides were washed with ethanol and the aptamer treated gold slides were washed by DI water and then vacuum dried at room temperature. Before sample loading, the sensing surface was normalized with corresponding binding buffer for ~5 min until the SPR response stabilized. Sample loading was performed by the Biacore 3000 auto-sampler using a flow rate of 5 µL/min. All experimental data was recorded and processed by BIAevaluation software.

6.3 Results and Discussion

6.3.1 SAMs Formation on Gold

To achieve optimal SPR performance, it is very important to create a uniform and stable receptor layer on gold to enable specific detection. Generally, thiol binding is the most widely used method for gold surface functionalization because thiol containing molecules are capable of direct binding to gold via a covalent bond. The SPR sensor is able to monitor the formation of the SAM on gold in real-time. Figure 6-2 presents sensorgrams showing SAM formation of MPBA on gold within the first 10 min (inner
plot showing the baseline shift of the SPR response over different sample loading cycles).
The baseline shift of the SPR response indicates the amount of molecules absorbed by surface. After the first cycle of sample injection, a ~ 200 RU in baseline shift was achieved. After the 10\textsuperscript{th} cycle of sample injection, the baseline shift only increased to ~ 370 RU. This indicated more than half of the MPBA layer was formed within the first minute of the injection. To monitor of the formation of the different SAMs on gold, MPBA in ethanol (the MPBA group in Figure 6-3), Reductacryl\textsuperscript{TM} resin reduced thiol aptamer in PBS buffer (the S-APT group in Figure 6-3) and non reduced thiol aptamer in PBS buffer (the S-APT-NR group in Figure 6-3) have been applied onto the sensing surface and the SPR baseline shift (measured as RU) were recorded, as shown in Figure 6-3. All SAMs formed much faster in the beginning since more free surface area was available for binding. The MPBA-based SAM had an overall faster formation than thiol aptamers. Considering the molecular weight of MPBA is significantly lower than the aptamers’, this also suggests a faster and tighter packing of MPBA layer on gold [200]. Non-reduced thiol aptamer SAMs also formed slower than those made with the reduced thiol aptamers. After 2 hours of immobilization time, only half as much of non-reduced thiol aptamer (~ 700 RU) had been adsorbed onto gold as the reduced thiol aptamer (~ 1700 RU). This indicates the need of reduction of the thiol-modified aptamers to guarantee a sufficient surface coverage.
Figure 6-2: SPR sensorgrams showing the formation of different MPBA SAM on gold for the first 10 min.

Figure 6-3: SPR response baseline shift during the formation of different SAMs on gold; S-APT=thiol-aptamer; NR= non-reduced.
6.3.2 Surface Binding: Immobilization Time Effect

After certain periods of immobilization, both GA and non-glycated human serum albumin (HSA) samples were loaded onto fresh prepared surfaces. The sensitivity of the sensor is reflected by the maximal sensor responses, which are the RU values in Figure 6-4. The selectivity is determined by the ratios of the sensor response to the target (GA) and non-target (HSA), which are the S values in Figure 6-4. In Figure 6-4a, for the 2 hours immobilization group, the MPBA-based sensor had the highest RU response for a given sample concentration, indicating the best sensitivity among all of the sensors. The S-APT2-based sensor had the best selectivity (highest S value). In Figure 6-4b, for the 12 hours immobilization group, however, the S-APT2-based sensor showed the highest RU response while the MPBA-based sensor had the best selectivity. These results indicate that the immobilization time can affect the sensor performance to a great extent. In both cases, the non-reduced aptamer showed the worst performance in both sensitivity and selectivity. The low selectivity might result from a non-specific immobilization of the aptamer onto the gold surface. The aptamers just aggregated onto gold instead of covalently binding, resulting in a non-uniformly organized layer. This demonstrated the need of reduction before immobilization of thiol modified aptamers. The 12 hour immobilization time group generally showed better sensor performance, which should result from more uniformly and completely packed SAMs. Therefore, for thiol-SAMs that directly formed on gold in this study, at least 12 hours immobilization time is needed to guarantee a good sensor performance. Considering the RU response of MPBA-based sensor (Figure 6-4b, MPBA group, brown bar) was only ~ 30% less than the S-APT2-based sensor (Figure 6-4b, S-APT2 group, brown bar) for the target (GA protein), while it
was almost four times better in selectivity, the MPBA-based sensor had the best overall performance for sensing pure GA/HSA. To test the sensing activity for complex samples, such as free sugars or other glycated proteins are presented in the sample, more experiments have been performed and the results show that aptamer is better choice for complex sample sensing, as shown in the following sections.

![Figure 6-4: SPR response baseline shift versus SAM immobilization time of 2 hours and 12 hours. The loaded samples were 1 µg/mL GA or HSA in 50 mM pH 9 phosphate buffer for MPBA-based sensors and 1 µg/mL GA or HSA in 50 mM pH 7.4 phosphate buffer for aptamer-based sensors. S=Selectivity (ratio of response of GA/HSA).](image)

6.3.3 Surface Binding: pH and Ion Strength Effect

The biological binding effects are highly dependent on binding temperature, pH, buffer type, and ion strength. In this section, how pH and ion strength affect the sensor response was investigated. From Figure 6-5, it is clearly shown that the sensors perform differently with different binding conditions. Since the APT2-based sensor has shown a slightly better sensitivity and selectivity for GA, only the APT2-based sensor was investigated.

It has been reported that the phenylboronate layer binds to sugar quicker and more stable in basic aqueous solutions [201]. So the MPBA-based sensors were mainly pH
dependent. Changing from pH 7 to pH 9, both the \( R_{\text{max}} \) (maximal binding) and baseline shift (abbreviated as BS in Figure 6-5), had been increased for more than two folds. The pH also impacted the aptamer-based sensor, but not as much as it affects the MPBA-based sensor. It is known that the pH affects the 3D conformation of both single strand DNA and proteins. Since the aptamer-protein binding is mainly conformation and charge dependent, pH 7 is best for aptamer / target binding (light green bar in pH 7 group has the highest RU among all groups) since it is close to the physiological pH in which the aptamers were developed. For all pH values, the aptamer-based sensor had better sensitivity (larger in \( R_{\text{max}} \) values) than MPBA-based sensors, indicating that aptamer-based sensors were more capable of wide pH range sensing applications. It also should be noted that in pH 11, which was far from the physiological pH, the aptamer-based sensor still showed higher \( R_{\text{max}} \) and more bound protein than pH 5 and pH 9. This might result from that negatively charged albumin aggregated onto the sensing surface and formed multiple layers [202].

For the ion strength test, to avoid other influence on the binding activity, only NaCl solution was involved to control the overall ion strength. As ion strength increased from 1 mM, the aptamer-based sensor sensitivity also increased. However, as the ion strength reached 50 mM (the pH 7 group), the sensor sensitivity started to decrease. Therefore, the optimal NaCl concentration for binding should be between 10 mM to 100 mM for APT2. For the MPBA-based sensors, the response increased slightly as the ion strength increased from 1 mM to 100 mM, and decreased by half as the ion strength increased to 1000 mM. This demonstrated that the ion strength did not impact the sensor response as it affected the aptamer-based sensor. In general, MPBA-based sensors had much smaller
standard errors (the error bars in Figure 3 are the standard errors calculated from 3 independent measurements) compared to the aptamer-based sensors. This should result from the simplicity and the uniformity of the MPBA SAM.

**Figure 6-5**: SPR response ($R_{\text{max}}$) and baseline shift (BS) in different binding conditions for 1 µg/mL GA. The $R_{\text{max}}$ values were obtained 5 min after the sample injection; the BS values were obtained 1 min after reloading the binding buffer. The pH binding buffer contains 50 mM NaCl. The ion strength binding buffer contains NaCl from 1mM to 1000 mM. The data were obtained from 3 independent measurements with standard deviation for the error bar. The inside plot is the real-time SPR response curve for S-APT2 pH 7 binding test. Sample Raw RU data for generating this plot is provided in Appendix I.

### 6.3.4 Sensor Sensitivity

The functionalized SPR sensor responses for GA concentrations from 0.2 µg/mL to 1µg/mL are shown in Figure 6-6. The APT2 calibration curves have higher slopes than
the MPBA calibration curves, indicating a higher sensor sensitivity for the aptamer-based sensor. As mentioned previously in chapter 2, both $R_{\text{max}}$ and baseline shift can be potentially treated as the sensor output. MPBA-based sensor generally had a more linear response in both $R_{\text{max}}$ and baseline, making it ideal for sensing pure GA samples. The $R_{\text{max}}$ of aptamer-based sensor was also linear. However, the baseline shift response of the aptamer-based sensor tended to be exponential, which should result from the limited availability of binding sites. The $R_{\text{max}}$ was also highly dependent on sample refractive index, so it could potentially be affected by other high concentration components in the sample. The baseline shift signal is mainly caused by the amount of specific binding, which is related to the target concentration. Therefore, the baseline shift should be a better choice as the signal for complex sample sensing and it will be adopted for the binding specificity study.

Figure 6-6: Sample concentration dependent SPR responses.
6.3.5 Sensor Selectivity

For complex samples containing free sugars such as glucose or other glycated proteins such as glycated hemoglobin (GH), the binding results for the MPBA and APT2-based sensors are shown in Figure 6-7 (the error bars are the standard error calculated from 3 independent measurements). For the MPBA-based sensor, although the binding condition was consistent, the sensor responses were greatly dependent on the overall free sugar and glycated proteins appearing in the sample. In Figure 6-7, the results show that the MPBA layer bound to GA, GH, glucose and a mixture of them, but not HSA (the small orange bar in Figure 5 MPBA group). This was due to MPBA having a general affinity to all free sugars and glycated proteins, and HSA did not contain any sugar. While for APT2-based sensor, the response was mainly determined by the primary target (GA) concentration. In Figure 6-7 APT2 group, the sensor had a very minimal response to glucose and GH, indicating that the specific binding resulted from the conformational change of the albumin introduced by glycation, not just the attached sugar molecules. The relatively high response to HSA (compared to the orange bar in MPBA group) should result from the physical adsorption of the protein onto the surface, rather than specific binding between the aptamer and the target. Since MPBA SAM was generally more uniform and dense, as discussed previously, the physical adsorption effect was not as significant for MPBA sensor as it for APT2 sensor. These results demonstrate that the MPBA functionalized sensor is not specific for complex samples (i.e., blood or plasma which contain free sugars and various of glycated proteins). Aptamer-based sensors, on the other hand, are more specific to their target even in complex samples. Even with the appearance of free sugars (i.e. glucose) and other glycated proteins (i.e. glycated
hemoglobin), the sensor response remained almost unchanged. This shows great potential for applying aptamer-based sensors in real world samples.

Figure 6-7: SPR response baseline shift versus different sample loaded onto MPBA and APT2-based sensors. The loaded samples were 1 µg/mL GA, HSA or GH in 50 mM pH 7.4 phosphate buffer. The glucose concentration was 1 mg/mL. The data were obtained from 3 independent measurements with standard deviation for the error bar. Sample Raw RU data for generating this plot is provided in Appendix I.

6.3.6 Further Optimization of the Aptamer-based Sensing Protocol

Ideally, thiol-terminated molecules can form SAMs on gold directly. However, larger molecules such as aptamers can form complex 3-D structures and the double thiol bond may not be exposed to the gold surface. Therefore, a pre-treatment of the single strand DNA can help shorten the immobilization time and generate a more uniform SAM for more reliable sensing [203]. For aptamer functionalization, the surface coverage is
mainly determined by the aptamer immobilization time and concentrations [172]. Closely packed aptamers might prevent the target molecules from approaching their respective binding sites efficiently and inhibit themselves from folding into secondary structures. While loosely packed aptamers will allow the non-targets to directly contact to gold surface, introducing undesired noise. Therefore, a two-step immobilization procedure, such as using amine terminated aptamer [204] or biotinylated aptamer with streptavidin as the supporting layer [205] could be adapted to further enhance the sensor performance, and also potentially to reduce the physical adsorption effect for HSA.

6.4 Conclusion

A direct real-time GA test has a potential to significantly impact diabetes diagnosis and monitoring. To our knowledge, for the first time, an aptamer-based SPR biosensor for the detection of GA has been demonstrated. The developed sensor has shown a better specificity and a broader working pH range over the well documented phenylboronate layer-based sensors for the detection of GA. The developed thiol surface functionalization methods and optimized binding conditions can also be directly applied to other surface binding-based biosensors such as quartz crystal microbalance, electrochemical sensor, and atomic force microscopy tip. Using only the SPR technique, the optimal immobilization time, binding condition, and binding specificity of different surface functionalization methods for the detection of GA have been studied. In blood, only a portion of the total protein will be glycated. Giving this fact, it is the percentage of the glycated protein with respect to the total protein, rather than the exact concentration
of the glycated protein, that is of interest. The next chapter will present a novel multi-
channel SPR setup for the real-time GA level sensing.
Chapter 7

Multi-channel SPR Sensor for the Real-time Detection of Glycated Albumin level

7.1 Introduction

As discussed in Chapter 6, in blood, only a portion of the total protein will be glycated and it is the percentage of the glycated protein with respect to the total protein, rather than the exact concentration of the glycated protein, that is of interest. In this chapter, a novel multi-channel aptamer-based SPR setup was developed using the BIAcore® 3000 SPR system for the real-time detection of the GA level. The flow cell path of the BIAcore® 3000 is shown in Figure 7-1. This system has 4 independent flow cells (Fc1, Fc2, Fc3 and Fc4) consisting of three internal switches and five in and out pathways. This specific design not only allows for use of four flow cells independently, but also enables the sample solution to be injected continuously though two or four of the flow cells (Figure 7-1 dark flow cells showing sample continuously flow though Fc1 and Fc2). Therefore, by functionalizing four flow cells with different bio-receptors, it is possible to measure up to four analytes in a complex sample with one injection. This forms the fundamental design of the multi-channel SPR sensor.
In chapter 5, HSA binding aptamers have been successfully developed by MB-SELEX. In chapter 6, GA binding aptamers have been characterized and have shown a better sensor performance compared to the well documented phenylboronate monolayer-based sensor. By immobilization of both GA and HSA binding aptamer to different flow cell channels, it is possible to measure both GA and HSA with one injection and the percentage of GA (GA level) can then be calculated.

![Figure 7-1: Biacore 3000 flow cell pathways [136].](image)

### 7.2 Materials and Experimental

#### 7.2.1 Materials and Instrumentation

To guarantee a quick and sufficient surface aptamer immobilization, biotinylated aptamers were adopted in this study. All the aptamers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The GA binding aptamer **G-APT**: 5’-biotin-
TAACTCACTCCATACTCACTTGGCTGATTCCACAACAAACACACCCCTAAACAGTCCTCCGCGGCTGACCCAGTTTGAGGGGGAGGAGGCCGGGC-3’ was the aptamer with better binding affinity in chapter 3; and the HSA binding aptamers **H-APT1**: 5’- Biotin-CTCTCCGGCCGCTGACCCAGTTTGAGGGGGAGGAGGCCGGGC-3’ and **H-APT2**: 5’- Biotin-ACGGGCACCTGGTTCCATCCGA-TGAGATTGTGATGTGTAACCTTAT-3’ were the SELEX end products obtained from table 5.5. Single glycated albumin was obtained from US Biological (Swampscott, MA). Streptavidin MBs (M-280) were purchased from Invitrogen (Carlsbad, CA). All other chemicals and fully glycated albumin were purchased from Sigma Aldrich (Carlsbad, CA) at the highest purity available.

SPR measurements were performed with a commercial grade BIAcore® 3000 system (BIAcore AB, Sweden) at 25 °C with the SIA kit sensor chips. The extra gold sensing slides were manufactured via physical deposition of 1 nm of thickness of titanium and 50 nm thickness of gold onto a pre-cleaned microscope glass cover slides.

### 7.2.2 Immobilization of Biotinylated Aptamers

The biotinylated aptamer immobilization process is very similar to the amine terminated aptamer immobilization as presented in Chapter 3, with the exception that after the EDC/NHS activation, streptavidin was immobilized onto the surface as the supporting layer before the aptamer injection. Figure 7-2 shows real-time monitoring of the formation of different layers on the gold using SPR. The gold slide was immersed in 5 mM ethanolic MPA solution for 2 hours to introduce carboxyl groups on the surface. It was then washed by ethanol and dried at room temperature. The cleaned slide was then
coupled to the sensor chip (see Appendix B.2 for details) to carry the following injections, which correspond to the injection steps shown in Figure 7-2.

1. Baseline (running buffer: water; flow rate: 5 µL/min)

2. EDC/NHS to activate the sensing surface (0.2M/0.05M in water, injection time: 7 min)

3. ~300 RU increase in baseline

4. Injection of streptavidin (400 µg/mL in 10 mM acetate buffer pH5)

5. ~4000 RU in baseline shift

6. Ethanolamine (EA) to deactivate excess reactive esters and remove non-specific binding (1M EA+HCl, pH 8.5, injection time: 7 min)

7. Immobilized SA = ~4000 RU.

8. Injection of Biotinylated aptamer (100 µg/mL in 1 X TE, 1 M NaCl, pH 7.5, injection time: 5 min).

9. Total immobilized biotinylated aptamer = ~650 RU
Figure 7-2: SPR sensorgram showing a typical immobilization sequence for biotinylated aptamer.

The non-specific bindings between the aptamers with the non-targets observed in Chapter 6 (i.e., G-APT to HSA non-specific binding) can introduce errors to the final calculated GA levels. To minimize these errors, in this study, two types of sensor setup were investigated to identify the flow path design that involves less error. For the type one setup, the flow cells 1 and 2 (Fc 1 and Fc 2) were functionalized with G-APT, and Fc 3 and Fc 4 were functionalized with H-APT1 and H-APT2, respectively. For the type two setup, the Fc 1 and Fc 2 were functionalized with H-APT1 and H-APT2 and the Fc 3 and Fc 4 were functionalized with G-APT. After the immobilization, different levels of GA (prepared by using the BIAcore® auto mixing function of 0.1 µg/mL of HSA and 0.1 µg/mL of GA v/v) were injected into all four flow cells and the baseline shift of the sensorgram was recorded. For reference, GA is fully glycated protein obtained from
Sigma, unless specified. Sensor regeneration was performed by the injection of 4 M NaCl for 5 min. Other sensor regeneration methods have also been tested and the results are discussed in Section 7.2.4.

**7.2.3 Sensing Results and Data Processing**

Assuming that the $F_{c1}$, $F_{c2}$, $F_{c3}$, $F_{c4}$ stand for the baseline shift of the RU after the injection, for the type one setup, the amount of total bound $GA = F_{c1} + F_{c2} = A$; the amount of total bound $HSA = F_{c3} + F_{c4} = B$. For the type two setup, the amount of $HSA = F_{c1} + F_{c2} = B$, and the amount of $GA = F_{c3} + F_{c4} = A$. The GA level can be given by:

$$
\%GA_{\text{Raw}} = \frac{A}{A+B} = t, \quad (7.1)
$$

This is the raw model of the multi-channel binding. However, as shown in Chapter 4, the GA binding aptamer sensor can non-specifically bind to a small amount of HSA. This could result in a higher measured GA value and introduce an increased error. Therefore, assigning the amount of non-specific binding of HSA to the first two flow channels as $n$, then the raw model can be revised as:

$$
\%GA_{\text{NR}} = \frac{A-n}{A+B}, \quad (7.2)
$$

This is the non-specific binding model (NR model), where $n$ is equal to the $A$ value obtained by a 0% GA injection (Table 7.1, 1st data line). More specifically, $n$ is determined with an assumption that the nonspecific binding ratio is fixed for a given concentration of GA. Since the $n$ value is also proportional to the total amount of HSA, for more accurate modeling, it is necessary to consider the total amount of HSA with
respect to the $n$ value. A feedback NR model-based on the raw model calculation result is given below:

$$\%GA_{F-NR} = \frac{A-n^{1-t}}{A+B}, \quad (7.3)$$

In this model, the $t$ value was first calculated from Equation 7.1. Then the amount of non-specific HSA binding was obtained based on the $t$ value. This is similar to the feedback concept in signal processing. This model should be well suited for GA and HSA mixed sample since regardless of the amount of nonspecific binding, the total amount of proteins A+B will be accurate. Therefore, only the acute value of GA needs to be adjusted. However, for more complicated samples such as blood, more advanced models should be incorporated for accurate data processing.

The raw data and the predicted GA levels of the type one setup are shown in Table 7.1.

<table>
<thead>
<tr>
<th>GA level</th>
<th>Fc1 (RU)</th>
<th>Fc2 (RU)</th>
<th>Fc3 (RU)</th>
<th>Fc4 (RU)</th>
<th>Raw</th>
<th>NR</th>
<th>F-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.21</td>
<td>10.28</td>
<td>168.82</td>
<td>32.68</td>
<td>0.139</td>
<td>0.011</td>
<td>0.019</td>
</tr>
<tr>
<td>0.1</td>
<td>29.87</td>
<td>13.31</td>
<td>142.25</td>
<td>31.42</td>
<td>0.199</td>
<td>0.061</td>
<td>0.079</td>
</tr>
<tr>
<td>0.2</td>
<td>36.98</td>
<td>21.53</td>
<td>127.44</td>
<td>32.27</td>
<td>0.268</td>
<td>0.131</td>
<td>0.160</td>
</tr>
<tr>
<td>0.3</td>
<td>41.56</td>
<td>40.1</td>
<td>94.52</td>
<td>12.15</td>
<td>0.434</td>
<td>0.274</td>
<td>0.335</td>
</tr>
<tr>
<td>0.4</td>
<td>62.51</td>
<td>29.62</td>
<td>72.56</td>
<td>14.53</td>
<td>0.514</td>
<td>0.347</td>
<td>0.426</td>
</tr>
<tr>
<td>0.5</td>
<td>82.63</td>
<td>35.12</td>
<td>64.61</td>
<td>12.34</td>
<td>0.605</td>
<td>0.451</td>
<td>0.538</td>
</tr>
<tr>
<td>0.6</td>
<td>100.31</td>
<td>66.25</td>
<td>44.21</td>
<td>25.02</td>
<td>0.706</td>
<td>0.579</td>
<td>0.665</td>
</tr>
<tr>
<td>0.7</td>
<td>121.52</td>
<td>32.94</td>
<td>23.37</td>
<td>17.94</td>
<td>0.789</td>
<td>0.636</td>
<td>0.754</td>
</tr>
<tr>
<td>0.8</td>
<td>134.14</td>
<td>25.21</td>
<td>19.52</td>
<td>9.77</td>
<td>0.845</td>
<td>0.686</td>
<td>0.818</td>
</tr>
<tr>
<td>0.9</td>
<td>125.36</td>
<td>12.53</td>
<td>14.51</td>
<td>11.32</td>
<td>0.842</td>
<td>0.659</td>
<td>0.811</td>
</tr>
<tr>
<td>1</td>
<td>142.58</td>
<td>11.24</td>
<td>4.82</td>
<td>5.63</td>
<td>0.936</td>
<td>0.754</td>
<td>0.925</td>
</tr>
</tbody>
</table>

The GA levels calculated from data in table 7.1 based on different models are presented in Figure 7-3. The Feedback NR model had the highest R square value,
indicating that it has the most accurate sensing results among the three models. It should be pointed out that for a normal physiological sample, the GA value would be below the 0.2 range. For GA level 0.1, the Raw model has a high error of 99%, where the NR model has a relatively lower error of 39% and the feedback NR model is most accurate one with the lowest error of 21%. For GA levels below 0.2, the NR models (both feedback and non-feedback) were slightly below the reference line, indicating that too much non-specific binding had been subtracted from the amount of \( A \) value. For GA levels above the 0.8 range, all predicted values were also below the references line. This might due to the reduced availability of the binding sites causing a smaller amount of non-specific bindings (too much non-specific binding \( n \) had been subtracted from the amount of \( A \) value). This also suggests that adjusting the non-specific binding constant (\( n \)) could further enhance the accuracy of the model.

Figure 7-3: Calibration curve using different models for the type one setup. (n=33)
The raw data and the predicted GA levels of the type two setup are shown in Table 7.2. The GA levels calculated from data in this table are presented in Figure 7-4. Although the overall R square values were lower than the type one setup, the GA levels below 0.2 tended to be more accurate. For GA level 0.1, the NR and feedback NR models have a low error of 12% and 23%, respectively. For GA level 0.2, the NR and feedback NR models have even lower errors of 10% and 8%. This should due to the non-specific binding of HSA binding aptamers to GA is much lower than the GA binding aptamer to the HSA. This also can be demonstrated by the $n$ values, where $n = 33$ for type 1 setup and $n = 14$ for type 2 setup. Lower $n$ value indicates less non-specific binding. Therefore, this type two setup produces less error. Although the overall R square values were lower, for physiological samples, the type two setup should be a better choice for its accuracy below the 0.2 GA level range.

Table 7.2: Raw data for the second type setup (HSA binding aptamer as Fc 1 and Fc 2, GA binding aptamers as Fc 3 and Fc 4). Raw, NR, and F-NR are the predicted GA levels using the Raw, NR, and feedback NR models.

<table>
<thead>
<tr>
<th>GA level</th>
<th>Fc1 (RU)</th>
<th>Fc2 (RU)</th>
<th>Fc3 (RU)</th>
<th>Fc4 (RU)</th>
<th>Raw</th>
<th>NR</th>
<th>F-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>191.52</td>
<td>21.2</td>
<td>9.82</td>
<td>4.23</td>
<td>0.062</td>
<td>0.000</td>
<td>0.004</td>
</tr>
<tr>
<td>0.1</td>
<td>152.12</td>
<td>20.64</td>
<td>16.23</td>
<td>21.2</td>
<td>0.178</td>
<td>0.112</td>
<td>0.123</td>
</tr>
<tr>
<td>0.2</td>
<td>134.25</td>
<td>21.54</td>
<td>29.32</td>
<td>24.6</td>
<td>0.257</td>
<td>0.190</td>
<td>0.208</td>
</tr>
<tr>
<td>0.3</td>
<td>112.49</td>
<td>17.21</td>
<td>40.96</td>
<td>11.27</td>
<td>0.287</td>
<td>0.210</td>
<td>0.233</td>
</tr>
<tr>
<td>0.4</td>
<td>101.3</td>
<td>9.4</td>
<td>40.28</td>
<td>15.36</td>
<td>0.335</td>
<td>0.250</td>
<td>0.290</td>
</tr>
<tr>
<td>0.5</td>
<td>74.21</td>
<td>5.17</td>
<td>52.91</td>
<td>17.82</td>
<td>0.471</td>
<td>0.378</td>
<td>0.434</td>
</tr>
<tr>
<td>0.6</td>
<td>41.6</td>
<td>8.54</td>
<td>49.81</td>
<td>26.92</td>
<td>0.605</td>
<td>0.494</td>
<td>0.566</td>
</tr>
<tr>
<td>0.7</td>
<td>37.8</td>
<td>8.34</td>
<td>71.5</td>
<td>13.56</td>
<td>0.648</td>
<td>0.542</td>
<td>0.611</td>
</tr>
<tr>
<td>0.8</td>
<td>42.6</td>
<td>7.47</td>
<td>81.24</td>
<td>24.6</td>
<td>0.679</td>
<td>0.589</td>
<td>0.650</td>
</tr>
<tr>
<td>0.9</td>
<td>35.6</td>
<td>6.26</td>
<td>87.5</td>
<td>36.9</td>
<td>0.748</td>
<td>0.664</td>
<td>0.721</td>
</tr>
<tr>
<td>1</td>
<td>9.22</td>
<td>9.54</td>
<td>89.21</td>
<td>34.2</td>
<td>0.868</td>
<td>0.770</td>
<td>0.855</td>
</tr>
</tbody>
</table>
Figure 7-4: Calibration curve using different models for the type two setup. (n=14)

The previous sample injections were performed by sequentially in order of increasing GA levels. To test the sensor performance for randomized sample injections, additional random series injections were performed and the raw data is shown in Table 7.3. The GA levels calculated from the data are presented in Figure 7-5. For randomly injected samples, the feedback NR model still maintained a relatively high R square value (i.e., 0.975), indicating that a reliable measurement could be achieved by a multi-channel setup and a proper data processing method. Since the binding time was limited, not all the analyte in the sample will be bound to the surface (i.e., in all the raw data tables, the Fc2 and Fc4 still have positive RU responses). Therefore, a second channel that captures the unbound analyte could significantly improve the accuracy of the sensing results.
Table 7.3: Raw data for type one setup sensor; random GA level samples injection. Raw, NR, and F-NR are the predicted GA levels using the Ras, NR, and feedback NR models.

<table>
<thead>
<tr>
<th>GA level</th>
<th>Fc1 (RU)</th>
<th>Fc2 (RU)</th>
<th>Fc3 (RU)</th>
<th>Fc4 (RU)</th>
<th>Raw</th>
<th>NR</th>
<th>F-NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.5</td>
<td>12.32</td>
<td>171.68</td>
<td>48.42</td>
<td>0.143</td>
<td>0.003</td>
<td>0.023</td>
</tr>
<tr>
<td>0.8</td>
<td>172.42</td>
<td>28.02</td>
<td>41.9</td>
<td>21.3</td>
<td>0.760</td>
<td>0.624</td>
<td>0.728</td>
</tr>
<tr>
<td>0.2</td>
<td>46.24</td>
<td>22.52</td>
<td>135.62</td>
<td>38.81</td>
<td>0.283</td>
<td>0.135</td>
<td>0.177</td>
</tr>
<tr>
<td>0.6</td>
<td>116.41</td>
<td>27.2</td>
<td>49.8</td>
<td>21.2</td>
<td>0.669</td>
<td>0.501</td>
<td>0.614</td>
</tr>
<tr>
<td>0.4</td>
<td>71.82</td>
<td>27.24</td>
<td>72.14</td>
<td>31.2</td>
<td>0.489</td>
<td>0.312</td>
<td>0.399</td>
</tr>
<tr>
<td>0.7</td>
<td>131.21</td>
<td>42.9</td>
<td>31.21</td>
<td>21.49</td>
<td>0.768</td>
<td>0.609</td>
<td>0.731</td>
</tr>
<tr>
<td>0.1</td>
<td>22.31</td>
<td>19.82</td>
<td>124.2</td>
<td>22.24</td>
<td>0.223</td>
<td>0.033</td>
<td>0.075</td>
</tr>
<tr>
<td>0.3</td>
<td>38.12</td>
<td>22.98</td>
<td>78.7</td>
<td>21.1</td>
<td>0.380</td>
<td>0.156</td>
<td>0.241</td>
</tr>
<tr>
<td>0.5</td>
<td>59.24</td>
<td>32.19</td>
<td>54.24</td>
<td>21.42</td>
<td>0.547</td>
<td>0.332</td>
<td>0.450</td>
</tr>
<tr>
<td>0.9</td>
<td>111.23</td>
<td>28.56</td>
<td>12.72</td>
<td>9.98</td>
<td>0.860</td>
<td>0.639</td>
<td>0.829</td>
</tr>
</tbody>
</table>

Figure 7-5: Calibration curve using different models for the type two setup. (n=36)
7.2.4 Sensor Regeneration

For current study, all the sensing surfaces were regenerated by 4 M NaCl as shown in Figure 7-6. After the sample injection, a baseline shift was observed, indicating a successful surface binding. A followed injection (NaCl regeneration) only removed ~ 80% of the bound sample in this case. From the raw data tables, using a same sensor chip with 4 M NaCl regeneration, the overall sensitivity of the sensor (i.e., the sum of RU of all four channels, \( A + B \)) was also decreasing. This is due to the limited availability of the binding sites and a less than 100% regeneration rate. The thrombin binding aptamers in Chapter 4 were much shorter ssDNAs that only formed simple 3D structures and the bindings were mainly charge dependent [206]. An acid solution (i.e., phosphoric acid) could successfully regenerate the sensing surface and prepare it for the next sample loading without losing the sensitivity. In this study, however, aptamers were much longer (> 40 bases) and formed more complex 3D structures. After testing different common regeneration methods, it is found that the longer aptamer-based sensing surface could not be 100% regenerated by acid (phosphoric acid), basic (NaOH), and high salt concentration (NaCl). This indicates that the binding between the longer aptamers and its target protein is both shape and charge dependent. Sodium dodecyl sulfate (SDS) is a commonly used surfactant that can be potentially applied for the regeneration of surface binding-based sensors. For investigation purpose, we injected 0.1% SDS to the protein bound surface, and the baseline shift became negative. This indicates that not only the proteins had been removed, but also part of the supporting layers. Based on this, SDS is too strong a surfactant for the sensor regeneration. Also, increasing the regeneration solution injection time for longer than 5 min did not help the regeneration rate. Although,
the design of a reusable sensor is not the primary objective of this study, for future commercialization, it will be very beneficial to develop a proper regeneration protocol. A mixed regeneration solution such as PPA mixed with NaCl, or low concentration of SDS mixed with NaCl may potentially regenerate the sensing surface sufficiently.

![Sensorgram](image)

**Figure 7-6:** An example sensorgram showing the sensor regeneration by NaCl after sample injection.

It is expected that using fresh sensors (no sensor regeneration) for each sample could result in a better accuracy. For the multi-channel sensor setup used in this chapter, it is the ratio of the two analytes that is of interest, not the overall concentration. Therefore, even with the reduced availability of the binding sites, for multiple sample injections with regeneration, the sensor still maintained a relatively high accuracy for detection of GA
levels. For example, for the GA level 0.1, all the predicted values (type one and type two setups) were below the ~25% error range.

7.2.5 Protein Glycation Rate vs. Glycation Level

An albumin molecule has multiple sites available for glycation [196]. Different from the glycation level which usually refers to the percentage of the albumin that has been glycated with regard to the total albumin, glycation rate refers to how many sites within a single albumin molecule have bound to glucose. All the previous data in this study was obtained using GA samples from Sigma, which is a fully glycated albumin sample (high glycation rate). For a comparison study, the lightly glycated albumin (i.e., single site glycated) purchased from US Biological (Swampscott, MA) was also used to test aptamer to protein binding. Since the GA binding aptamer was developed using fully GA as the target, the aptamers were able to specifically bind to the target as demonstrated in Chapter 6. It is expected that the 3D conformation and the charge distribution are significantly different between full GA and HSA, but very similar between the lightly glycated albumin (single GA) and HSA. This has been investigated by the MB-based binding capacity test as the results shown in Figure 7-7. The experimental method of the MB-based binding capability test was similar to that presented in Section 4.2.2 using streptavidin magnetic beads.

In Figure 7-7, for the HSA binding aptamer group (H-APT), full GA had a relatively low binding ratio to the H-APT (~ 3%), and a high binding ratio (~ 85%) to the single GA and HSA. This indicates that the H-APT can distinguish full GA and HSA, but not single GA and HSA. However, the binding ratio was slightly different between the single
GA and HSA, suggesting a slight difference in the 3D conformation. This was as expected since the aptamer was developed for the full GA binding.

Figure 7-7: Aptamer to protein binding ratio determined by the MB coupling method. The fully glycated albumin (Full GA) was obtained from Sigma. The lightly glycated albumin (Single GA) was purchased from US biological.

For the GA binding aptamer group (G-APT), full GA had a relative high binding ratio to the G-APT (~ 84%), while the binding ratios to the HSA and single GA were lower. Although G-APT should not specifically bind to HSA, almost half of the single GA and HSA were bound to the protein. This corresponds to the previous observation that HSA can aggregate onto the aptamer surface. In both groups, the binding activities of single GA and HSA were very similar to each other, indicating that there was only a slight conformational difference between the two. Since most specific bindings between
aptamers and the target proteins occur relatively quickly (~ 5 min as shown from the SPR results), and a 12 hours incubation time was used for the MB-based binding study, more non-specific bindings were expected. The real binding between the HSA and G-APT should be much lower.

The 3D conformation difference between the single GA and HSA is small. Therefore, as mentioned in Section 3.2 and Section 5.4, the development of a specific single GA binding aptamer could be challenging. For the future development of highly GA specific aptamers, the counter-selection SELEX method should be introduced to generate aptamers that have ability to distinguish samples with similar chemical structures, as discussed in details in Section 5.4.

7.3 Conclusion

In this chapter, a multi-channel SPR sensor setup using both GA and HSA binding aptamers has been presented and characterized. A feedback data processing model was developed to further improve the accuracy of the measurement. To our knowledge, this is the first reported real-time GA level sensor that does not need full sensor regeneration for multiple sample measurements. It has great potential to be further tuned towards product commercialization to help the diagnosis and treatment of diabetes. The aptamer development protocol and the multi-channel detection setup can also be directly applied in other protein biomarker analysis applications.
Chapter 8

Conclusion and Future Research

8.1 A Comparison of Aptamer Modification for Immobilization

In this study, three different modifications have been used for aptamer immobilization. Table 8.1 summarizes the advantages and differences for each modification method.

Table 8.1: A comparison of different aptamer modifications.

<table>
<thead>
<tr>
<th>Modification method</th>
<th>Amine</th>
<th>Thiol</th>
<th>Biotin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter</td>
<td>4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>cost</td>
<td>low</td>
<td>high</td>
<td>medium to high</td>
</tr>
<tr>
<td>immobilization time</td>
<td>fast (~ 10 min)</td>
<td>slow (overnight)</td>
<td>fast (~ 10 min)</td>
</tr>
<tr>
<td>ease of immobilization</td>
<td>3 steps</td>
<td>1 step direct immobilization</td>
<td>4 steps</td>
</tr>
<tr>
<td>reduction</td>
<td>not needed</td>
<td>needed</td>
<td>not needed</td>
</tr>
<tr>
<td>suitable for long aptamers (&gt; 40 bases)</td>
<td>not suitable, or needs pre-treatment</td>
<td>suitable</td>
<td>very suitable</td>
</tr>
<tr>
<td>amine containing immobilization buffer</td>
<td>not applicable</td>
<td>applicable</td>
<td>applicable</td>
</tr>
<tr>
<td>acidic immobilization buffer</td>
<td>not applicable</td>
<td>not applicable</td>
<td>applicable</td>
</tr>
</tbody>
</table>

For shorter aptamers (< 20 bases) such as thrombin binding aptamers in Chapter 4, the amine modification is well suited (low cost and fast immobilization time). However, for longer aptamers (> 40 bases), the amine modified aptamer has a low immobilization rate.
This is most likely due to the complicated 3D structure of the longer aptamer which prevents the exposure of the amine group to the surface. A pre-treatment of the single strand DNA could help shorten the immobilization time and generate a more uniform monolayer for more reliable sensing [203]. For the thiol modified aptamer used in Chapter 6, although the immobilization requires the longest time, it is the simplest process among all the modification methods (i.e., no need of MPA pre-immobilization and EDC/NHS treatment). Therefore, to evaluate newly developed aptamers, thiol-modification is preferred due to its simplicity. However, since the surface coverage is not guaranteed and the aptamer distribution is not optimized, the sensor performance may be hindered. Using a direct immobilization process, thiol modification lacks a supporting layer (i.e., MPA layer as used in Chapter 4 and Chapter 7). Therefore, small non-relevant molecules could potentially enter the detectable range of the sensor, introducing noise to the sensing result. This could be improved by using a hybrid self-assembly monolayer (SAM) such as thiol aptamers mixed with other small thiol containing molecules (i.e., cysteine and glutathione). Since thiol modified aptamers need a relatively long immobilization time, for multi-channel SPR setup, it is not practical to functionalize multiple channels individually using the auto sampler (i.e., functionalize all four channels using the BIAcore® system will need more than 48 hours). Therefore, biotin modified aptamers were adopted in Chapter 7 for multi-channel SPR sensing. A biotin molecule has four specific binding sites available, and the binding has an ultra-high affinity (i.e., $K_D = \sim 10^{-14} \text{ M}$) in a wide range of pH and ion strength [207]. Figure 8-1 shows the molecular structure of biotin. The size of the biotin molecule is much larger than the thiol and amine groups. Therefore, it is much easier to reveal itself to the coupling sites. In
most cases, biotin modified aptamers do not need specific pre-treatment for the immobilization. The main disadvantage of biotin modification is the high cost and the need of specific sample handling. Streptavidin is a relatively expensive protein which needs to be freshly prepared and properly stored. The experimental process is also more complicated than other methods. The immobilization process needs four steps for four different layers and each step needs a specific buffer.

![Figure 8-1: Molecular structure of biotin.](image)

**8.2 Future Research Recommendations**

**8.2.1 Aptamer Modifications**

In this study, all the aptamer modifications are performed by attaching certain groups to the 5’ end of the aptamer, which allows for the immobilization of the aptamers. It is also known that inserting a hydrophilic group such as ethylene oxide onto the 5’-end of the aptamer may help reduce nonspecific protein binding and a longer hydrocarbon spacer tends to further increase the sensor sensitivity [171]. Figure 8-2 shows an example aptamer modification to enhance sensor performance. However, the two-step immobilization method as described in Section 4.2.2 should be a more cost effective and controllable method compared to adding all of the modifications to the aptamer monomer at once. Also, as discussed in Section 6.3.6, a pre-treatment such as high salt
concentration or heat can help reveal the binding group to the surface to achieve a better surface coverage.

![Hydrocarbon modification](image)

Figure 8-2: Aptamer modification for enhanced sensor performance.

### 8.2.2 Multi-layer / Mix-layer Functionalization

Better performance of the sensor may possibly be achieved by using a mixed length spacer layer [189], such as 11-mercaptoundecanoic acid (MUA) combined with 3-mercaptopropionic acid (MPA) to increase the sensitivity and specificity [190]. Figure 8-3 shows an example setup for planar surface and AuNP-based mixed length SAM. This may be due to the fact that such a configuration can potentially help to form and maintain the specific shapes of the immobilized aptamers. By specifically designing the SAM (i.e., optimizing the hydrocarbon length and introducing co-adsorbents [191]), the sensor performance can be further improved in terms of the sensitivity and selectivity.

![Multi-layer functionalization](image)

Figure 8-3: An example setup for planar and AuNP surface-based mixed length SAM [208].
8.2.3 Further Optimize the Binding Buffer, SELEX Procedure, and Data Processing Models

The binding buffer can be further optimized by introducing different concentrations of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) ions. Albumin is the main \( \text{Ca}^{2+} \) binding protein in blood [209]. It is necessary to consider the \( \text{Ca}^{2+} \) involved binding since the plasma sample contains \( \text{Ca}^{2+} \). In future SELEX runs, physiological levels of \( \text{Ca}^{2+} \) (~ 10 mg/dL) could be included in the protein-aptamer binding buffer to select aptamers better suited for physiological sample sensing applications. As discussed in Section 5.4, the 3D conformation difference between the lightly glycated and non-glycated HSA is small. The SELEX protocol needs to include a counter-selection process. Moreover, other glycated proteins (i.e., HbA1c) binding aptamers can also be developed to cover a broader long-term glycation control time frame. As mentioned in Section 7.2.3, the current developed non-specific binding models probably will not be well suited for complex samples such as blood since there will be more types of different non-specific binding involved. More non-specific binding parameters should be taken in consideration for future model modification is more complicated samples are used.

8.2.4 Increase Sensitivity by Introducing Gold Nanoparticles (AuNP)

As mentioned in chapter 2, AuNP has been applied to SPR to enhance the sensor sensitivity. Figure 8-4 shows different sizes of spherical AuNPs and color change from blue to red as the size decreases. Figure 8-5 shows the monitoring of the formation of Au-nanorods (AuNRs) using UV-Vis absorption spectroscopy and the formed AuNRs. These
nanoparticles were previously synthesized by our group and the experimental protocol is provided in Appendix J. For aptamer-based SPR sensor, the aptamer can be immobilized onto the AuNP and then mixed with the analyte for detection. The binding of the target to the surface aptamer will also bring the AuNP within the detection range, and thus significantly enhance the PSPR sensor sensitivity. Since the binding will introduce the overall change of the size, or an aggregating effect of the AuNPs, the absorption peak is expected to be change in the UV range (i.e., color change as shown in Figure 8-4). Simple colorimetric sensors can also be developed based on aptamer immobilized AuNP. However, due to the low sensitivity of the UV detection method, the performance of this sensor would not be comparable to the SPR-based sensor.

Figure 8-4: Size dependent color change of the AuNP. (a) TEM image showing AuNPs ~ 500 nm in diameter; (b) TEM image showing AuNPs ~ 50 nm in diameter; (c) AuNP color change from blue to red as the size decreases.
8.2.5 Using SPRi or LSPR to Enable High Throughput Sensing

Similar to the multi-channel setup, SPRi can enable simultaneous detection of multiple analytes and require only a small amount of sample. This is well suited for aptamer-based detection since multiple aptamers can be easily developed by SELEX. The feedback data processing method introduced in Section 7.2.3 can also be directly applied to SPRi. LSPR, as discussed in Section 2.2.3, is less sensitive than the conventional PSPR, however, it can be directly coupled with 96 wells to enable high throughput colorimetric sensing. Figure 8-6 illustrates the LSPR slides in a 96 UV well plate. The SEM image of the uniformly distributed AuNP coated LSPR slide is shown in Figure 2-8.
Figure 8-6: An illustration of LSPR slides in the 96 UV wells.

Figure 8-7: (a) An actual LSPR sensor setup
(b) An illustration of a fiber optics-based LSPR setup
Portable and low cost LSPR can also be achieved by the use of fiber optics. Figure 8-7 shows a prototype fiber optics-based LSPR developed by our group. The white light source (i.e., broad spectrum in visible range) was connected to an optical fiber which contained six single fibers to transfer the light to the LSPR surface and a center fiber to direct the back reflected light to a portable spectrometer. The absorption peaks related to the surface RI information can be obtained. Through the use of a proper fluidics design and a specific surface functionalization method, it is possible to perform biosensing using this setup in real-time.

8.2.6 Hybrid Sensor to Further Enhance Performance

SPR has also been reported in combination with other sensing technique to further enhance the performance such as Raman spectroscopy [133], electro-chemical (EC) [210], mass spectroscopy (MS), and high performance liquid chromatography HPLC [134]. For aptamer-based SPR, the aptamers can also be immobilized in a chromatography column to separate different analytes in the sample and each analyte can then be measured by SPR with general affinity to the analytes (i.e., carboxyl group functionalized surface). For chromatography, even though the affinity of the aptamer to different targets is only slightly different, as long as the flow path is long enough, different analytes can still be separated and detected by SPR with high sensitivity. When a aptamer with high specificity cannot be developed, the combination of chromatography technique with SPR can be a practical solution. Conventionally, chromatography uses UV-Vis spectrometer for detection. UV-Vis spectroscopy is not a sensitive detection technique which requires relatively high sample concentration and sample volume. Since SPR is much more
sensitive (see Table 2.1 for sensitivity comparison), the resolution is expected to be much higher for the chromatography and SPR hybrid sensor than the conventional HPLC. In the EC and SPR hybrid sensor [210], for SPR detection, the light and the prism are on the backside of the gold layer. Therefore, it will not interfere with the electrodes of the EC sensor attached to the gold surface. EC and SPR sensors generally provide similar information about the surface binding. The combination of the two sensing techniques could obtain the EC and SPR data simultaneously without the need for additional surface modifications. The two different types of data can be further characterized for more accurate sensing results.

8.3 Conclusion of This Research Project

In this research project, the feasibility of using an aptamer-based surface plasmon resonance (SPR) sensors for the real-time detection of glycated blood proteins (i.e., GA) has been studied. For the purpose of establishment, the well documented thrombin binding aptamers were immobilized onto a gold sensing surface using a two-step amine coupling method. The experimental results show that the aptamer functionalized SPR is a well suited sensing platform for selective blood protein detection applications.

To obtain the specific receptors that enable selective detection, the human serum albumin (HSA) binding aptamer has then been developed using the magnetic beads-based Systematic Evolution of Ligands by EXponential amplification process (MB-SELEX). The experimental protocol of the in vitro selection process have been standardized (i.e., target protein immobilization, DNA binding and releasing, PCR process, and DNA
cloning). The developed aptamers have shown a common pattern in sequence and a similarity in secondary structures.

Furthermore, the glycated albumin (GA) binding aptamers have been identified and the sensor performance has been characterized under different binding conditions. The aptamer functionalized sensor has shown a better performance in both sensitivity and selectivity for the detection of GA compared to the well documented phenylboronate monolayer-based sensor, indicating that aptamers are a better receptor choice for GA detection applications.

Finally, a novel multi-channel aptamer functionalization sensor and a feedback data processing model have been developed based on the glycated and non-glycated albumin binding aptamers. The experimental results demonstrate that the aptamer-based SPR sensor has the ability to detect GA levels within a 25 % error range. This method also has potential to be further developed into a reusable sensor.

This dissertation provides the essential foundation experiments to demonstrate the feasibility of using aptamer-based SPR sensor for the detection of glycated protein. It is expected that the further engineering and improvement of the sensor will eventually lead to a fast, reliable, label-free, and cost-effective glycated protein detection sensor, which may prove useful in the diagnosis and treatment of diabetes.
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Appendix A

Matlab Codes for PSPR Simulation

```matlab
%%%Method: rigorous Fresnel reflection theory
%%% Different thickness of Au layer

np=1.51;                       %%%BK7 glass
nd=1.328;                      %%%water
ep=np^2;                        %%%n=sqrt(e), since u-->1
ed=nd^2;
em=-25+1.44i;                  %%%Au
lamda=800;                      %%%wavelength in nm
theta=60:0.001:70;              %%%incident angle
kz=2*pi./lamda*np.*sind(theta); %%%incident wave vector
for q=40:10:70
    %%%Au thickness in nm
    kpx=sqrt((2*pi./lamda).^2*ep-kz.^2);
    kdx=sqrt((2*pi./lamda).^2*ed-kz.^2);
    kmx=sqrt((2*pi./lamda).^2*em-kz.^2);
    rpm=(em.*kpx-ep.*kmx)./(em.*kpx+ep.*kmx);
    rmd=(ed.*kmx-em.*kdx)./(ed.*kmx+em.*kdx);
    rpmd=(rpm+rmd.*exp(2i*q.*kmx))./(1+rpm.*rmd.*exp(2i*q.*kmx));
    R=abs(rpmd).^2;
    phi=phase(rpmd)+pi;            %%%phase of the complex number rpmd
end

%%%Result:
%%% q=40nm  SPR=66.080
%%% q=50nm  SPR=65.924
%%% q=60nm  SPR=65.868
```

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To find SPR angle vs sample refractive index

```matlab
np=1.51; % BK7 glass
nd=1.328; % water
ep=np^2; % n=sqrt(e), since n->1
ed=nd^2;
em=-25+1.44i; % Au

lamda=800; % wavelength in nm
theta=60:0.001:70; % incident angle
q=50; % Au thickness in nm
kz=2*pi./lamda*np.*sind(theta); % incident wave vector
x=1.300:0.001:1.333
for m=1:length(x)
    nd=nd+0.001
    ed=nd^2;
    kpx=sqrt((2*pi./lamda).^2*ep-kz.^2);
    kdx=sqrt((2*pi./lamda).^2*ed-kz.^2);
    kmx=sqrt((2*pi./lamda).^2*em-kz.^2);
    rpm=(em.*kpx-ep.*kmx)./(em.*kpx+ep.*kmx);
    rmd=(ed.*kmx-em.*kdx)./(ed.*kmx+em.*kdx);
    rpmd=(rpm+rmd.*exp(2i*q.*kmx))./(1+rpm.*rmd.*exp(2i*q.*kmx));
    R=abs(rpmd).^2;
    phi=phase(rpmd)+pi; % phase of the complex number rpmd
    [C,I]=min(R)
end
```

SPR=59.999+I/1000

```matlab
a(m)=SPR
end
plot(x,a)
plot(theta,R)
```
Appendix B

SPR Systems Extra Notes

B.1 SensiQ® SPR system:

Common Trouble Shooting:

1. No RU response; the response curve is a flat line

   Possible cause: the gold slide is placed in the opposite side or the gold slide is not clean

   Solution: replace a new gold slide and make sure the gold side is facing up towards the flow cell; run a normalization process following the guidance in the SensiQ® control software

2. SPR dip profile is not normal

   Possible cause: the gold slide is cracked or the gold slide is not clean

   Solution: check if the gold slide is cracked; run a normalization process following the guidance in the SensiQ® control software; wash the injectors using ultrasonic bath.

   When couple a gold slide to a stripped sensor, cut the slide to exactly fit the sensing
area. Unfit sized slide is very likely to be cracked as pushing the sensor towards the flow cell

3. The response curve is continues increasing or decreasing

Possible cause: the temperature is not stable; the flow cell is not clean; air bubble in flow cell

Solution: wait until the room temperature is stable; before injecting any sample, make sure they are in room temperature; run a normalization process following the guidance in the SensiQ control software; repeat this step if problem still exist. Use only DI water as the running buffer; inject binding buffer first to establish a baseline, then inject binding buffer containing sample for evaluations.

Experimental Notes:

1. Removing the original Au surface from the SensiQ sensor:

To remove the gold layer from the original SensiQ® sensor, prepare a clean 96 well plate, a beaker of water (~ 1 L), and saturated HCl and HNO₃. Transfer 150 µL of HCl and 50 µL of HNO₃ to a well plate; pipette to mix well. Apply ~ 50 µL of the mix to the gold surface and using the pipette tip to gently scratch the surface. This will gradually dissolve and remove the gold, leaving a black optical window as shown in Figure B-1. Repeat this step multiple times to make sure all the gold has been removed. The acid mix solution (known as the aqua regia) is extremely corrosive and need to be handled carefully in fume hood. The used solution need to be collected and diluted with water before disposal.
2. SensiQ® expert settings:

In case of the SPR dip is shifted out of the detection range or sensor performance is not stable. Adjusting the parameters in SensiQ® expert setting could potentially fix the problem. Open the SensiQ® software and press the hot key “Ctrl + Shift + E” to call the expert setting window. The default parameters are shown in Figure B-2. Please note that changing those parameter will not do physical damage to the system, however, it might introduce potential problems such as inaccurate flow control and difference in sensitivity. When the lab-made gold slide is coupled onto the sensor, the SPR dip might show in a different position. The “Left pixel” and “Right Pixel” should be adjusted accordingly to keep the dip position in the middle of the detection range.
Figure B-2: The default parameters for the SensiQ® Expert Settings

B.2 BIAcore® 3000 SPR System:

Common Trouble Shooting:

1. **No RU response, the response curve is a flat line.**
   
   **Possible cause:** the gold slide is placed in the opposite side or the gold slide is not clean

   **Solution:** replace a new gold slide and make sure the gold side is facing up towards the center square hole of the holder.

2. **Sample injection doesn’t affect the RU response curve or liquid coming out from the injection site.**

   **Possible cause:** flow cell clogged or disconnected tubing.

   **Solution:** first check all the tubing in the cabinet are correctly connected; reconnect the disconnected tubes in the cabinet;
If problem still exists, run an unclogging process following the guidance in the working tool menu;

If problem still exist, run a superclean process following the guidance in the working tool menu; Repeat this step until problem fixed.

3. **The response curve is continues increasing or decreasing.**

   **Possible cause:** the temperature is not stable or the flow cell is not clean.

   **Solution:** wash the flow cell system with DI water using a high flow rate (~100 µL/min); Use the build-in temperature control; Use just DI water as the running buffer and a separate channel running the binding buffer only as the reference to eliminate the RI change introduced by the buffer.

4. **The response curve in the injecting channel will affect the response curve in other channels.**

   **Possible cause:** the gold slide is not positioning correctly; The gold slide has been cracked; Improper double tape size.

   **Solution:** see experimental note 1 2 and 3.

**Experimental Notes:**

1. Cut the gold slide to exactly fit the mold (0.37 inch X 0.45 inch); Note that the width of the slide CANNOT be any larger than 0.37 inch. Otherwise the slide WILL be cracked during docking process. This would potentially damage the internal microfluidic system. The size of the gold slide should be checked using the coupling molder provided in the SIA kit before mounting.
2. Use the gold slide holder in a good condition (no bent; surface is clean); Use the double tape matching the size of the gold slide; DONNOT use oversized double tape.

3. Cut out all the double tape in the open window area of the slide holder using a sharp blazer.

4. To obtain quality binding affinity data, use the binding buffer as the running buffer. Run a prime process before every binding. Run a sensorgram with the binding buffer only to normalize the sensing surface until the response curve is stable. This usually takes 3-5 min.

5. For concentration measurements, use only DI water as running buffer. Inject binding buffer only to establish a background. The final RU response with regard to the background is the final RU reading. Measure the baseline shift as the amount of binded analyte.

6. DONOT use any pH buffer or ion strength buffer higher than 20 mM as the standby buffer, which could potentially block the internal flow system.

7. To keep the system running properly, run a superclean process with the BIAcore® maintenance chip before leaving the system in the standby mode; especially after running a sticky protein (i.e., albumin) binding experiments.

8. The EDC/NHS solution needs to be freshly prepared. However, storing the aqueous solution of EDC and NHS separately in -20 °C and mixing them right before usage is also feasible. Make sure the mixed solution is used within 30 min after mixing.
9. For thiol-aptamer modification, using the flow cell to load the reduced aptamer solution continuously allows for the real-time monitoring the layer growth on gold. However, the sensing surface developed by this method has a poorer sensing performance compared the immersing functionalization method as described in Section 6.2.2.
Appendix C

SPR Au Slides Manufacturing Protocol

1. Pre-clean all glass slides (25 mm by 25 mm microscope cover slides) before deposition. For this glass preparation procedure the following materials are needed: four 1 L beakers, Micro-90® concentrated cleaning solution, bristle brush, sonicator with temperature controlled water bath, deionized (DI) water, glass slides (25 mm by 25 mm), glass slide holder, compressed nitrogen gas or air.

1.1 Fill the water bath of the sonicator with tap water and set the temperature to 66 °C. Allow the temperature to stabilize.

1.2 Don clean gloves. Clean the four 1 L beakers by scrubbing them with a bristle brush using a solution of 2% (v/v) Micro-90® and DI water. After scrubbing, rinse the beakers with DI water to remove any remaining Micro-90® solution.

1.3 Load glass slides onto the glass slide holder so that they are isolated from each other. The flat surfaces of the slides should not be touching each other.

1.4 Label the four 1 L beakers as Beaker 1, Beaker 2, Beaker 3, and Beaker 4.

1.5 Take Beaker 1 and fill it with 15 mL of Micro-90® and 750 mL of DI water.

1.6 Submerge the glass slide holder with the glass slides into Beaker 1.
1.7 Place Beaker 1 into the water bath of the sonicator.

1.8 Allow the cleaning solution in Beaker 1 to equilibrate to the temperature of the water bath. It should take about 5 minutes to reach 66 °C.

1.9 After Beaker 1 has reached 66 °C, turn on the sonicator and allow it to run for 30 minutes.

1.10 While Beaker 1 is in the sonicator, fill Beaker 3 with 900 mL of DI water and place it in the sonicator water bath to bring its temperature up to 66 °C.

1.11 While Beaker 1 is in the sonicator, fill Beaker 2 with 900 mL of DI water.

1.12 After 30 minutes in the sonicator, remove Beaker 1 from the sonicator. Remove the glass slide holder from Beaker 1. Discard the cleaning solution in Beaker 1. Rinse both the glass slide holder containing the glass slides and Beaker 1 with DI water.

1.13 Place the glass slide holder with glass slides into Beaker 2. Dunk the glass slide holder into Beaker 2 approximately 15 times.

*Note: do not allow water to dry on slides during transfers.*

1.14 Remove the glass slide holder with glass slides from Beaker 2 and place into Beaker 3.

1.15 Beaker 3 should be at 66 °C in the sonicator at this time. Turn on the sonicator and allow it to run for 10 minutes.

1.16 While Beaker 3 is in the sonicator, discard the contents of Beaker 2, rinse Beaker 2 with DI water, and fill up Beaker 2 with 900mL of fresh DI water.

1.17 After 10 minutes in the sonicator, remove Beaker 3 from the sonicator. Remove the glass slide holder from Beaker 3 and place it into Beaker 2. Dunk the glass slide holder into Beaker 2 approximately 15 times.

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Note: do not allow water to dry on slides during transfers.

1.18 Discard the contents of Beaker 3, rinse Beaker 3 with DI water, and fill Beaker 3 up with 900 mL of fresh DI water.

1.19 Remove the glass slide holder with glass slides from Beaker 2 and place it into Beaker 3. Dunk the glass slides holder into Beaker 3 approximately 15 times.

Note: do not allow water to dry on slides during transfers.

1.20 Fill Beaker 4 with 900 mL of DI water.

1.21 Remove the glass slide holder with glass slides from Beaker 3 and place into Beaker 4. Dunk the glass slide holder into Beaker 4 approximately 15 times.

Note: do not allow water to dry on slides during transfers.

1.22 Change to new gloves as clean gloves are needed for the next step. Remove glass slides one by one from Beaker 4 and blow the water off of the slides with compressed nitrogen.

Note: Do not allow the water to dry on the surface of the slides. “Push” the water off of the slides using the compressed nitrogen.

1.23 Place each clean dry slide into a clean, covered container to prevent dust from falling on the surfaces.

Note: This procedure can be used to clean and prepare many different surfaces for use in many different thin film deposition applications. This method has the advantage of being free from toxic or harmful chemicals and all waste is safe to go down the drain.

2.1 Either E-beam or thermal-based PVD can be used for the gold deposition. For Surface Plasmon Resonance (SPR) sensing surfaces, deposit 1 nm of Ti as the adhesion layer and 50 nm of Au as the sensing layer onto the glass slides with a deposition rate of 0.1 nm/s.
2.2 For LSPR slides, deposit 4 - 10 nm of Au layer followed by heat treatment. See reference [74] for details.

2.3 Store all slides in proper containers to prevent contaminations.
Appendix D

Matlab Codes for SensiQ® Discovery SPR Dip Profile

Evaluation

```matlab
%%input=y (23 numbers)%%adjust the file name accordingly
m=csvread('file path folder\sample.csv',1,1)
for c=1:2
    data=m(:,c)
for i=40:100
    data1(i-39)=data(i)
end
[o q]=min(data1)
q=q+39
l=16
z=q-l/2
x=z:.001:z+l
for j=1:l+1
    y(j)=data(z+j-1)
end
p=polyfit(x,y,20)
x1=z:.001:z+l
f=polyval(p,x1)
[o n]=min(f)
n=n/1000+z
subplot(3,1,c)
plot(x,y,'--',x1,f)
title(['CH' int2str(c)])
subplot(3,1,3)
plot(x,y,'--',x1,f)
title('CH1&CH2')
```

158
hold on
dip(c)=n  %%% CH1 and CH2 dip position saved
end
Appendix E

Experimental Protocol for MB-SELEX

E.1 Coupling Target Protein to MB

1) Re-suspend 165 µL of MBs in a 0.65 mL micro centrifugation tube; wash with 500 µL buffer A twice.

*Tip: For washing the MBs, put the tube on a strong magnet, remove as much supernatant as you can using a 200 µL pipette, and then remove the rest of supernatant using a 10 µL pipette carefully.*

2) Add 100 µL 1 mg/mL target protein (HSA in this study) in buffer A and 50 µL buffer A; Mix well.

3) Incubation in 37 °C for overnight.

*Note: It is better to incubate the solution in a slow shaker. If no slow shaker is available in the incubator, hand-shake the solution every 1 hour or so.*

4) Wash the beads with buffer A twice or more times.

5) Add 10 µL 1 mg/mL HSA in buffer A and 240 µL buffer A; Mix well.
Note: Every SELEX round needs 4 µL beads/sample. 240 µL is only good for 60 runs. Prepare double volume if needed for more sample runs.

6) Store the protein coupled MBs in 2-8 °C for future use.

Note: Before using the protein coupled MBs, wash the MBs twice with proper buffers to remove free proteins.

E.2 Binding and Amplification

1) Transfer 4 µL of protein coupled MBs to a PCR tube; wash with 100 µL of DI water twice. Assume a 100% binding efficiency, 4 µL of MBs can bind ~1.5 µg protein.

2) For the 1st SELEX round, add 1 uL of ~ 10 µg/mL (a total of ~ 10 ng of ssDNA) random ssDNA pool, and 49 uL of binding buffer to the washed MBs PCR tube; Mix well.

Note: shake the tube every 4-5 min to keep an equally suspension of the MBs during the mixing. When adding small quantities of the solutions to the PCR tube, make sure the tip is all the way into the solution but not reach the bottom of the tube and then inject the solution all at once. Do not re-inject even a small trace of solution is left in the pipette tip. Also, make sure all liquid is sitting in the bottom of the tube (no liquid sticking on the side).

Tip: After mixing, prepare a 95 °C water bath for step 4).
3) After 20 min of mixing, wash the MBs 3 times with binding buffer and then add 10 µl DI water

*Note:* The binding time here is an important control parameter which is mainly determined by the initial concentration of the ssDNA. Generally, the ssDNA with a high affinity to the target protein will bind to its target protein in several minutes as proven by the SPR study in chapter 3. Since the ssDNA concentration is relatively low in this step, a longer mix time is needed to ensure the sufficient bindings. However, if the incubation time is too long, after all the active binding sites been occupied, nonspecific binding could be introduced, which would result in ssDNAs with lower affinity to the protein bind to the MBs. So a proper incubation time is essential in this step. A 20 min incubation time was chosen to start with. In later SELEX rounds, since the overall aptamer affinity to protein is becoming higher, the optimal incubation time will need to be adjusted accordingly. On the other hand, increasing the initial ssDNA concentration in step 2 will also ensure the sufficient bindings to guarantee the aptamer product. However, it is found that high ssDNA concentration will also cause an aggregation effect which introduces non-specific tight bindings. This will be shown in the results section.

4) Place the tube in a 95 °C water bath with gentle shaking for 3 min

*Note: if the MBs are not equally suspension, shake the tube before putting it in the water bath*

5) In a new PCR tube, add the following pre-PCR mix:
Table E.1: PCR-1 formula

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Supernatant from the tube in step 4)</td>
<td>7.5 µL</td>
</tr>
<tr>
<td>Forward Primer (10 µM)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Reverse Primer (10 µM)</td>
<td>2.5 µL</td>
</tr>
</tbody>
</table>

6) PCR run-1(exponential magnification phase): Place the PCR tube in the sample holder in the iQ5 system, set up the parameters as follows:

Table E.2: PCR temperature cycles

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>94 °C</th>
<th>2 min</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30 sec</td>
<td>20 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>65 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>extension</td>
<td>72 °C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>Chill</td>
<td>4 °C</td>
<td>Indefinite</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

**Note:** The standard SYBR PCR protocol needs 30 cycles for the magnification step, however, in this SELEX process, it is found that all the PCR products were formed within 20 cycles. So only 20 cycles is adopted.

**Note:** In the plate setup page in the iQ5 software, make sure the volume, tube and seal types are matching with the actual samples. Otherwise the final real-time PCR plot would not be correct.

**Tip:** To help an equally heat distribution, place water containing PCR tubes to surround the sample PCR tubes in the sample holder. Otherwise it is very likely to overheat and melt the tubes. If the software is not communicating with the iQ5 system, turn off the iQ5 system (both detector and temperature control units) and the control software. Then turn on the iQ5 system, log in, press “shift+F2”, then “F5”, and reopen the control software.
**Important note**: if the software shows a warning message stating that the background file is not founded, **DO NOT** start the PCR run. Reboot the hardware and software following the above “Tip” to solve the problem.

7) After the PCR run-1, take out the PCR tube and add the following solution:

Table E.3: PCR-2 formula

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>Forward Primer (10 uM)</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>DI water</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

8) PCR run 2 (linear magnification phase): this step uses only forward primer to generate ssDNA and a same temperature cycles as shown in Table E.2. Assume a prefect amplification condition, after 20 cycles PCR, 95% of the DNA will be single stranded.

**Note**: make sure the sample volume is changed to 50 µL in the plate setup page.

9) Take out the PCR tube, transfer 10 µL of the solution to a UV 96-well and then add 90 µL of DI water (a 10:1 dilution). Record the absorption in 260 nm and 280 nm. Calculate the final PCR end product concentration from the 260 nm O.D. standard curve as provided in Figure 5-5. The ratio of O.D. value of 260 nm/280 nm greater than 1.5 indicating a purified DNA sample.

**Tip**: For optimal UV absorption measurement, keep the O.D. range in 0.1-1. The final PCR concentration after 20 cycles amplification is usually around 100µg/mL – 1000 µg/mL.
10) Dilute the PCR end product concentration to ~ 10 µg/mL with DI water as the new DNA pool. Add 1 uL new DNA pool, and 49 uL of binding buffer to the washed MBs containing PCR tube; Mix well.

*Note*: the protein coupled MBs should be reusable after releasing the bound DNA and washed. However, to make sure best binding results, only fresh MBs were used in this study.

11) Follow steps 1) through 10) using the new DNA pool for the next SELEX rounds.

12) For the last SELEX round, stop in step 6) PCR run 1, and increase the “final extension” time from 5 min to 15 min. There is no need to run PCR run 2 for ssDNA.

*Note*: This step is to ensure 3’ adenylation of the PCR generated DNA which is a key point to successfully clone the DNA to the TOPO vector in the cloning step.

*Note*: Since to start each SELEX round, only a small amount of new DNA pool is needed. Keep the unused PCR end product from last round in 2-8 °C. In case the PCR result is not correct, it will be a good idea to go back from last round and run the SELEX round again and also run an agarose gel electrophoresis to verify the result. An agarose gel electrophoresis protocol is provided in appendix 4.

---

**E.3 LB (Luria-Bertani) Medium Plates Preparation for Culturing Cells:**

Composition: 1% Tryptone; 0.5% Yeast Extract; 1% NaCl; 1.5% Agar; pH 7.0

Adjust pH to 7.0 and bring the total volume to 1 liter.
1) Add 15 g agar and dissolve.

2) Autoclave on liquid cycle for 20 minutes at 15 psi.

3) After autoclaving, cool to ~ 55 °C (comfortable holding in hand), add antibiotic if needed (final concentration 50 µg/mL ampicillin) and pour ~ 15 - 20 mL to each 10 cm plate.

4) Let harden (~5 min); then seal; invert; and store at cooler in dark.

**E.4 Cloning and Sequencing**

*Note: Before using, transfer the plates to 37 °C incubator for at least 1 hour.*

Before cloning, make sure the last run PCR was using both forward and reverse primers with *Taq* polymerase (the enzyme included in the Bio-rad SYBR is *Taq*) and a final extension step of 15 min to ensure 3’ adenylation. The PCR product can be verified using agarose gel electrophoresis. Then set up a 6 µL cloning reaction using the TOPO cloning kit. The PCR product concentration can be measured by UV absorption of 260 nm using the calibration curve in Figure 5-4.

*Note: all the tubes, tips and beads used in this section need to be sterilized.*

<table>
<thead>
<tr>
<th>Table E.4: TOPO cloning reaction formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product (diluted to ~ 26 pg)</td>
</tr>
<tr>
<td>Salt solution (from the TOPO kit)</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>TOPO Vector</td>
</tr>
</tbody>
</table>

1) Mix the above solution and incubate for 5 minutes at room temperature.

2) Place the tube on ice.
3) Add 2 µL of the TOPO Cloning reaction to a vial of One Shock *E.Coli* or equivalent competent cells and mix gently.

*Note:* Only swirl gently, do not vortex. The One Shock *E.Coli* cells are very fragile.

4) Incubate on ice for 5 min.

5) Heat-shock the cells for 30 -90 seconds at 42 °C without shaking.

*Note:* Increase the heat-shock time accordingly if a low vector transformation rate is observed, see results and discussion for details.

6) Place the cells on ice immediately after heat-shock for 2 min.

7) Add 250 µL of room temperature S.O.C. medium to the cells.

8) Cap the tubes and place at 37 °C water bath for 1 hour while shaking at 200 rpm

9) Beads shaking to spread 10-50 µL from each transformation onto 37 °C pre-warmed LB plates containing X-gal and 50 µg/mL ampicillin.

*Note:* for X-gal containing plates, using beads shaking method to spread 40 µL of 40 mg/mL X-gal in DMSO and incubate at 37 °C until ready for use. DMSO is recommended for the X-gal solvent because it is less toxic than the widely used dimethylformamide (DMF).

10) Incubate plates overnight at 37 °C.

11) Pick the white colonies and culture in LB medium containing 50 µg/mL ampicillin in labeled lines. After the cells grow well on the plates (~ 4 to 12 hours), the plates are ready to be shipped out for sequencing.

12) We used functional biosciens Inc. (http://functionalbio.com/web/) for the commercial sequencing service. When filling out the order form online, choose
TOPO cloning kit and M13 (-20) primer. Seal the plates with an air tight container and ship the package with ice bags.
Appendix F

Matlab Codes for Processing Raw data from the Molecular Device Spectra Max 384 Plus UV-Vis Spectrometer

```matlab
clear all
data=xlsread('file path folder\sample.xls',3,'E2:G2709')
%%%make sure there is data in the excel file and the size is matching
pt=input('Input the 96well plate label (2 digits, array first):    ')
blank=input('Input the blank plate label:      ')
col=rem(pt,10)  %%%column data
arr=(pt-col)/10 %%%array data
colb=rem(blank,10)
arrb=(blank-colb)/10
st=400     %%%spectra start and end
ed=700
z=420     %%%fitting start
l=200     %%%fitting length
i=arr
ib=arrb
m=1
for n=st:ed-1
AA(m)=data(i,col);  %%%raw data
B(m)=data(ib,colb);   %%%blank data
A(m)=AA(m)-B(m);  %%%background substration
i=i+9;
m=m+1;
end
%%%input=y
```
x=z:z+l
for j=1:l+1  \text{y=raw fitting data}
    y(j)=A(z-st+j)
end
p=polyfit(x,y,20) \text{fitting order (at least 20)}
x1=z:.001:z+l
f=polyval(p,x1)
[m n]=max(f)
n1=max(y)
n=n/1000+z
plot(x,y,'--',x1,f)
hold on
intr=25 \text{integration range}
a=n-intr
b=n+intr \text{integration start and end}
p1=polyint(p)
sum1=polyval(p1,b)-polyval(p1,a)
Appendix G

Agarose Gel Electrophoresis Protocol for PCR End Product Verification and Purification

After the last SELEX round, it is necessary to run an agarose gel electrophoresis to verify and purify the PCR end product before cloning it into the vector. If the RT-PCR result is not as expected, an agarose gel electrophoresis can also be performed to evaluate the DNA contents in the PCR end products. The TBE buffer, which is also the gel running buffer, is the 0.5X Tris-borate-EDTA prepared as follows:

1) An EDTA (ethylenediamine tetraacetic acid) solution is prepared ahead of time. EDTA will not go completely into solution until the pH is adjusted to about 8.0. For a 500 mL stock solution of 0.5 M EDTA, weigh out 93.05 g EDTA disodium salt (FW = 372.2). Dissolve in 400 mL deionized water and adjust the pH with NaOH. Top up the solution to a final volume of 500 mL.

2) Prepare a Stock Solution of TBE. Make a concentrated (5X) stock solution of TBE by weighing 54 g Tris base (FW = 121.14) and 27.5 g boric acid (FW = 61.83) and dissolving both in approximately 900 mL deionized water. Add 20 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L. This solution can be stored at
room temperature but a precipitate will form in older solutions. Store the buffer in glass bottles and discard if a precipitate has formed.

3) Prepare a Working Solution of TBE. For agarose gel electrophoresis, TBE should be used at a concentration of 0.5X (1:10 dilution of the concentrated stock). Dilute the stock solution by 10x in deionized water. Final solute concentrations are 45 mM Tris-borate and 1 mM EDTA.

A standard 1.2% agarose gel electrophoresis protocol is listed as below:

1) Agarose Gel Preparation
   a. For small gels, use 100 ml of 0.5X TBE in 100 ml bottle
   b. Add 1.2 grams agarose to bottle
   c. Microwave for 1.5 minutes, stopping every 30 seconds to swirl bottle. Continue heating until agarose is completely dissolved.
      
      Note: Important!! Do not allow agarose to boil when heating. Pay close attention to the bottle as it is heated and stop microwave at first sign of boiling.
      Swirl bottle gently and resume heating
   d. Carefully remove bottle from microwave and allow to cool to approximately 50 °C; or until you can comfortably hold bottle in hand.
   e. Add 10 L of 10 g/ml Ethidium Bromide solution to cooled agarose and gently swirl to mix. Dispose of pipet tip in 50 ml centrifuge tube for disposal with hazardous chemicals.

2) Assembly of casting basin and pouring of gel
   a. Place casting tray in casting basin and clamp tightly.
b. Place appropriate combs

c. Level basin

d. Slowly pour cooled agarose into the casting tray.

e. Remove any bubbles within the gel, particularly those around the combs using a clean, dry pipette tip.

f. Allow to cool till solid. Agarose will become translucent when cooled (~20 min).

g. After completely cooled, gently remove combs by pulling them straight up and out.

3) Assembly of running basin

a. Remove casting tray from casting basin and place in running basin

b. Samples run BLACK TO RED – be sure the gel is in the running basin in the correct configuration to run BLACK TO RED!!

c. Completely submerge gel in 0.5S TBE. All wells must be submerged, but be careful not to overfill the basin.

4) Preparation of samples

a. Combine 1 L of 6X agarose loading dye with 5 L PCR sample in small micro-centrifuges tube.

b. Combine appropriate volume of molecular weight markers with 1 L 6X agarose loading dye.

5) Loading of samples

a. Using a standard pipette tip, carefully load all 6 L of sample + loading dye into wells of gel.

b. Be very careful not to puncture the bottom of the well.

c. Add samples to well very slowly and allow to settle into bottom of well.
d. Load Molecular Weight Markers in the first and last well. If numerous samples are loaded, add several additional MWM throughout to allow easier comparison between samples and MWM.

6) Running the Gel

a. After loading the final sample, place cover on running basin making sure the electrodes are firmly seated in the lid and that there is a good connection.

b. Plug basin into power supply – again – pay close attention to connection (BLACK to BLACK, RED to RED)

c. Turn on Main Power Supply Control

d. Set parameters for run

   i. Agarose should be run at 5 V/cm – the distance is measured from positive to negative electrode, not the actual gel size. For the small size basin, use 110 V

   ii. Set mAmps for 35

   iii. Run time = 1 hour for most gels. Larger gels can be run longer, but should be checked every hour. You can hit START again without resetting any parameters if you want more distance in your gel

e. Hit START button on power supply

f. Visually confirm function by identifying “bubble curtain” which will appear within 1 minute on the negative electrode wire at the top of the gel.

7) Viewing Gels

a. Turn main power supply OFF

b. Unplug running basin from power supply.
c. Remove cover and remove gel tray.

d. Cover UV light board with Saran Wrap

e. Slide agarose gel off casting tray to Saran Wrap on UV light board

f. Replace protective covering to prevent personal damage from UV light. UV goggles and/or face shield are available if needed.

g. Turn on UV light and turn off room lighting.
Appendix H

HSA Binding Aptamers Identified from the 2nd SELEX Run

In the second SELEX run, 75 total aptamer candidates have been sequenced and the 45 positive results are listed below. Lower case base pairs indicate low accuracy; Upper case base pairs indicate high accuracy. The number after each sequence shows the total base pairs.

Forward Aptamers:

1. CCAGCTCGTAGTGGCGTCTTTTTTCATTTTGGTACTTATCGCAA (44)
2. CATGACagtaaATATTTAAATTGttgATGTCGCCGCGAGGcagC (44)
3. CAaatgccttgaagctcaactgttgacgccccctggctttgagge (45)
4. AAATTTCATGTTCACACCGTCCATGCGCCCTCTTCGGAGTGC (44)
5. TAAACACAGTTTTTCTTTGTAATACAATCtgggcCTAGGTTTTT (46)
6. TCAATGATGCAGACATGATATCAATGTGACTAATCTGAACACAC (44)
7. TAGAGCGTTAAACGCGGATTTGCATGTAtcnetcGGTACGTCCAACtgc (46)
8. cagttagggtccaaTcgtctggTgaaggTCTTCCTagcgcacA (45)
9. agcgactatgatctatctacctccactgcgcgaAacccgcgaT (44)
10. TCTCGCGAACGGCTCGTCTCCTGGAAGGTTTTCAGCGCCAAT (44)
11. ACATTTTAAATTAGCATAGAGGGTATCCATTCATATTATAC (44)
12. GGGATTCAATTCTCGAAAATGTATAATCGCTTGTTCAAGTAATA (44)
13. TATATCTTAGGTTACTGTCCTGTAAAAATGATTTTTTTCTCCCCTC (44)
14. TTTATAAGGGTTTAGACAATAGCGCATGATTACGTCTGGCTCA (45)
15. CCTCGCAATTAAGAGAGTATTGGGTCATAGCCGTCACAGTCAC (44)
16. tgcgcgcgacactgttgtgatCAtccacgctgcccaacacatt (44)
17. tcTgcgcgacACTTACCGGCGGCGAAGGCGACGCGACGTCCGA (44)
18. GCTATCGGTCACCGTCAACAGTCTTTCTCCATCTTTTGTATTTTACA (44)
19. CTAATTATGTGTGTAGTTTTGTCGTTTTATCGTACCATTACATCA (44)
20. ctgctacgagcatggactctttcgcacgcctccacacccGcttga (45)
21. ttcagcctccgcgtggtgatccgcacgccgcgtccacctcT (44)

Backward Aptamers (the actual aptamers are the complementary sequences):
22. TTTCTGAATTcctatggACGCActagtgtcAgtggtcGAcgtc (44)
23. AAATTGAATATGGTAGGTATATCCAGTTACTTATTGGCCAGCA (44)
24. GGTAGGTTGAATATATGAGACTCTTTACGAGAATGCAGACTAA (44)
25. AGCATCGTCCGATCAGTATTTATACGATTAGTTTTCTGCAACG (44)
26. TACACAGtcttgacCtagtgaATGCGcataCAGGCCGACTGActGC (47)
27. GCAATCTACTTAGAAAtngnTTGcanataATATAATCTGGATAAAA (46)
28. anncCCTAATGGAACGGTGTanWCTGCGCATAATGACT (48)
29. GccnggcAagnntgGGCCaancceGCCgaatAGAGGTACCTAGTGTCT (48)
Aptamers with High Accuracy and 44 Base Pairs:

1. CCAGCTCGTAGTGGCGTCTTTTTTCATTTGTGACTTATCGCAA
2. AAATTTCATGTTCCACACGTTCCATGCGCCCTCCTTCGAGTGC
3. TCAATGATGCAGACATATCATATGCGACTATCTGAACACAC
A simple sequence counting method was used to find the common patterns within these 20 aptamer candidates and the 4 aptamers listed in Table 5.2 (24 aptamers total). Every possible three DNA base pairs combination were treated as a group. The total numbers of the appearance of these groups among all 24 aptamers are listed in Table H.1. Note that this method was used to identify the potential aptamers sharing the most
common pattern. It is not guaranteed that the chosen aptamers have higher affinities.

Table H.1: Common pattern counting results of the developed 24 aptamer candidates

<table>
<thead>
<tr>
<th></th>
<th>AAA</th>
<th>AAT</th>
<th>ACC</th>
<th>ACG</th>
<th>AGA</th>
<th>AGT</th>
<th>ATC</th>
<th>ATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>TAA</td>
<td>18</td>
<td>21</td>
<td>12</td>
<td>11</td>
<td>25</td>
<td>23</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>TCA</td>
<td>19</td>
<td>14</td>
<td>19</td>
<td>20</td>
<td>13</td>
<td>19</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>CAA</td>
<td>17</td>
<td>22</td>
<td>12</td>
<td>9</td>
<td>8</td>
<td>15</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>CCA</td>
<td>18</td>
<td>13</td>
<td>9</td>
<td>18</td>
<td>10</td>
<td>18</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>CAA</td>
<td>15</td>
<td>19</td>
<td>9</td>
<td>11</td>
<td>15</td>
<td>23</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>GCA</td>
<td>12</td>
<td>9</td>
<td>16</td>
<td>11</td>
<td>15</td>
<td>13</td>
<td>11</td>
<td>10</td>
</tr>
</tbody>
</table>

From Table H.1, there are 8 DNA groups that have more than 20 times of appearance, 5 of which contain double “T”. All double “T” containing groups (shown in red in the above table) have relatively higher times of appearance. Therefore, the 9 aptamer candidates that contain most multiple “T”s were identified and listed as below:

1. CAATACCGATTTGTCTAAGGGAAAACGTGTAACTTTTGGATCCTT (0)
2. AAATTTTCATGTTCCCACACGTTCCATGCGCCCTCCTTCGAGTGC (1)
3. ACATTTAATTAGCATAGAGGGTGATCCATTCAATATTATATAC (7)
4. GGGATTCAATTCTCGAAATGTATAATCCGCTTTGTTCAAGTAATA (2)
5. TATATCTTAGGTTACTGTCCTGTTTAATGATTCTTTTTCCCTCTC (4)
   GCTATCGGTCCAACGTCAACAGTTCCATCTTTTGATTGTATTTACA (1)
6. CTAATTTATGTGAGGTAGTTTTGGTCGTTTTATCGTACCATTACATC (5)
7. TTAGGTCTTGCAATTCTCGTAAGAGTGCTCATTATATATCACCCTACC (5)
It was also found that an “A” (as shown in yellow above) was commonly appeared in conjunction with the multiple “T”s (as shown in red above). The number behind each sequence is the total of “A”s that next to red region. Sequence 3 and 9 has the most “A”s in yellow and they were chosen for the binding affinity test.

A quick binding affinity test was performed using the MB binding capability method as described in Section 4.2.2 and Section 7.2.5. The results are shown in Figure H-1. The H-APT1 was the HSA binding aptamers developed in the first SELEX run as listed in Section 7.2.1. The H-APT2 and H-APT3 are the aptamers developed in the second SELEX run as discussed above. It shows that the developed aptamers all have a higher binding capability of the target (i.e., HSA) than the non-target (i.e., GA), indicating they could be treated as the potential receptors for biosensor development. The H-APT1 has the best binding performance over the three aptamer candidates. It should be noted that the incubation time for H-APT1 was ~ 12 hours and for H-APT2 and H-APT3 was ~ 48 hours. Thus more non-specific binding is expected for the H-APT2 and H-APT3 groups. This should partially result in the relatively high binding ratio to the non-target. Due to the limited availability of the binding sites, more non-specific binding will also lower the aptamer to target bindings. This might explain the relatively lower binding ratio to the target compared to the H-APT1 group. More specific binding affinity test can be performed using the functionalized SPR sensor as described in Chapter 6 and Chapter 7.
Figure H-1: Aptamer to protein binding ratio determined by the MB coupling method.
Appendix I

Raw SPR RU data for Figure 6-5 and 6-6

Figure I-1. Raw RU data for generating Figure 6-5. All the data injection starting RU are normalized to zero. The signal jump is Figure 4 should result from air bubble, not the sample binding. Plot 1-4 are aptamer binding sensorgrams for GA in pH 5, pH 7, pH 9 and pH11; Plot 5-8 are aptamer binding sensorgrams for GA in Na$^+$ concentration 1mM, 10mM 100mM and 1000mM; Plot 9-12 are MPBA binding sensorgrams for GA in pH5, pH 7, pH 9 and pH 11; Plot 13-16 are MPBA binding sensorgrams for GA in Na$^+$ concentration 1mM, 10M 100mM and 1000mM. Detailed binding buffer information can be found in Chapter 6.
Figure I-2. Raw RU data for generating Figure 6-7. All the data injection starting RU are normalized to zero. Plot 1-6 are aptamer binding sensorgrams for GA, HSA, Glucose, GA+Glucose, GH and GA+GH. Plot 7-12 are MPBA binding sensorgrams for GA, HSA, Glucose, GA+Glucose, GH and GA+GH. Detailed binding buffer information can be found in Chapter 6.
Appendix J

Protocols for Gold Nano-rods (AuNRs) and Gold Nano-Particles (AuNPs) Synthesis

J.1 Au NRs Synthesis using Seed-mediated Method

Briefly, the seed solution was made by adding a freshly prepared, ice-cold NaBH₄ solution (0.3 mL, 0.01 M) into a liquid mixture composed of HAuCl₄ (0.125 mL, 0.01 M) and cetyltrimethylammonium bromide CTAB (3.75 mL, 0.1 M). The NR growth solution was prepared by adding ascorbic acid (1.152 mL, 0.1 M) into a solution composed of HAuCl₄ (7.2 mL, 0.01 M), AgNO₃ (1.08 mL, 0.01 M), and CTAB (171 mL, 0.1M). The original NRs were obtained by adding the seed solution (0.189 mL) into the growth solution (45.1 mL). The Au NRs solution was washed by centrifugation at 14,000 rpm for 12 min and re-suspended in 1 mL of DI water. This step was repeated for 3 times and the formed Au NRs solution can be stored in refrigeration for up to 3 months.

J.2 AuNPs Synthesis using Conventional Turkevich’s Method

Briefly, for a total of 100mL standard aqueous AuNPs, 95mL of an aqueous chloroauric acid solution containing 5 mg of Au was first brought to boil with a slow
magnetic stir, then 5 mL of 1% sodium citrate solution was added to the boiling solution. The solution first changed to bluish color, then purplish and eventually to ruby red. After ~30 min of boiling, the color was stable, then left the solution to cool to room temperature. The yielded spherical particles have a diameter of ~29 nm, as determined from TEM images. This method is suitable for larger quantity (> 100 mL) of AuNP synthesis without the requirement of high uniformity in size.

J.3 AuNPs Synthesis using Fast NaBH₄ Reduction Method

Briefly, first prepare 50 mM aqueous chloroauric acid in a glass vial. An aqueous stock solution of 50mM borohydride anions (BH₄⁻) in a glass beaker was made by dissolving NaBH₄ granules with the same molar amount of NaOH, guaranteeing stability for several hours at room temperature. For the nanoparticles ~ 3.2 nm in diameter, add 9.4 mL of DI water and 100 µL of the aqueous chloroauric solution to a glass vial. While stirring the glass vial on a mechanical shaker for uniform mixing, inject 300 µL of the BH₄⁻/OH⁻ solution all at once. The total weight of the aqueous solution was controlled to be 10 g so that the concentration of gold ions is 0.50 mM. The solution changed color from light yellow to orange immediately, and then to red while. Keep the vial stirred for 1 min to release hydrogen gas molecules. For nanoparticles of other sizes, the amount of the BH₄⁻/OH⁻ solution was increased from 300 to 650 µL followed by heating for 2-3 min at the boiling temperature of water on a hot plate. The AuNPs generated by this method is more uniform than the Turkevich’s method and a general smaller sizes. Binding a target to a smaller AuNP would introduce a larger percentage size change, resulting a bigger absorption peak shift. Therefore it is more suitable for colorimetric-based sensing
applications. The main drawback of this method is that the total amount of AuNP is limited (~ 10 mL per run) comparing to the Turkevich’s method (~ 1 L per run).

J.4 Tune the Size of AuNPs and the Use of AuNPs for Colorimetric Sensing

We found that acid solution and basic solution can cause AuNPs to aggregate each other, resulting a blue shift in the absorption spectrum. Caffeine and theophylline molecules tend to protect the AuNP to maintain its original size. This might due to the caffeine and theophylline molecules can bind to the surface of AuNPs to prevent them to aggregation. This finding could potentially lead to a development of AuNP-based colorimetric biosensors for the detection of caffeine and theophylline. With the introduction of caffeine or theophylline, more acid or basic solution will be needed to achieve a same level of blue shift in the absorption band. Therefore, the amount of the acid or basic solution added corresponds to the concentration of the caffeine or theophylline. This forms the basis of the AuNP-based colorimetric caffeine and theophylline biosensor.