Development of Electrochemical Immunosensors for the Detection of Clinically Important Biomolecules

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemistry

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Abstract

IUPAC defines “biosensor” as a device that uses biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect compounds usually by electrical, thermal or optical methods. The objective of this thesis was to develop cost-effective, sensitive, and selective electrochemical immunosensors (biosensors using immunosystems as the biorecognition layer) for the detection of clinically important biomolecules. Immunoassays are frequently applied in medical, pharmaceutical and food industries. Our hypothesis was that “Immunosensors based on disposable screen-printed electrodes would allow the quantification of Legionella pneumophila, adrenocorticotropic hormone and growth hormone in biological samples”. In order to prove our hypothesis, we utilized different surface reactions to immobilize the biorecognition layers on electrode surfaces, and challenged our immunosensor in clinically relevant biological samples. There is an increasing need for sensitive, selective, and rapid detection platforms and the outcome of our work would address this demand.
Herein, we reviewed the electrochemical principles and electrochemical techniques, also introduced screen-printed electrodes and surface characterization techniques (Chapter 1). Then, we introduced analytes including *Legionella pneumophila*, adrenocorticotropic hormone, and growth hormone, and the detection methods were explained with illustration (Chapter 2). The detailed protocols were described including reagents, experimental time, and instrumentation parameters (Chapter 3). We discussed the results for the detection of *L. pneumophila* bacteria in water samples, and compared the Nyquist plots with fluorescence microscopy images (Chapter 4.1). Then, we discussed the results of enzyme-immunosensors for detecting adrenocorticotropic hormone in plasma and blood samples. The alkaline phosphatase that was attached to adrenocorticotropic hormone could process substrates to form insoluble product that would precipitate on the electrode surface. This precipitation film significantly increased the charge transfer resistance, which enables ultrasensitive detection of adrenocorticotropic hormone (Chapter 4.2). Finally, we described the results of using EIS to detect rat growth hormone in plasma and blood samples (Chapter 4.3). We concluded that the development of immunosensors towards clinical and environmental assays was a lengthy process that would require further optimization studies and improvements (Chapter 5). As our hypothesis indicated, it is promising that these biosensors may soon be commercially available with the rapid advances in detection technologies.
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Chapter 1

1 Introduction

1.1 Principles of electrochemistry

1.1.1 Faradaic processes

There are two types of electrochemical processes occurring at electrode surfaces, the faradaic processes and non-faradaic processes.\(^1\)

Faradaic processes are monitored through the transfer of electrons during the redox process of the analyte\(^1\):

\[
O + n e^- \rightleftharpoons R
\]

**Equation 1.1\(^1\)**

Where \(O\) is the oxidized species, \(n\) is the number of moles of electrons \(e^-\), and \(R\) is the reduced species.

The reaction in Equation 1.1 would take place under thermodynamically or kinetically favourable potential region. The faradaic process has several steps, electroactive species being mass-transferred to the electrode surface, electron transfer across the electrode/solution interface, and the product is mass-transferred back to the bulk solution. The net rate of this simplest reaction is determined by the rate determining step, which could be either controlled by a mass transport step or an electron transfer step.\(^1\)

Mass-transport controlled reactions are controlled by the rate at which the electroactive species reach the
surface through diffusion, convection, and/or migration. These reactions obey thermodynamic relationships, and the relationship between potential ($E$) at the electrode and the concentration of electroactive species ($C_{Ox}$ and $C_{Red}$ for oxidized species and reduced species respectively) at the electrode surface ($x = 0$) is described by the Nernst equation:

$$E = E^0 - \frac{RT}{nF} \ln \frac{C_{Ox}(x = 0)}{C_{Red}(x = 0)}$$

Equation 1.2

In Equation 1.2, $E^0$ is the standard potential for the redox reaction, $R$ is the universal gas constant (8.314 J·K$^{-1}$·mol$^{-1}$), $T$ is the temperature in Kelvin, $n$ is the number of electrons transferred in the reaction, and $F$ is the Faraday constant (96,485 C). Such faradaic process obeys Faraday’s law, that $n \times 96,485$ C corresponds to 1 mole of redox species change in oxidation state.
The mass transport of molecules penetrating a unit area of an imaginary plane in a unit of time, in another flux, is dependent on several factors, including diffusion coefficient, concentration gradient, potential gradient, and charge of analyte. When diffusion, convection and migration all take place at the same time, the mass-transport process is very complicated making it difficult to study the relationship between current and analyte concentration.\(^1\) For a planar electrode, convection could be eliminated by using a quiescent solution, and migration could be minimized by adding a high concentration of supporting electrolyte to reduce the electrical field of charge. If the rate of reaction is limited only by diffusion, the relationship between current and analyte concentration would be described by the Cottrell equation:

\[
\iota(t) = \frac{nFAD_{\alpha}^{\frac{3}{2}}C_{\alpha}^{*}}{\pi^{1/2}t^{1/2}}
\]

**Equation 1.3\(^1\)**

In Equation 1.3, \(n\), \(F\), \(A\), \(D_{\alpha}^{\frac{3}{2}}\), \(C_{\alpha}^{*}\), \(t\) represent the number of electrons per ion, Faraday’s constant, active electrode area, bulk concentration of oxidized electroactive species and time, respectively. However, Cottrell equation must be used with taking the experimental and instrumental limitations into account.
The other process besides mass-transport, is the electron transfer from solution to electrode. The electron transfer process at the electrode interface for a reaction:

\[ O + n e^- \rightleftharpoons R \]

**Equation 1.4**

The energy of the electrons in the donor orbital of reduced species must be equal to or higher than the energy of electrons in the electrode for oxidation, and the energy of the electrons in the electrode must be higher than the energy in the receptor orbital of R for reduction. The relationship between current i and potential \( E \) for electron transfer limited process is described by Butler-Volmer equation:

\[
i = n F A \kappa \left[ C_{OX} e^{\frac{\alpha n F}{R T} (\varepsilon - \varepsilon^0)} - C_{Red} e^{\frac{(1-\alpha) n F}{R T} (\varepsilon - \varepsilon^0)} \right]
\]

**Equation 1.5**

In Equation 1.5, \( n, F, A, R, T, C_{OX}, \) and \( C_{Red}, \) were the same as described before. \( \kappa \) is the heterogeneous rate constant. A smaller \( \kappa \) means slower electron transfer kinetics, in a situation where there is
perturbation in \( C_o \) and \( C_{\text{red}} \), the recovery time to reach equilibrium will be longer, such system is considered to be irreversible. On the other hand, a larger \( k_0 \) means the system is considered to be in a reversible state.\(^2\) The term \( C_{\text{OX}} e^{\frac{\alpha F}{RT}(E - E_0)} \) corresponds to the anodic current, and the term \( C_{\text{Red}} e^{\frac{(1-\alpha)F}{RT}(E - E_0)} \) corresponds to the cathodic current. \( \alpha \) is the transfer coefficient with the symmetry between forward and reverse reaction. For example \( \alpha \) is 0.5 when \( C_{\text{OX}} \) is the same as \( C_{\text{Red}} \) at the beginning of the reaction, and \( \alpha \) may change as the acceleration and deceleration of cathodic and anodic current. When \( E = E^0 \), no net current is flowing, but the cathodic and anodic current component is still dynamic.\(^2\)

### 1.1.2 Non-faradaic process

Although faradaic process is usually the primary investigation process of an electrode reaction, non-faradaic process must be taken into account. Non-faradaic processes such as adsorption or desorption could change the electrical double layer, thus changing the electrode area, potential and current as described in the following section.\(^1\)
1.1.3 Electrical double layer

There is a dipole composed of charged particles at every material interface. In electrochemistry, the electrical double layer reflects the ionic zones formed in the solution as a result to compensate for the excess charge on the electrode.\(^1\)

---

Figure 1. An electrical double layer region model, where anions are specially adsorbed. (Figure 1.2.3 from *Electrochemical Methods: Fundamentals and Applications, 2nd Edition, with permission*)\(^1\)
As shown in the Figure 1, the electrical double layer has several components. The closest layer is called inner Helmholtz plane (IHP) with $x_1$ distance away from the electrode.\(^1\) IHP is the locus of point of specifically adsorbed ions and solvent molecules.\(^1\) The second layer is called outer Helmholtz plane (OHP) with $x_2$ distance away from the electrode.\(^1\) OHP is an imaginary plane passing through the center of closest solvated ions that are non-specifically adsorbed and attracted by the columbia force. After OHP, it is the diffuse layer, a three dimensional region from OHP to bulk solution. The charge density at the electrode/solution interface is given by:\(^1\)

$$\sigma^S = \sigma^i + \sigma^d = -\sigma^M$$

**Equation 1.6\(^1\)**

In Equation 1.6, $\sigma^S$ is the total excess charge density on the solution side of the electrical double layer, $\sigma^i$ is the total charge density of IHP, $\sigma^d$ is excess charge density in diffuse layer, and $\sigma^M$ is the charge density of the electrode.\(^1\)
Figure 2. The relationship between potential $\phi$ in electrical double layer with thickness $x$, when there are no specially adsorbed ions. (Figure 13.3.6 from *Electrochemical Methods: Fundamentals and Applications*, 2nd Edition, with permission) \(^1\)
As shown in Figure 2, the relationship between electrical potential $\varphi$ across the double layer region is shown. The symbols $\varphi_2$ is the inner potential at the OHP, and $x_2$ is distance from the electrode to the OHP. The potential drops linearly until OHP, and then decreases exponentially in the diffuse layer. The electrical double layer resembles an ordinary capacitor:

$$q = CE$$

**Equation 1.7**

Where $q$ is charge, $C$ is the capacitance (in farads), and $E$ is the potential. The capacitance of the double layer includes the capacitance of the compact layer $C_H$ and diffuse layer $C_G$ in series:

$$\frac{1}{C} = \frac{1}{C_H} + \frac{1}{C_G}$$

**Equation 1.8**

The charging of a capacitor, or change in electrode area give rise to a non-faradaic response known as charging current. In a potential-step experiment applied to an RC circuit, the charging current $i_c$ is:
\[ i_\varepsilon = \frac{E}{R_s} \frac{-\varepsilon}{e^{R_s C_{dl}} dt} \]

**Equation 1.9**

Whereas \( R_s \) is uncompensated solution resistance, \( t \) is time, and \( C_{dl} \) is double layer capacitance.

Measurement of double-layer capacitance gives information about the adsorption and desorption processes, also about the structure of modified electrodes.

### 1.2 Electrochemical transduction methods

#### 1.2.1 Cyclic voltammetry

Voltammetry is a technique that utilizes a potential ramp, or potential shift, and measures a resultant current. Cyclic Voltammetry (CV) is the most popular voltammetric technique that is being utilized today, because it provides information on the thermodynamics of the redox process and kinetics of the heterogeneous electron transfer.\textsuperscript{4}
As shown in Figure 3, the negative potential began to ramp at time 0 until certain potential $E_\lambda$ is reached. CV is a reversal technique, the time corresponding to $E_\lambda$ is called switching time, and that is time when the scan potential ramps in the opposite direction.
Figure 4 demonstrates a cyclic voltammogram, the potential scan would oxidize the reduced form of analyte from $E^0$ to $E_\lambda$ for $E_\lambda > E^0$. The reverse scan is from $E_\lambda$ back to $E^0$ and reduces the analyte from oxidized form to reduced form.

The capacitive current at the beginning of a scan is the charging current caused by the electrical double layer at the electrode surface, and the faradaic current is the current response related to the concentration of the target analyte. When the potential ramped up from a low value $E^0$ to a high value $E_\lambda$, a current $i_{pa}$ corresponds to the oxidation peak potential ($E_{pa}$) shown in Figure 4. $I_{pa}$ is observed due to the loss of an electron from reduced form analyte. Once the potential is reversed from a higher value $E_\lambda$ to lower one $E^0$, a peak current ($I_{pc}$) would be observed corresponding to the reduction peak potential ($E_{pc}$).
1.2.2 Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) is a useful diagnostic tool to characterize physicochemical processes. EIS allows time-dependent quantitative data about interface characteristics; thus, has been applied in various fields such as electrodeposition, corrosion, batteries, and solar cells.5-8

Impedance is shown as following equation:

\[ Z = \sqrt{R^2 + X^2} \]

**Equation 1.10**

Impedance is denoted by Z in equation 1.10, like resistance (R), it measures the ability of a circuit to resist electrical current flow, but unlike resistance, impedance is a complex number and measured by applying a small AC potential.15

\[ X = X_L - X_C \]

**Equation 1.11**

Resistor, capacitor, and inductor all have impedance. In Equation 1.10 resistance is denoted by R, and in Equation 1.11 reactance is denoted by X, reactance is further composed of inductive reactance \( X_L \) and capacitive reactance \( X_C \).

\[ Z = R \]
As shown in Equation 1.12 and 1.13, the relationship between impedance and resistance is positive, because they are in-phase, and between impedance and capacitive reactance is negative, because they are out-of-phase.¹

**Figure 5.** Nyquist plot represented as real impedance ($Z'$) versus imaginary impedance ($-Z''$). The equivalent circuit at the bottom represents the Randles’ circuit.¹⁻⁹
Nyquist and Bode plots are the two most common data presentations for EIS. In this thesis, we will introduce Nyquist plots, as the results in the later chapters were interpreted using Nyquist plots. Nyquist plot is represented with a real impedance component $Z'$ on the x-axis, and real impedance corresponds to the resistive impedance. The imaginary impedance component $-Z''$ is on the y-axis, which corresponds to the capacitive impedance. A shortcoming of Nyquist plot is that the frequencies for the data points are not shown. Different electrical circuits could be used to fit this plot and give useful information on different parameters of interest. As shown in Figure 5, Randles’ circuit is the most commonly used circuit in EIS-based biosensors, it has four parameters, bulk solution resistance ($R_s$), charge transfer resistance ($R_{ct}$), double-layer capacitance ($C_{dl}$), and Warburg impedance ($Z_w$). In a three electrode system $R_s$ is mainly contributed by electrolyte resistance, the electrolyte resistance between counter electrode and reference electrode is already minimized, but the electrolyte resistance between working electrode and reference electrode needs to be modelled in a cell. The $R_s$ is from the origin to the beginning of the semicircle, and would depend on the property and environment of the electrolyte such as the type of electrode, electrolyte concentration, electrolyte ionic strength, temperature, and geometry of the electrode. $R_{ct}$ is responsible for the kinetically controlled electrochemical reaction, shown as the diameter of the semicircle on x-axis in Figure 5. When the electrons cross the electrode-electrolyte interface, the charge is being transferred, and the resistance impeding the charge transfer is $R_{ct}$. The $C_{dl}$ corresponds to the electrical double layer capacitance, a positively charged electrode would attract a layer of negative ions and vice versa and mimics a capacitor, where an array of charged particles and oriented dipoles exist at the electrode interface.
Nyquist plot begins with high frequencies and extends into low frequencies. Open boundary finite-length Warburg impedance would apply to most biosensors, where $Z_w$ is not prominent in high frequencies, because the oscillating diffusion layer is inversely proportional to the frequency.\textsuperscript{16} Diffusion of the reactant is close to the electrode interface at high frequencies, but at low frequencies, the reactants would diffuse in a longer distance from the electrode, and it would become a mass-transport controlled reaction;\textsuperscript{16} thus $Z_w$ would become more prominent and shown as the tail after the semicircle in Figure 5.

1.3 Screen-printed electrodes

Disposable screen-printed electrodes can be mass produced at low cost.\textsuperscript{17} The process normally require a substrate that is made of inert materials such as glass fibre, PVC, polycarbonate or ceramic.\textsuperscript{18} Then a layer of ink or paste is deposited on this substrate through a patterned mesh and pushed through by a squeegee.\textsuperscript{18} The ink or paste are made of thixotropic fluid, such that it undergoes shear thinning allowing it to penetrate through the screen mesh which defines the final shape/design. Upon contact with the substrate, typically a ceramic or plastic material, the ink returns to its viscous state forming the intended shape/design with definition. The pattern of the mesh could be designed using lithographic techniques made of materials such as photosensitive gels or polyesters.\textsuperscript{18} The screen-printed electrodes (SPEs) allow each experiment to be performed on a fresh and analogous surface to prevent possible cross-contamination errors.\textsuperscript{18} Each electrode can be disposed of after use, and that can eliminate carry-over contamination from tedious cleaning.\textsuperscript{19}
Tremendous amount of research activities have been carried out in the past decade toward the miniaturization of electrochemical chips and toward the development of hand-held devices.20-23 Furthermore, these immunochips can be compatible with portable devices that are comparable to the size of a cell phone for convenient on-site measurements.24-28

1.4 Surface characterization techniques

1.4.1 Surface plasmon resonance (SPR)

Surface Plasmon Resonance (SPR) provides a non-invasive, real-time, and label-free method to study binding interactions between immobilized biomolecules and injected analytes.29-31 The technique has a range of applications, such as affinity analysis,32-33 kinetics analysis,34 epitope mapping,35 binding stoichiometry,36 concentration assay,37 and more. In this thesis, Biacore SPR X100 system was used to study the binding affinity of antibodies with antigens.

Figure 6. Surface plasmon resonance detection principle, Kretschmann Configuration. (Figure 1-2 from Surface Plasmon Resonance Methods and Protocols, with permission)38
As shown in Figure 6, Biacore SPR instruments use the Kretschmann Configuration, a gold chip is inserted between the prism and flow cell. We will define the “bottom” of the gold chip medium as the side that is in contact with the prism, and “surface” of the gold chip as the side that is in contact with the sample. The incident light of p-polarization strikes the “bottom” of the lower refractive index gold metal medium through the higher refractive index prism to achieve total internal reflection. Surface plasmon resonance is an optical phenomenon, it occurs when a polarized incident light strikes the metal surface at a certain angle under conditions of total internal reflection. When the light is reflected, an evanescent wave travel through the metal medium that exponentially decays with distance from the metal/prism interface. Metal films such as gold has delocalized electrons in the conduction band, and the evanescent wave promote the delocalized electrons to oscillate. This delocalized electron oscillation is called surface plasmon. If the momentum of the incident light matches the momentum of the surface plasmon, they would resonate; thus surface plasmon resonance (SPR) occurs. When SPR occurs, the energy of incident light would be transferred to the surface plasmon, and the delocalized electron oscillation would propagate along the interface of metal and its dielectric medium. The energy transfer process from photon to plasmon must conserve momentum and energy. Since resonance only occurs when momentum of the incident light is equal to the momentum of the plasmon, the momentum of incident light and plasmon could be described by vector functions.

The surface plasmon wave vector ($k_{sp}$) between the metal and dielectric medium is given as
In Equation 1.14, $\lambda$ is the wavelength of the incident light, $\varepsilon_{\text{metal}}$ and $\varepsilon_{\text{dielectric}}$ are the dielectric permittivity of the thin metal film and dielectric medium. The incident light wave vector ($k_i$) is given as:

$$k_i = \left( \frac{2\pi}{\lambda} \right) \times n_{\text{prl}} \sin \theta_i$$

In Equation 1.15, $\lambda$ is the wavelength of the incident light, and $n_{\text{prl}}$ is the refractive index of the prism used to polarize lights, and $\theta_i$ is the angle of incidence light.
Thus, as shown in Figure 7, SPR only occurs if the incident light strikes the gold surface at $\theta_i$, with wavelength $\lambda$ when $k_{zp} = k_i$. This would result in an SPR curve with a sharp decrease in reflectance,
and the lowest reflectance would occur at $\theta_i$ among measured incident angles.\textsuperscript{38}

![SPR sensorgram](image.png)

Figure 8. SPR sensorgram when solution passes over the sensor surface, represented as SPR angle in millidegree ($\text{m}^\circ$) versus time. \textsuperscript{38} (Figure 1-1 Surface Plasmon Resonance Methods and Protocols, with permission)\textsuperscript{38}

As shown in Figure 8, when a binding event occurs between the surface-immobilized ligand and the analyte on the gold surface, the associated increase in the mass leads to a change in the dielectric constant, thus a change in the plasmon wave vector, and further changes the shift in the resonance angle. In Biacore instruments, this shift in resonance angle is represented in a sensorgram of resonance unit (RU) versus time, where roughly 1000 RU corresponds to an angle change of $\sim 0.1^\circ$.\textsuperscript{38}
Since the surface plasmon field is affected by evanescent wave in the direction perpendicular to the interface, a change of the dielectric constant $\varepsilon_{\text{dielectric}}$ and surface plasmon resonance characteristic is only detectable if the change occurs within the penetration depth of the surface plasmon field. An SPR instrument will only be sensitive to molecular processes that occur at a distance to the metal surface that is roughly half the wavelength of the used light, usually within 200 nm from a metal surface.\textsuperscript{44}

For this thesis, Biacore SPR system was used to determine the affinity constants between antibodies and antigens. There are three approaches to measure the binding affinity, first is affinity from kinetic constants, second is steady-state affinity, and third is affinity in solution. For the purpose of our experiments, we will focus on affinity from kinetic constants.

In a reaction:

$$A + B \rightleftharpoons AB$$

\[ k_a \quad k_d \]

**Equation 1.16**

Where A is the ligand, B is the analyte, and AB is the interaction complex, $k_a$ is the association rate constant ($M^{-1}S^{-1}$), and $k_d$ is the dissociation rate constant ($S^{-1}$). The net rate of $AB$ forming is given by:
\[
\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]
\]

**Equation 1.17**

And the net dissociation rate is given by:

\[
\frac{d[AB]}{dt} = k_d[AB]
\]

**Equation 1.18**

Now, if we substitute antigen \((Ag)\) and antibody \((Ab)\) to replace \(A\) and \(B\):

\[
Ag + Ab \rightleftharpoons AgAb
\]

**Equation 1.19**

The concentration of \(AgAb\) complex formed could be demonstrated by RU in SPR response. The total binding capacity of \(Ab\) is an estimated value \(RU_{max}\), and the unoccupied \(Ab\) on the surface is
$RU_{\text{max}} - RU$, with. Analyte $Ag$ flows through the flow cell with known concentration. Now we could rewrite the net association rate constant in RU as:

The net association rate:

$$\frac{d[AgAb]}{dt} = k_a[Ag][Ab] - k_a[AgAb]$$

**Equation 1.20** would become:

$$\frac{d[RU]}{dt} = k_a[Ag][RU_{\text{max}} - RU] - k_a[RU]$$

**Equation 1.21**

The net dissociation rate would become:

$$\frac{d[RU]}{dt} = -k_d[RU]$$

**Equation 1.22**

If we rearrange the net association rate constant, to y=mx+b relationship, then:
\[ \frac{d[RU]}{dt} = k_{a}[Ag][RU_{max}] - (k_{a}[Ag] + k_{d})[RU] \]

**Equation 1.23**

Where \( y \) is \( \frac{\sigma[RU]}{dt} \), \( m \) is \( -k_{a}[Ag] + k_{d} \), \( x \) is \( [RU] \), and \( b \) is \( k_{a}[Ag][RU_{max}] \). To solve for \( k_{a} \) and \( k_{d} \), we need to first find out \( k_{a} \) from the y-intercept, because we know \( [Ag] \) and the constant \( [RU_{max}] \). Once we find out \( k_{a} \), we could find out \( k_{d} \) from the slope. It is important to use increasing concentration of \( [Ag] \) to obtain a more accurate \( k_{a} \) and \( k_{d} \) value.

### 1.4.2 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is extensively used to study the elemental composition of a surface, chemical or electronic state of the elements in a surface. XPS is based on photoelectric effect. When a solid emits electrons upon adsorbing the energy of light, the emitted electron is called photoelectron with identical mass and charge as other electrons.\(^{45-47}\)

In XPS, the notation of photoelectrons is usually labelled with orbital angular momentum and principal quantum numbers of the electron, both are described by integer values. The orbital angular momentum describes the shape of the orbital, it is usually noted by letters s, p, d, f, corresponding to 0, 1, 2, 3;
whereas the principal quantum numbers designates the size and energy level of an electron, it is denoted by K, L, M for electron shell 1, 2, 3. Electrons with a different orbital angular momentum, and principal quantum number would have a different binding energy towards the nucleus.⁴⁸

As shown in Figure 9, the x-ray with energy \( h\nu \) causes photoexcitation of a 1s electron of at a solid surface with binding energy \( B_E \). The photoelectron travels to the solid valence band and overcomes the
work function $\phi_s$, which is the minimum thermodynamic energy required to move from valence band to vacuum. Finally, the photoelectron is emitted into the vacuum with kinetic energy $K_E$.\[^{48}\]

The kinetic energy in this process is described in the following equation:

$$K_E = h\nu - \mathcal{E}_E - \phi_s$$

**Equation 1.24\[^{49}\]**

In order to measure the kinetic energy of a photoelectron, it has to travel through the kinetic energy analyzer to the detector, which often has an electron multiplier component with a work function $\phi_a$. Usually, the electron multiplier has a smaller work function, such that the kinetic energy of the electrons is increased by $\phi_s - \phi_a$. The kinetic energy detector would finally detect kinetic energy of a photoelectron with the following equation:

$$K_E = h\nu - \mathcal{E}_E - \phi_s + \phi_s - \phi_a = h\nu - \mathcal{E}_E - \phi_a$$

**Equation 1.25\[^{49}\]**
The XPS data is laid out as counts/s as the number of electrons detected per second versus binding energy, which is calculated based on the rearranged equation as described above. In this thesis, XPS is used to evaluate the elemental composition of the modified screen-printed electrodes.

1.4.3 Contact angle goniometry

Wettability is defined by the contact angle of the liquid phase with the solid. The analysis of contact angle could be used to determine surface energy.\textsuperscript{49,50}

Such analysis is useful in many fields, for example it is applied in erosion control studies for surface water repellence, bio-compatibility with human-implants, or glass panel cleanliness.\textsuperscript{51,52} In this thesis, contact angle measurements were performed to characterize the surface modifications on electrodes.
As shown in Figure 10, contact angle $\theta$ is at the intersection of liquid-solid, liquid-vapour, and vapour-solid interface. This particular angle is between the surface and a tangent line between liquid-vapour interfaces. There are three forces that determine the contact angle, as described by Thomas Young known as the Young’s equation:

$$\cos \theta = \frac{f_{VS} - f_{LS}}{f_{LV}}$$
Equation 1.26\textsuperscript{53} \\
where $f_{vs}$, $f_{ls}$, $f_{lv}$, and $\theta$ is the interfacial force of vapour-solid, interfacial force of liquid-solid, interfacial force of liquid-vapour at a particular angle $\theta$.\textsuperscript{53}

Interfacial force of liquid-solid, and vapour-solid are in opposite directions, and interfacial force of liquid-vapor is in the direction of the tangent line. The simple theoretical shape analysis could be used to estimate the contact angle. As shown in Figure 11, the liquid drop is assumed to be a part of a perfect sphere.\textsuperscript{53}

![Diagram](image)

Figure 11. The liquid drop illustrated with radius, tangent-line, contact angle, and height of the drop.
As shown in Figure 11, the height of the droplet could be measured, and radius of the sphere is estimated. Thus, the contact angle $\theta$ could be calculated using Equation 1.27.

\[ \theta = 90 - \tan^{-1} \frac{r - h}{\sqrt{2rh - h^2}} \]

**Equation 1.27**

In reality, the surface tension minimizes the surface area to make the sphere shape, but gravity would flatten the drop. This simple analysis is only feasible to estimate contact angle in small drop volume, where gravitational force is negligible. Besides gravitational forces, there are also other types of interactions that would affect the shape of a drop, such as dipole-dipole interactions, acid-base interactions, or hydrogen bonding. In these cases, a more accurate estimation of the contact angle would need to apply other equations and models.\(^{55}\)
1.5 Objectives and hypothesis

The objective of this thesis was to design cost-effective, sensitive, and selective electrochemical immunosensors for the detection of clinically important biomolecules. To achieve this, we utilized different surface modifications to immobilize the biorecognition layers, and test our system in clinically relevant real samples. Our hypothesis is that immunosensors based on disposable screen-printed electrodes would allow the quantification of *Legionella pneumophila*, adrenocorticotropic hormone and growth hormone in real samples.

1.6 References

48. Watts, F.; Wolstenholme, J. *An Introduction to Surface Analysis by XPS and AES*. Willey, 2005


Chapter 2

2 Analytes and detection methods

2.1 Connecting text

I started my graduate study at University of Toronto in September of 2011, my research plan was to continue the work of a former graduate student Kathy Li to develop electrochemical immunosensors for pituitary hormones detection. However, the outbreak of Legionnaires’ disease in Quebec, Canada in the year 2012 led to an collaboration project with Dr. Guyard from The Ontario Agency for Health Protection and Promotion (OAHPP) and Dr. Terebiznik from University of Toronto – Department of Biological Sciences. There are commercialised kits for *L. pneumophila* serogroup 1, but reports have suggested other serogroups could also cause Legionnaires’ disease. We developed screen-printed electrode based method that could be easily adapted to detect different serogroups of *L. pneumophila*. After the publication of our manuscript related to *L. pneumophila* detection, I continued my research plan to develop electrochemical immunosensors for the detection of adrenocorticotropic hormone (ACTH) and growth hormone (GH). In this thesis, we have demonstrated the detection of *L. pneumophila*, ACTH, and GH using screen-printed electrodes, and these analytes are introduced further in the following sections of this chapter.

2.2 Legionella pneumophila

*Legionella pneumophila* comprises more than 70 serogroups that inhabit natural and human engineered aquatic environments. A review of drinking water-associated diseases in United States showed that *L.
pneumophila accounted for 29% of outbreaks from 2001 to 2006.² L. pneumophila is parasitic in protozoan organisms and infects humans through the inhalation of contaminated aerosolized droplets of water. This opportunistic pathogen targets respiratory tissue causing a severe pneumonia known as Legionnaires’ disease and the lesser form, Pontiac fever.³, ⁴ According to World Health Organization (WHO), mortality rate associated with Legionnaires’ disease is up to 40% among average patients and up to 80% among immuno-suppressed patients. Since death by L. pneumophila infection depends on the early antimicrobial treatment, rapid diagnosis of this disease is critical for efficient treatment and patient survival. Epidemiological data indicates that L. pneumophila is responsible for 91.5% of diagnosed Legionellosis cases and that serogroup 1 (Lp1) is the predominant serotype found in North America and Europe.⁴ Studies by the Ontario Agency for Health Protection and Promotion (OAHPP) showed that 39% of the culture-confirmed Legionellosis reported over the last three decades in Ontario were caused by serogroups different from Lp1.⁵, ⁶ Conventional detection tests use Legionella culture, direct immunofluorescence assay, urinary antigen test, serology testing, or polymerase chain reaction.¹ However, these methods have turnaround times measurable in several hours to days, are expensive, are technically demanding, and have been developed to only focus on serogroup 1. Here, we propose a low-cost and miniaturized electrochemical system that requires an incubation time of approximately 1 h, does not require a strong technical expertise, and has the potential to be easily modified to detect various L. pneumophila serogroups. Intensive research activities have been carried out in the past decade toward the miniaturization of electrochemical chips and toward the development of hand-held devices.⁷-¹⁴
Electrochemical impedance spectroscopy (EIS) has been extensively applied as the method of choice for biosensor designs due to its well-described and highly sensitive surface characterization capabilities.\textsuperscript{15-19}

The EIS behaviour of our immunochip was interpreted using the Randles’ model equivalent circuit, consisting of ohmic resistance ($R_s$), of electrolyte, double layer capacitance ($C_{dl}$), charge-transfer resistance ($R_{ct}$), and Warburg impedance ($Z_w$) of the electrode.\textsuperscript{20} The electrode accessibility to the solution-based redox probe was effectively reduced upon attachment of biomolecules. Throughout the modification steps of the immunochip, fluorescently labeled biomolecules were preferred to allow microscopic imaging of the surface-bound antibodies and bacteria.
As shown in Figure 12, EIS characterization was performed after each surface modification step. The thiol group of 6-mercaptohexanoic acid (6-MHA) formed thiolate-gold bond to self-assemble onto the electrode surface, and the carboxyl group of 6-MHA was reacted with EDC/NHS to form the covalent amide bond with the primary amines on the secondary antibodies. The secondary antibody would then bind with primary antibody for the detection of L. pneumophila.

2.2.1 References
2.3 Adrenocorticotropic hormone

Adrenocorticotropic hormone (ACTH) is a 39-amino acid peptide hormone (4.5 kDa) released from the anterior pituitary gland, serving multiple functions in many tissue targets.\textsuperscript{1} ACTH is an essential component of the hypothalamic-pituitary adrenal axis, regulating corticosteroid hormone production which has important functions in a myriad of critical physiological functions.\textsuperscript{2-4} Dysregulation of ACTH secretion, resulting from conditions such as hypopituitarism\textsuperscript{5} or Cushing’s Syndrome,\textsuperscript{6} can be life threatening if not diagnosed and treated properly, and in the most severe cases, it can cause death because of vascular collapse.\textsuperscript{7} For early and accurate assessment of altered ACTH secretion, advances in its detection are required; however, there are challenges associated with the diagnosis for altered ACTH level. ACTH deficiency is often misdiagnosed due to the general symptoms such as weight loss, vomiting, nausea, and muscle weakness. Furthermore, the fluctuation of ACTH in serum (<4.1 to 51.4 pg/mL) makes diagnosis even more problematic.\textsuperscript{8} To address these challenges in diagnosis, a rapid, sensitive, and selective detection method is needed. Various detection methods exist for the detection of ACTH, such as radioimmunoassays,\textsuperscript{9-10} chemiluminescence assays,\textsuperscript{11} and enzyme-linked immunosorbent assays.\textsuperscript{12} Current issues concerning these methods include sensitivity and specificity along with bench-top instrumentation that requires skilled technicians and time-consuming procedures.\textsuperscript{13} In this report, we present a proof-of-principle study on impedimetric detection of ACTH using disposable screen-printed gold electrodes (SPGEs). Our immunoassay is a cost-effective approach that requires low sample volumes. SPGEs can be mass produced at low-cost, and each experiment can be performed on a fresh and analogous surface to prevent possible cross-contamination errors between
surfaces that were exposed to biological samples.\textsuperscript{14} Each SPGE can be discarded after use and eliminate carry-over contamination from tedious cleaning, and also require less reagents for detection.\textsuperscript{15-17}.

Figure 13. Illustration for the SPGE-based detection of ACTH. (a) Self-assembled film of 3,3΄-Dithiobis[sulfo succinimidyl]propionate (DTSSP) was formed on nanostructured gold surface; (b) NHS-moieties of DT-SSP enabled the covalent immobilization of antibodies on the surface; (c) The biotinylated adrenocorticotropic hormone (biotin-ACTH) was captured by immobilized antibodies; (d) Streptavidin-conjugated alkaline phosphatase (Streptavidin-ALP) was then coupled to biotin-ACTH; (e) Amplification reaction was initiated by adding the water-soluble substrate mixture, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), that produced insoluble indigo dimer; (f) Electrode interface was monitored using electrochemical impedance spectroscopy (EIS) after each surface modification step.
The detection of ACTH is shown in Figure 13: (a) a film of 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP) forms through Au-S interactions, and (b) acts as the linker molecule to immobilize the antibodies covalently on the nanostructured surface (the scanning electron microscope image of the nanostructure topology was shown in our previous publications). (c) The antibodies interact with the biotinylated ACTH (biotin-ACTH), and (d) biotin moieties capture the streptavidin-conjugated alkaline phosphatase (Streptavidin-ALP). (e) The surface-anchored streptavidin-ALP processes the substrate containing disodium 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and forms insoluble 5,5′-dibromo-4,4′-dichloro indigo that significantly increases the surface thickness, as it precipitates and forms an insulating layer on the surface. (f) As non-biotinylated ACTH from spiked samples displaces the biotinylated ACTH, streptavidin-ALP is also detached from the surface, decreasing the formation of insoluble product. The changes in charge transfer resistance (Rct) are measured using electrochemical impedance spectroscopy (EIS).

2.3.1 References

### 2.4 Growth hormone

Growth hormone (GH) has two isoforms (22 kDa and 20 kDa) released from the somatotrophic cells in the anterior pituitary gland, serving multiple functions in many tissue targets. GH plays a critical role in
the regulation of blood glucose, longitudinal bone growth, and enhancement of muscle mass.\textsuperscript{2} Deficiencies in GH can have severe developmental consequences such as hypoglycemia in newborns, stunted growth in childhood, and physical/psychological symptoms well into adulthood.\textsuperscript{3,4} On the other hand, over expression of GH can result in gigantism, acromegaly, and impaired glucose tolerance.\textsuperscript{5-7} Beyond a clinical setting, GH doping is observed extensively in professional athletes to enhance overall tissue maintenance, repair, and muscle growth.\textsuperscript{8} The use of synthetic GH as an ergogenic drug has become increasingly prevalent, which resulted to its ban in professional athletes by the World Anti-Doping Agency (WADA).\textsuperscript{9} It is important to monitor the growth hormone level in order to face these major global health challenges, advances in GH screening are required to facilitate early detection and treatment.\textsuperscript{10}

In an immunosensor, the integration of the recognition element with the signal transducer is usually achieved by chemical modifications that enable the immobilization of antibodies. Aryl diazonium salts have become increasingly popular given their ease of use for modifying a wide variety of surfaces and also their stability as a chemical linker.\textsuperscript{11-14} Recently, Eissa et al.\textsuperscript{15} reported the electrochemical modification of graphene-modified screen-printed carbon electrodes (SPCEs) with 4-nitrophenyl diazonium salt, which enabled the covalent attachment of antibodies for the detection of a milk allergen β-lactoglobulin. Eissa and Zourob\textsuperscript{16} have also reported the development of an electrochemical competitive immunosensor for the detection of okadaic acid in shellfish. Graphene modified SPCEs were functionalized by the electrochemical reduction of in situ generated 4-carboxyphenyl diazonium salt in acidic aqueous solution.\textsuperscript{16} The sensitive detection of egg allergen ovalbumin was also achieved
with a detection limit of 0.83 pg/mL in PBS using graphene modified SPCEs with a carboxyphenyl film on the graphene surface. The optimal applied voltage and concentration needs to be carefully determined for the reductive adsorption of diazonium salt linkers, to avoid multiple layer formation caused by aryl radicals and azo radicals. At present, reported GH screening methods include enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), mass spectrometry, and surface plasmon resonance (SPR). Current issues concerning these methods include sensitivity, specificity, cost and time. There is an urgent need for an ultrasensitive screening procedure for the detection of GH that is rapid and low cost. Our immunosensor provides an economical approach to a miniaturized electrochemical system that can detect GH in clinical samples, without strong technical expertise.
Figure 14. Conceptual illustration of the impedimetric detection of GH. (A) A film of 4-methoxybenzenediazonium tetrafluoroborate (4-MBD) was immobilized on an electrode surface by electrodeposition (i); Surface-confined 4-MBD molecules were electrochemically oxidized to carboxylic acid groups (ii) that were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to allow subsequent covalent immobilization of antibodies (iii); GH is captured with antibodies on the surface (iv). (B) Screen-printed carbon electrodes of dimension 4 × 12 mm in length. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were employed to characterize the electrode surfaces.

As shown in Figure 14(A), (i) 4-methoxyphenyl film is formed through reductive adsorption. Upon applying an electrical potential, an aryl radical intermediate is formed because the electron withdrawing power of the diazonium group. The radical then forms a C-C bond with the carbon surface with high
stability over time.\textsuperscript{29-31} (ii) Conditioning potential of +1.0 V (vs. Ag/AgCl) is applied to oxidize the functional groups on the 4-methoxyphenyl film to form carboxylic acids; (iii) subsequently, the carboxylic acid groups are activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinamide (NHS) to immobilize the antibodies through amide bonds; (iv) various concentrations of GH could be detected using the antibodies immobilized on the surface through antibody-antigen interactions. Both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) are used to characterize the electrode surface, and carbon working electrode (WE) surface can be characterized by changes in peak potential and quantitated by changes in the charge transfer resistance.

2.4.1 References

Chapter 3

3 Experimental

3.1 Connecting text

In this chapter, we described the experimental procedures in detail, including reagents and instruments.

3.2 Detection of *Legionella pneumophila*

3.2.1 Reagents

Alexa Fluor®555 anti-rabbit secondary IgG antibody was purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti-*Legionella pneumophila* primary antibody was kindly donated by the Ontario Agency for Health Protection and Promotion (OAHPP). 16% paraformaldehyde (PFA) was obtained from Canemco Inc. (Quebec, ON, Canada). 6-mercaptohexanoic acid (6-MHA), N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich USA. The SPGE were kindly donated by the BioDevice Technology Inc. (Ishikawa, Japan). Phosphate buffer saline (PBS, 50 mM, pH 7.4) was prepared with ultra-pure water (18.2 MΩ.cm) from the Cascada Pall water purification system.

3.2.2 Instrumentation

The EIS measurements were performed in 50 mM PBS (pH 7.4) containing 10 mM of ferri/ferro cyanide ([Fe(CN)₆]³⁻/⁴⁻) with a frequency between 0.1 Hz and 10 kHz and an AC amplitude of 5 mV using the Eco Chemie μAutolab Type III potentiostat/galvanostat (Metrohm Autolab, Utrecht, The Netherlands), and analyzed with Frequency Response Analyzer (FRA) software.
3.2.3 Bacteria culture

$L. pneumophila$ serogroup1 strain Lp02 was used in this study. GFP expressing Lp1 was described previously. Bacteria were cultured in buffered charcoal-yeast extract (BCYE) agar at 37 °C for 3 days before use. Bacterial colonies were resuspended in PBS, and fixed in 4% PFA for 1 h at room temperature. Bacterial concentration was determined using a Cell Density Meter Model 40 (Fisher Scientific) at 600 nm. Before treatment, bacterial suspension was washed twice and resuspended using PBS. Serial dilutions were done using PBS to obtain different bacterial concentrations.

3.2.4 Electrode surface modifications and detection

Various concentrations of 6-MHA were incubated with Au electrode surface for 48 h, followed by a stringent washing process in blank PBS. The electrode surface was then reacted with 2 mM EDC and 5 mM NHS for 1 h, followed by a wash. Subsequently, secondary antibody was incubated with activated 6-MHA for 24 h at 4°C followed by a wash step to remove the non-specifically adsorbed antibodies, then attachment of primary antibody continued for another 24 h at 4 °C, followed by washing process as described earlier. Finally, desired concentrations of Legionella were detected using EIS after 30 min of incubation at room temperature on the chip surface followed by the stringent washing process.

3.2.5 Immunofluorescence microscopy

Images were acquired using a Hamamatsu EM-1K, EMCCD camera mounted Quorum Optigrid microscope (Leica DMI6000B) equipped with 20x and 40x oil air objective lenses. Image acquisition and post-acquisition processing were performed using Volocity 4.3 software (Improvision). DIC images were acquired using 210 ms exposure and 255 unit sensitivity without auto-contrast. Fluorescence
images were acquired using 40 ms exposure and 100 units sensitivity without auto-contrast to visualize the secondary antibody. GFP expressing bacteria were imaged using 580 ms exposure and 68 units sensitivity without auto-contrast.

3.2.6 References


3.3 Detection of adrenocorticotropic hormone

3.3.1 Reagents

Anti-ACTH polyclonal antibody was purchased from EMD Millipore (Darmstadt, Germany). Biotin-labelled adrenocorticotropic hormone (ACTH) (1-39, Human) and non-labelled ACTH (1-39, Human) were purchased from AnaSpec (Fremont, CA). Streptavidin-conjugated alkaline phosphatase (Streptavidin-ALP), disodium 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 3,3’-dithiobis [sulfosuccinimidylpropionate] (DTSSP), were purchased from Sigma-Aldrich (Oakville, ON). The screen-printed gold electrodes (SPGEs) were obtained from BioDevice Technology, (Ishikawa, Japan). Follicle-stimulating hormone (FSH), human growth hormone (hGH), rat blood and plasma samples were kindly pretreated and prepared by Dr. Le Tissier. Other reagents were of analytical grade, and were used as received.

3.3.2 Surface modifications

First, the working electrode of the SPGE was incubated with 80 μL of 2 mM DTSSP in 100 mM Na₂CO₃ for 48 h at -4°C. Then, an aliquot (15 μL) of anti-ACTH antibodies at a desired concentration in
50 mM phosphate buffer solution with 100 mM NaCl (pH 7.4) was spotted onto the working electrode surface and incubated for 12 h at -4°C. Next, an aliquot (5 μL) of the desired concentration of biotin-ACTH was incubated on the surface for 30 min. An aliquot (20 μL) of streptavidin-ALP was spotted on the electrode surface and incubated for 1 h. After each modification step, SPGEs were washed with Milli-Q water rigorously. Finally, an aliquot (20 μL) of BCIP was spotted on the surface and incubated for 10 min to form the insoluble indigo product. For the competitive detection of ACTH, known concentrations of non-labelled ACTH were mixed with 100 pg/mL biotin-ACTH and exposed to the antibody-modified SPGEs followed by the same experimental steps as described above.

3.3.3 Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) was performed using a μAutolab II Electrochemical Analyzer (Metrohm, Switzerland) in conjunction with Frequency Response Analyzer (FRA) software. EIS were recorded using 10 mM of [Fe(CN)₆]³⁻/⁴⁻ as the redox probe in 50 mM PBS with 100 mM NaCl (pH 7.4) in the frequency, ω, ranging from 1 Hz to 10 kHz at a dc potential of 0.30 V, corresponding to the recorded [Fe(CN)₆]³⁻/⁴⁻ standard reduction potential, with a superimposed root mean squared AC voltage amplitude of 5 mV. Analysis of the raw impedance data was based on complex non-linear least-squares (CNLS) regression fitting to the Randles’ equivalent circuit.

3.3.4 Scanning electron microscopy (SEM)

SPGE surfaces were observed using a Hitachi S530 scanning electron microscope (Hitachi, Japan) in the Centre for the Neurobiology of Stress at UTSC. The surfaces were electrically connected to the sample
stub by smearing silver paste between the SPGE and the metallic stub. The surfaces were monitored at an acceleration voltage of 20 kV with a working distance of 5.0 mm.

3.4 Detection of growth hormone

3.4.1 Reagents

Rat growth hormone (GH) full length protein (ab51232) and monoclonal mouse anti-growth hormone (anti-GH) antibody (ab9821) were obtained from Sigma-Aldrich (Oakville, ON). Potassium dihydrogen orthophosphate (KH$_2$PO$_4$), dipotassium orthophosphate (K$_2$HPO$_4$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinamide (NHS), potassium ferricyanide (K$_4$Fe(CN)$_6$), potassium ferrocyanide (K$_3$Fe(CN)$_6$), 4-methoxybenzenediazonium tetrafluoroborate, acetonitrile (CH$_3$CN, HPLC grade), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Oakville, ON). Tetraethylammonium tetrafluoroborate was purchased from Alfa Aesar (Mississauga, ON). All solutions were prepared using ultra-pure water from a Cascada LS water purification system (Pall Co., NY) at 18.2 MΩ. Male Sprague Dawley rats (Charles River) were anesthetised with isoflurane and then maintained under isoflurane by vaporizer with a nose cone attachment. Blood was drawn by cardiac puncture in the right ventricle. The blood samples were subsequently analyzed for growth hormone levels. All procedures using animals were approved by the Animal Care Committee of the University of Toronto and were in accordance with the guidelines established by the Canadian Council on Animal Care.
3.4.2 Electrode modifications

Glassy carbon electrode (GCE, CH Instruments, TX) was polished for 2 min using 1.0, 0.3, and 0.05 μm alumina. The electrode was rinsed with ultrapure water and sonicated for 5 min between each polishing step to remove any alumina present on the surface. Aryl diazonium salt (1 mM) in acetonitrile was prepared and de-aerated for 15 min. Tetraethylammonium tetrafluoroborate was used as the electrolyte with CV. A scan rate of 100 mV/s was applied for two cycles between +1.0 V and -1.0 V, and then rinsed with copious amounts of electrolyte and water. Next, the electrode was exposed to a biasing potential of +1.0 V for 1 min in 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ (PBS) at pH 7.4. The activation of exposed carboxylic acid groups on the surface was accomplished using 10 mM EDC and 40 mM NHS for 1 h and rinsed with PBS. The antibodies were incubated on the electrode surface for 18 h at 4°C, then rinsed with PBS. GH sample solutions were prepared at various concentrations in PBS and used as the target analyte. Each of the sample solutions was added to the electrode surface, and incubated for 30 min at room temperature with moderate shaking to ensure contact of GH with the surface-confined antibodies.

3.4.3 Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS)

CV and EIS were performed using a μAutolab III Electrochemical Analyzer (Metrohm Autolab, Utrecht, The Netherlands) in conjunction with its general-purpose electrochemistry software (GPES) and frequency response analyzer (FRA). CV and EIS were measured with 10 mM [Fe(CN)$_6$]$_{3-}$/4- in PBS with 100 mM KCl using a three-electrode system at room temperature. CV measurements were performed
before and after aryl diazonium salt modification at a scan rate of 100 mV/s between -0.5 V and +0.5 V. EIS was performed with a frequency range from 100 kHz to 100 mHz at a biasing voltage of 5.0 mV.

3.4.4 Surface plasmon resonance (SPR)

All SPR experiments were performed using Biacore X100 system (GE Healthcare, QC) with a CM5 sensorchip. Experiments were conducted at 25 ºC and the SPR running buffer (SPR running buffer, 0.01 M HEPES, 0.15 M NaCl, 0.05 mM EDTA, 0.05% surfactant P20, pH 7.4) was sterile filtered (0.2 μm). Two flow cells of the sensorchip were used, one (reference flow cell, FC-1) to detect the non-specific adsorption for background subtraction, and the other one (detection flow cell, FC-2) was used to detect the specific binding of target analyte. The anti-GH antibody was covalently coupled onto the Au sensorchip using the Amine Coupling Kit (GE Healthcare, QC) following the standard protocol described by the manufacturer. The target GH samples were injected to both flow cells using four different concentrations at 30 μL/min for 2 min. The setup was fully-automated using the Biacore X100 software. All concentrations of the target GH were performed in triplicates with zero concentration blanks before and after each injection of the sample. The dissociation constant was determined to be 1.44 (±0.15) nM using the Biacore Evaluation Software.

3.4.5 X-ray photoelectron spectroscopy (XPS)

XPS spectra were recorded using a Physical Electronics (PHI) Quantera II spectrometer equipped with an Al anode source for X-ray generation and a quartz crystal monochromator for focusing the generated X-rays. A monochromatic Al K-α X-ray (1486.7 eV) source was operated at 50 W and 15 kV, and a pass energy of 280 eV was used to obtain all collected survey data. All spectra were obtained at 45º take
off angles, and a dual beam charge compensation system was used to neutralize all samples. The system base pressure was no higher than $1.0 \times 10^{-9}$ Torr, with an operating pressure that did not exceed $2.0 \times 10^{-8}$ Torr. The instrument was calibrated using a sputter-cleaned piece of Ag, where the Ag 3d5/2 peak had a binding energy of $368.3 \pm 0.1$ eV and full width at half maximum for the Ag 3d5/2 peak was at least 0.52 eV. Data manipulation was performed using PHI MultiPak Version 9.5.1.0 software.

3.4.6 Contact angle goniometry

Contact angle was measured on a Future Digital Scientific OCA35 system. Milli-Q water was used to test the contact angle on the various surfaces. The static sessile drop method was used and a small droplet of 0.2 µL Milli-Q water was placed on the sample surface. An electronic picture of the droplet was taken and the contact angle was calculated using Young's Equation based on the shape of the droplet on the electrode.

3.4.7 ELISA-based detection kit

The rat/mouse GH detection kit was purchased from EMD Millipore (Etobicoke, ON). In the sandwich-based assay, the GH samples were captured by the pre-tittered anti-GH polyclonal antibodies on the 96-well plate, and then the binding of a second biotinylated anti-GH polyclonal antibody would form a “sandwich” by capturing the target protein on the surface. Streptavidin-conjugated horseradish peroxidase was then exposed to the biotinylated antibodies. Upon addition of a substrate, the quantitative detection was achieved with the formation of yellow-colored product at 450 nm. The concentration of GH in blood and plasma samples was derived by interpolation from a calibration curve.
generated in the assay with reference standards of known concentrations of rat GH at 0.07, 0.21, 0.62,
1.9, 5.6, 16.7, and 50 ng/mL.

3.4.8 References


Chapter 4

4 Results & Discussion

4.1 Disposable immunochips for the detection of *Legionella pneumophila* using electrochemical impedance spectroscopy

4.1.1 Preface

This chapter has been adapted from the following article:


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*Author contributions:* NL, MT and KK designed experiments. AB, AP, MT, and CG provided antibodies and bacteria samples and assisted with optical measurements. VWSH, CXR, and AJV assisted with electrochemical measurements. NL, MT and KK discussed the results and wrote the manuscript.

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As mentioned in our objectives, our goal was to design cost-effective, sensitive and selective electrochemical immunosensors for clinically important proteins. Here, we compared the EIS results
with fluorescence microscopy to optimize our surface modifications, which enabled us to detect *L. pneumophila* bacteria in spiked water samples.

### 4.1.2 Summary

The rapid diagnosis of Legionellosis is crucial for the effective treatment of this disease. Currently, most clinical laboratories utilize rapid immunoassays that are sufficient for the detection of Legionella serogroup 1, but not other clinically relevant serogroups. In this chapter, the development of a disposable immunochip system is described in connection with electrochemical impedance spectroscopy and fluorescence microscopy. The immunochips were prepared by covalently immobilizing fluorophore-conjugated *L. pneumophila* antibodies on Au chips. The analytical performance of the immunochips was optimized as a prescreening tool for *L. pneumophila*. The versatile immunochips described here can be easily adapted for the monitoring of all Legionella serogroups in clinical and environmental samples.
4.1.3 Results & Discussion for the detection of *Legionella pneumophila*

The $R_{ct}$ characteristics of the 6-mercaptohexanoic acid (6-MHA) film were studied at the following concentrations as shown in Figure 15.

![Graph showing dependence of 6-mercaptohexanoic acid concentration on charge transfer resistance at a blank Au chip.](image)

Error bars indicate the standard deviation of triplicate measurements ($n = 3$).

As expected, 6-MHA acted as an insulating layer and the highest $R_{ct}$ corresponded to 1.0 mM 6-MHA, thereby, suggesting the best coverage of the chip surface. The decreasing trend of $R_{ct}$ could be caused by two reactions. First, the aggregation and auto-oxidation of the molecule at such high concentrations could prevent thiolate-gold interactions. Second, the thiols could precipitate at higher concentrations and occupy electrode surface to prevent thiolate-gold interactions. For the next step, the chips modified with 6-MHA were used to optimize the concentration of the secondary antibody. As shown in Figure 16, both...
the blank and the lowest concentration 1.0 μg/mL had the highest R_{ct}, but the fluorescence image shown in Figure 21 (Supporting Information) suggests that the secondary antibody concentration at 10.0 μg/mL had the densest coverage on the chip.

The immobilization of Alexa-555 labeled secondary antibody allowed dense coverage of the surface but also decreased the charge transfer resistance. The increase in the average charge transfer resistance value for 1.0 μg/mL might not indicate there was a drift in the R_{ct} values, because the error overlapped with that of 6-MHA modified chip. Since the secondary antibodies were labelled with Alexa-555 dye. Alexa fluorophores are made of sulfonation of rhodamine. Although the negative charges on sulfonates could repel the negatively charged ferri/ferrocyanide redox probes. But the π interactions among the Alexa dye could also promote the antibodies to aggregate. The promoted aggregation of antibodies
would lead to an uneven distribution of antibodies on the electrode surface producing “pin holes” on the surface, which would allow ferri/ferrocyanide to interact easily with the electrode surface. After the optimization of secondary antibody, primary antibody concentration was optimized to 190 μg/mL (data not shown). Finally, different concentrations of *L. pneumophila* were tested using the antibody-modified chips in connection with EIS. As shown in Figure 17a, the impedance was presented as the sum of the $Z'$ (real $Z$) and $Z''$ (imaginary $Z$) components that originate mainly from the double layer capacitance. The circuit in the inset of Figure 17a was selected to show the electrochemical process and fit the accurate values. As shown in Figure 17b, increasing concentrations of *L. pneumophila* resulted in increasing $R_{ct}$, which suggested that attachment of bacteria created insulating properties on the chip surface. Furthermore, the EIS signal began to plateau at $2.0 \times 10^2$ cells/mL. The observed detection limit of $2.0 \times 10^2$ cells/mL for our immunochip was suitable for detection in samples, where *L. pneumophila* contamination could be moderate to severe. The immunochips were also stored at 4°C for 6 weeks in order to test their stability.
Figure 17. Electrochemical sensing of *L. pneumophila*. (a) Dependence of *L. pneumophila* concentration on Nyquist plot (Z‘ vs −Z") using Randles’ circuit for a primary antibody modified Au chip. (b) Dependence of *L. pneumophila* concentration on charge transfer resistance for a primary antibody modified Au chip. Error bars indicate the standard deviation of triplicate measurements (n = 3).
As shown in Figure 21 (Supporting Information), neither the average $R_{ct}$ nor the standard deviation changed significantly after 6 weeks of storage, suggesting the immunochips were stable over a limited amount of time. We have also challenged the immunochips with tap water samples spiked with *L. pneumophila* as shown in Figure 18.

![Nyquist plot](image)

Figure 18. Electrochemical sensing of *L. pneumophila*. (a) EIS results of samples with *L. pneumophila* spiked in both tap water and PBS shown on Nyquist plot ($Z'$ vs $-Z''$) using Randles' circuit.
Samples with $2.0 \times 10^5$ cells/mL of *L. pneumophila* were spiked in both tap water (pH 7.7) and PBS (pH 7.0); the detection of *L. pneumophila* had a small discrepancy in charge transfer resistance for both samples. The average $R_{ct}$ was 97 kOhm for tap water sample and 88 kOhm for PBS sample, suggesting a strong potential for on-field detection, and the small error bars indicated the robustness of the immunochip after exposure to samples from different sources. Furthermore, the immunochip demonstrated strong specificity toward *L. pneumophila*. The immunochip was challenged with a sample that contained 5% (w/v) BSA instead of the bacteria. The $\Delta R_{ct}/R_{ct}^0$ ratio was significantly higher for the specific binding of *L. pneumophila* as shown in Figure 23, Supporting Information. The fitting errors were less than 5% as shown in one of the representative graph in Figure 24, Supporting Information, which demonstrated that the Randles’ circuit allowed for accurate fitting.
As shown in Figure 19, we compared the detection of *Legionella pneumophila* versus the non-specific adsorption of *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus epidermidis*. We find the signal for *Legionella pneumophila* was significant higher, suggesting our system was selective towards *Legionella pneumophila*. The signal for *Escherichia coli* was higher than the signal for *Bacillus subtilis* and *Staphylococcus epidermidis*. We think this is due to the structural difference between gram positive and
gram negative bacteria. Even though gram positive bacteria has a thicker peptidoglycan layer, the gram negative bacteria has an extra outer membrane composed of lipopolysaccharides and proteins, and an extra layer of periplastic space. Both of these layers would contribute to a higher charge transfer resistance.

Differential interference contrast (DIC) and fluorescence imaging confirmed Alexa-555 secondary antibody binding on Au chips treated with the primary antibody (Figure 20B,C) and subsequent binding of GFP expressing Lp1 on chips treated with bacteria (Figure 20C). In contrast, secondary antibody and Lp1 signals were not amply visible on control chips (Figure 20A). The Au chip surface was fabricated with the electro-deposition of Au nanoparticles. Upon the immobilization of antibodies and bacteria on Au nanoparticles, the surface roughness of the chips changed significantly; thus, the irregularities observed in the topology of Au surface were common throughout all the batches of chips.
Figure 20. Fluorescence characterization of Au Chip. DIC and immunofluorescence of Alexa-555 anti-rabbit secondary antibody and GFP *L. pneumophila* on an untreated control Au chip (A), Au chip with antibody (B), and Au chip with antibody and bacteria (C). Arrows in inset indicate a *L. pneumophila* filament. Scale bars represent 40 um. (A) and (B) were imaged at 20× and (C) at 40×.

4.1.4 References

4.1.5 Supporting information

Figure 21. Immunofluorescence of different secondary antibody concentrations. Scale bars represent 40 µm at 20x. Images were taken at 102 ms exposure and 22 unit sensitivity.

Fluorescence images were taken for blank and different concentrations of secondary antibody, the immobilized secondary antibody on the SPGE surface increases with increasing concentration of the secondary antibody.
Figure 22. The same sets of chips that were measured in Figure 17 were tested after six weeks of storage at 4°C to demonstrate the stability over time.

Neither the average $R_{ct}$ nor the standard deviation changed significantly, the increasing concentrations of bacteria still resulted in increasing $R_{ct}$, suggesting the charge transfer resistance values were stable over time.
Figure 23. $\frac{\Delta R_{ct}}{R_{ct}^0}$ ratio comparison for the (a) non-specific adsorption (NSA) of 2.0E+05 cells/mL, (b) NSA of 5% BSA (w/v), and (c) specific binding of 2.0E+05 cells/mL.

The layers in (a) $\Delta R_{ct}$ were calculated from $R_{ct}$ of [bare Au + L. pneumophila] – [bare Au], and $R_{ct}^0$ was from [bare Au]. (b) $\Delta R_{ct}$ was calculated from $R_{ct}$ of [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody + BSA] – [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody], and $R_{ct}^0$ was from [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody]. (c) $\Delta R_{ct}$ was calculated from $R_{ct}$ of [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody + specific binding of L. pneumophila] – [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody], and $R_{ct}^0$ was from [bare Au + 6-MHA + covalent binding of secondary antibody + primary antibody]. The $\frac{\Delta R_{ct}}{R_{ct}^0}$ ratio was significantly higher for the specific binding of the bacteria, which demonstrated that non-specific adsorption was
successfully suppressed and the immunochip displayed selectivity towards the detection of \textit{L. pneumophila}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{nyquist_plot.png}
\caption{Schematic representation for curve fitting for a Nyquist plot using FRA software. The Nyquist plot corresponds to a screen-printed gold electrode modified with 6-mercaptohexanoic acid and secondary antibody.}
\end{figure}

All of the fitting errors were less than 5\%, which demonstrated that the Randles’ equivalent circuit allowed accurate fitting.

4.1.6 Supporting information references

4.2 Miniaturized impedimetric immunosensor for competitive detection of adrenocorticotropic hormone

4.2.1 Preface


*Author contributions:* NL and KK designed the experiments. NL performed the experiments. EML assisted in electrochemical measurements. NL and KK discussed the results and wrote the manuscript.

*Acknowledgements:* Authors thank Professor Paul Le Tisser (University of Edinburgh, UK) for kindly providing the antibodies and blood samples. This research was carried out with the financial support from NSERC Discovery Grant.

In the last chapter, we developed an immunosensor for the detection of *Legionella pneumophila* in spiked water samples using screen printed gold electrodes. The applications of immunosensor are often performed using more complicated plasma and blood samples. In this study, we modified the gold electrode with dialkylsulfide as the linker. Furthermore, the immobilized alkaline phosphatase could process 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to produce precipitating products that would form a film on the electrode surface to increase charge transfer resistance. This increase in charge transfer resistance would improve the sensitivity of electrochemical impedance spectroscopy (EIS)-based adrenocorticotropic hormone (ACTH) detection in plasma and blood samples.
4.2.2 Summary
In this proof-of-concept study, a miniaturized immunosensor was developed for the highly sensitive detection of adrenocorticotropic hormone (ACTH) using electrochemical impedance spectroscopy (EIS) in connection with disposable screen-printed gold electrodes (SPGEs). A film of 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) was prepared to immobilize the anti-ACTH antibodies covalently on the nanostructured SPGE surface. The surface-immobilized anti-ACTH antibodies captured the biotinylated ACTH (biotin-ACTH) and non-labelled ACTH for the competitive immunoassay. After coupling of a streptavidin-alkaline phosphatase conjugate (Streptavidin-ALP), the bio-catalyzed precipitation of an insoluble and insulating product onto the sensing interface changed the charge transfer resistance ($R_{ct}$) characteristics significantly. The detection limit of 100 fg/mL was determined for ACTH in 5-µL sample volume, which indicated that this versatile platform can be easily adapted for miniaturized electrochemical immunoensing of clinically important biomolecules.

4.2.3 Results & Discussion for the detection of adrenocorticotropic hormone
The electrochemical detection relied on the formation of the insoluble product on the electrode surface, which significantly increased the $R_{ct}$ in a short time. First, calibration studies were performed using varying concentrations of antibody and streptavidin-ALP (Fig. 28 & 29, Supplementary information). The optimum concentrations for antibodies and streptavidin-ALP were determined as 10% (v/v) and 100 ng/mL, respectively. As shown in Figure 25a, concentration between 10 fg/mL to 1 ng/mL of biotin-ACTH was calibrated. Since 1 ng/mL had the highest $R_{ct}$, suggesting at 1 ng/mL biotin-ACTH would occupy most of the antibody binding sites. Concentration dependence of $R_{ct}$ was studied using various concentrations of non-labelled ACTH in the presence of 1 ng/mL biotin-ACTH. The binding ability of
non-labelled ACTH should be stronger than the binding affinity of biotin-labelled ACTH, because the antibodies used are polyclonal antibodies for non-labelled ACTH. We should observe a decrease in the Rct, because the non-labelled ACTH does not have affinity for streptavidin-ALP.

Figure 25. (a) Plot for the Rct of various biotin-ACTH concentrations after fitting the raw data to equivalent circuit model using CNLS regression. (b) Plot for the Rct ratio of various non-labelled ACTH concentrations after fitting the raw data to equivalent circuit model using CNLS regression. Error bars indicate the standard deviation of six replicate measurements (n=6). Other conditions were as described in the Experimental section.
As non-labelled ACTH displaced the biotin-ACTH on the antibody-modified surfaces, the difference between Rct in the presence and absence of non-labelled ACTH increased significantly, thus the Rct ratio also increased as we increase the concentration of non-labelled ACTH. As shown in Figure 25b, the Rct ratio ΔRct/Ri was calculated using the formula ΔRct = Rf-Ri, where Rf displays the Rct that was detected after exposure of antibody-modified SPGEs to non-labelled ACTH, and Ri correspond to the Rct of the biotin-ACTH modified electrodes. The Rct began to plateau beyond 100 pg/mL ACTH suggesting a dynamic range between 100 fg/mL and 100 pg/mL in PBS. This dynamic range is 10^2 magnitude larger than the dynamic range of the only ACTH sensor published, offering a more versatile detection platform. A detection limit was observed as 100 fg/mL, this detection limit is about 100-fold more sensitive than a commercially available ELISA-based kit, which has reported a detection limit in the 10 pg/mL range. Next, the selectivity of our immunosensor was challenged in the presence of interfering proteins.
Figure 26. (a) Nyquist plots for the detection of ACTH, hGH, and FSH at 100 fg/mL in PBS fitted with the Randles’ equivalent circuit. (b) Plot for the $R_{ct}$ ratios calculated for ACTH, hGH and FSH after fitting the raw data to equivalent circuit model using CNLS regression. Error bars indicate the standard deviation of six replicate measurements ($n=6$). Other conditions were as described in the Experimental section.
Two pituitary gland hormones, human growth hormone (hGH) and follicle stimulating hormone (FSH) that might be present in real samples were used in control experiments as shown Figure 26a. The Rct ratios ΔRct/Ri (Figure 26b) were calculated using the formula ΔRct = Rf-Ri, where Rf displays the Rct that was detected after exposure of antibody-modified SPGEs to ACTH, hGH, or FSH as the target protein in the competitive assay, and Ri displays the Rct that was detected in the absence of target proteins with the rest of the experimental steps performed as described in the Experimental section. The ΔRct/Ri ratio was significantly high for ACTH at 0.64 in comparison with the non-target proteins hGH and FSH. Relatively low ΔRct/Ri ratios of 0.23 and 0.18 for hGh and FSH, respectively, were attributed to much lower non-specific adsorption of those proteins on the electrode surface. Control experiments were performed to further challenge the immunosensor using various plasma and whole blood samples.
Figure 27. (a) Representative Nyquist plots for the detection of ACTH, hGH, and FSH in undiluted plasma as fitted with the Randles’ circuit. (b) Plot for the $R_{ct}$ ratios calculated for ACTH, hGH and FSH in whole blood (red) and plasma (blue) samples after fitting the raw data to equivalent circuit model using CNLS regression. Error bars indicate the standard deviation of six replicate measurements ($n=6$). Other conditions were as described in the Experimental section.
After spiking 100 fg/mL ACTH, hGH and FSH in undiluted plasma (Figure 27a and 27b-blue bars) and blood (Figure 27b-red bars), those samples were used as the target protein solutions in the experimental steps as described above. There was a large increase in the responses as expected from the non-specific adsorption of various biomolecules from plasma and blood. The calculated F-value is 3.95 which is smaller than the table F-value at 95% confidence interval 5.05; thus, detection of ACTH in plasma does not offer more precision than the detection of ACTH in blood. However, ΔR_{cd}/R_{i} ratio was high for ACTH in both plasma and blood samples compared to those obtained with hGH and FSH. There are many reports on the methods to minimize non-specific adsorption which could be applied to our study, such as using alkanethiols with a longer carbon chain and polyethylene glycol linkers.\textsuperscript{3, 4} The normal ACTH level fluctuates between 9-52 pg/mL, our results suggested that the proof-of-concept immunosensor could provide a promising platform to detect ACTH in undiluted samples.

4.2.4 References

4.2.5 Supplementary information

(a)

Figure 28. Electrochemical characterization of antibody immobilized to a 3,3'-Dithiobis[sulfosuccinimidyl]propionate modified miniaturized gold electrode phosphate buffer saline. (a) Nyquist plot ($Z'$ vs $-Z''$) of various antibody concentrations, fitted with Randles' circuit. (b) Charge transfer resistance of various antibody concentrations. Error bars indicate the standard deviation of triplicate measurements ($n=3$).
Figure 29. Electrochemical signal amplification using alkaline phosphatase for adrenocorticotropic hormone sensing on an antibody modified miniaturized gold electrode phosphate buffer saline. (a) Nyquist plot ($Z'$ vs $-Z''$) of various alkaline phosphatase concentrations, fitted with Randles’ circuit. (b) Charge transfer resistance of various alkaline phosphatase concentrations. Error bars indicate the standard deviation of triplicate measurements ($n=3$).
4.3 Diazonium-based immunosensor for the impedimetric detection of growth hormone

4.3.1 Preface

- **Li, N.;** Chow, A.M.; Ganesh, H.S.V.; Ratnam, M.; Said, H.; Brown, I.R.; Kerman, K.


*Author Contributions:* N.L., A.M.C., I.R.B., and K.K. designed the experiments and wrote the manuscript. N.L. A.M.C. H.V.S.G., M.R. and H.S. performed the experiments. A.M.C., P.L.T., I.R.B. and K.K. discussed and analyzed the experimental data. N.L., I.R.B. and K.K. discussed the results and wrote the manuscript.

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In the last chapter we developed an immunosensor for the detection of ACTH using screen-printed gold electrodes. While the system demonstrated great potential for detection in plasma and blood, the surface modifications of gold nanostructured surfaces were not readily reproducible. In this chapter, we used screen-printed carbon electrodes to lower the cost of our immunosensors, and also put more effort in surface characterizations using X-ray photoelectron spectroscopy, contact angle goniometry, and surface
plasmon resonance to verify our surface modifications. Finally, we detected growth hormone in both plasma and blood, and compared our results with those obtained using a commercially available detection kit.

4.3.2 Summary
Altered growth hormone (GH) level is a major global health challenge that would benefit from advances in screening methods that are rapid and low cost. Here, we present a miniaturized immunosensor using screen-printed carbon electrodes (SPCEs) for the detection of GH with high sensitivity. The diazonium-based linker layer was electrochemically deposited on SPCE surfaces, and subsequently immobilized monoclonal anti-GH antibodies as the sensing layer. The surface modifications were monitored using contact angle measurements and X-ray photoelectron spectroscopy (XPS). The affinity constant of the anti-GH antibodies was also determined as 1.44 (±0.15) nM using surface plasmon resonance. The immunosensor was able to detect GH in the 10 – 1000 pg/mL range using a 20-µL sample volume. The selectivity of the immunosensor was also challenged with rat and mouse blood samples collected at various developmental stages of the animals, demonstrating the potential applicability for detection in real samples.

4.3.3 Results and Discussion for the detection of growth hormone
Both CV and EIS were used to electrochemically characterize the GCE before and after electrodeposition of diazonium salt film. In Figure 30A, the bare GCE had prominent oxidation and reduction peak at 0.28 V and 0.16 V using [Fe(CN)_6]^{3-/4-} (i); whereas electrodeposition of 4-methoxyphenyl resulted in an irreversible reduction in current (ii). The peak current reduction suggested a film of 4-methoxyphenyl was immobilized on the electrode surface. To minimize the denaturation of antibodies, CV was not used in further experiments.
Figure 30. Surface characterization of electrode surface modifications. (A) Cyclic voltammogram of (i) bare glassy carbon electrode (GCE) and (ii) 4-methoxyphenyl (4-MP) film modified GCE using 10 mM [Fe(CN)₆]³⁻/⁴⁻ in PBS with 100 mM KCl at 100 mV/s as described in the Experimental section. (B) EIS measurements demonstrated with Nyquist plot (|Z''| vs |Z'|) and fitted with Randles’ equivalent circuit for the characterization of bare GCE, 4-MP film modified electrode, and antibody modified electrode. (C) XPS-based characterization of bare screen-printed carbon electrode (SPCE), 4-MP film modified electrode, and antibody modified electrode. (D) Contact angle measurements of bare SPCE, 4-MP film modified electrode, and antibody modified electrode. (E) SPR-based immunosensor measurements to determine the dissociation constant of antibody with targeted GH as the analyte.

EIS was used to detect the interfacial properties for bare GCE, 4-MP film modified GCE, and antibody modified GCE. As shown in Figure 30B, the charge transfer resistance (R_{ct}), depends on the dielectric and insulating properties at the interface. R_{ct} values continued to increase with the step-wise
modifications with 4-MP film and then antibodies on the electrode surface. The increase in $R_{ct}$ was consistent with the previous CV data suggesting the aryl diazonium salt film had an insulating property. Further increase in charge transfer resistance suggested that antibodies were successfully immobilized onto the GCE surface. EIS results were confirmed using XPS as shown in Figure 30C, the increase in O 1s counts shown in green lines and redlines was contributed by the oxygen atoms in the 4-MP film and antibodies, and the significant increase in N 1s counts was attributed to the primary amine groups of the antibodies, suggesting the successful immobilization of the antibodies on the SPCE surface. As shown in Figure 30D, the contact angle measurement had a small decrease after the immobilization of 4-MP film, and then, a significant decrease was observed after the immobilization of antibodies. The contact angle measurements were in agreement with the XPS results (Figure 30C), where the small increase in O 1s after immobilization of 4-MP film followed by a significant increase in O 1s after the immobilization of antibody would contribute to the decreasing hydrophobicity of the electrode surface. In order to determine the binding affinity constant, $K_d$ value of the anti-GH antibody, SPR (Figure 30E) was performed using CM5 chips. The anti-GH antibody displayed a $K_d$ value of 1.44 (±0.15) nM.
Figure 31. Impedimetric detection of GH using antibody-modified carbon electrodes; (A) Nyquist plots for the detection of various concentrations of GH on GCE; (B) Dependence of GH concentration on the charge transfer resistance values obtained from the Randles' equivalent circuit on GCE. Error bars indicate the standard deviation of triplicate measurements (n=3). (C) Nyquist plots for the detection of various GH concentrations on SPCE. (D) Dependence of GH concentration on the charge transfer resistance values obtained from the Randles' circuit on SPCE. Error bars indicate the standard deviation of triplicate measurements (n=3).

As shown in Figure 31A, the $R_{ct}$ ratios between blank and a range of GH concentrations between 100 to 1000 pg/mL are summarized in Figure 31B. The initial (Ri) and final (Rf) resistance values were measured before and after the GH binding to the antibody-modified electrodes. The antibody modified GCE was able to detect GH with a dynamic range between 100 pg/mL to 1000 pg/mL. The immunosensor was improved using SPCEs for miniaturized and portable detection capabilities. In Figure 31C, the Nyquist plot for SPCE started at a much larger x-axis value compare to the one of the GCE, because SPCE electrode surface is much rougher than the GCE surface. $R_{ct}$ values corresponding
to each modification of the SPCEs continued to increase, the $R_{ct}$, fitting error, and $R_s$ are shown in Table 1.

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Table 1. Charge transfer resistance $R_{ct}$, fitting % error, bulk solution resistance $R_s$, for the surface modifications on SPCE.

This trend agreed with GCE modifications, suggesting successful modification of antibody-modified surfaces. The Nyquist plots increased with increasing concentrations of GH, and the charge transfer resistance ratios are plotted in Figure 31D. The linear range of GH detection using glassy carbon electrode was from 100 pg/mL to 1000 pg/mL, with a regression line formula $y = 0.0021x + 1.1805$, $R^2 = 0.982$. Even though the dynamic range using SPCE was similar to that of glassy carbon, the linear range of GH detection was smaller, from 10 pg/mL to 100 pg/mL, with a regression line formula $y = 0.0035x + 0.2431$, $R^2 = 0.9944$. The linear range has a calibration curve has slope of 0.0035, using the formula limit of detection = $3\times\sigma_{blank}/m$ where $\sigma_{blank}$ is the standard deviation of blank with a value of 0.0056 and $m$ is the slope of the calibration curve, thus the calculated detection limit is 4.8 pg/mL, which was 5000 times lower than the detection limit (25 ng/mL) reported in connection with an evanescent-cascaded waveguide coupler designed by Ozhikandathil et al.\textsuperscript{1} 740 times lower than the detection of GH using integration of gold nanoparticles in microfluidics (3.7 ng/mL) as reported by Sadabadi et al.\textsuperscript{2} In order to demonstrate applicability of our GH detection method to biological samples
containing high concentrations of potentially interfering proteins, the immunosensor was challenged in various blood and plasma samples from male rats as shown in Figure 32.

Figure 32. (A) Nyquist plots for the detection of GH and BSA in diluted blood; (B) $R_\text{ct}$ ratio comparison between attachment of BSA and GH in diluted blood. (C) Nyquist plots for the detection of 100 pg/mL GH in various concentrations of blood. (D) Nyquist plots for the detection of 100 pg/mL GH in various concentrations plasma; (E) $R_\text{ct}$ ratios for the detection of GH in various concentrations of blood and plasma. Error bars indicate the standard deviation of triplicate measurements ($n=3$).
As shown in Figure 32A, the Nyquist plot radius increased with 4-MP film modification and immobilization of antibodies, and suggested successful modification of the surface. As shown in Figure 32B, BSA (5%, w/v) and GH (100 pg/mL) were spiked into 10-fold diluted blood samples. The charge transfer resistance $R_i$ and $R_f$ were measured before and after the binding of BSA or GH at the antibody-modified SPCEs. A p-value of $1.9 \times 10^{-5}$ was calculated, by conventional criteria, the difference between charge transfer resistance ratio from the binding of BSA and the binding of GH was considered to be statistically significant. The $R_{ct}$ ratio was significantly higher for GH spiked blood samples, suggesting that the antibody-modified SPCEs were effective in facilitating the ultrasensitive detection of GH in the presence of complex protein mixtures present in blood. Hence, our immunosensor has a promising potential for applications using clinical samples.

As shown in Figure 32C and D, GH (100 pg/mL) was detected in 10-fold and 5-fold diluted as well as non-diluted whole blood and plasma samples. In Figure 32E, the average $R_{ct}$ ratio increased by 18% from 10-fold to whole blood samples, and 15% from 5-fold diluted to the whole blood sample. The increase in $R_{ct}$ ratio was due to the non-specific adsorption of proteins in the blood matrix. The p-value was 0.046 between 10-fold and 5-fold diluted blood samples, and it was not considered to be statistically significant by conventional criteria. The p-value was 0.24 between 5-fold diluted and whole blood samples, also not considered to be statistically significant by conventional criteria.

Blood plasma samples having a simpler protein composition resulted in lower $R_{ct}$ than whole blood samples in 5-fold diluted and non-diluted samples. The p-value was 0.056 between 10-fold and 5-fold diluted plasma samples, and 0.086 between 5-fold diluted and non-diluted plasma samples. Both p-values were not considered to be statistically significant by conventional criteria, and suggested increasing plasma concentrations did not result in a significant increase with non-specific adsorption and
surface fouling. The calculated F-value between blood and plasma samples were 2.07 at 10X dilution, 0.57 at 5X dilution, and 1.41 at no dilution. All of these values were smaller than the table F-value at 95% confidence interval and 5 degree of freedom, suggesting the detection of GH hormone is not necessarily more precise than the detection of GH in blood samples.

The immunosensor was also applied to monitor GH in blood samples collected male rats aged from 2 weeks to 19 months. EIS results were compared with those obtained using a commercial GH kit. As shown in Figure 33, GH levels decreased with advancing age rats using both detection systems. In general, the EIS data indicated that the rats had higher or similar GH concentration compared to the data.
obtained using the commercial kit. The lower GH concentration determined by the commercial kit may be attributed to the complicated sample preparation procedures, as well as the short life-time of the signalling dye, which was observed to decrease in absorbance rapidly over time.

4.3.4 References


Chapter 5

5 Conclusions and future directions

In our effort to develop electrochemical immuno-sensors based on disposable screen-printed electrodes for the quantification of *L. pneumophila*, ACTH, and GH, we used gold and carbon screen-printed electrodes with various surface modifications for the immobilization of antibodies. In the following sections, the issues and proposed solutions regarding each analyte are discussed.

5.1 Detection of *L. pneumophila*

In conclusion, Chapter 4.1 demonstrated a promising chip-based detection platform of *L. pneumophila* serogroup1 strain Lp02 with an observable detection limit of 200 cell/mL. The system also demonstrates potential for robustness, there was not significant change in charge transfer resistance even after 6 weeks. Although the charge transfer resistance was much higher for the specific binding of *L. pneumophila*, there is still a significant charge transfer resistance of non-specific binding of *Escherichia coli*, *Bacillus subtilis*, and *Staphlococcus epidermidis*. The primary antibody was poly-clonal antibody, and the binding mechanism with *L. pneumophila* was not clearly understood, which means it could have affinity for parts of the cell wall, proteins, or lipopolysaccharides. One possible method to minimize the non-specific binding of other bacteria and biomolecules is to use a monoclonal antibody that is specific for the *Legionella pneumophila* peptidoglycan-associated lipoprotein (PAL), a lipoprotein that is conserved among *Legionella pneumophila* species, such antibodies could be produced using PAL fusion proteins. However, targeting PAL protein would not allow differentiation of different *Legionella pneumophila* serogroups.¹
Fluorescence microscopy not only allowed us to visualize the electrode surface, it was also useful to optimize the concentration of Alexa-555-conjugated secondary antibody that was utilized in our experiments. The increasing concentration of Alexa-555-conjugated secondary antibody led to an unexpected decreasing charge transfer resistance, I hypothesize that the π-interactions among the Alexa-555 dye molecules promoted antibody aggregation, which lead to an uneven distribution of antibodies on the electrode surface, producing “pin holes” on the surface, which allowed the redox probe, ferri/ferrocyanide to interact easily with the electrode surface. This could be further understood with scanning tunneling microscopy to study the surface topology at a molecular level. Our detection limit is feasible for moderate to severely contaminated environments, another PhD student will continue this project to work on the linearity over the dynamic range, also work with more biological samples such as detection of L. pneumophila in lung mucus samples. Development of this technology will allow more cost-effective preliminary screening of this pathogen, an essential for the prevention of infection and administration of effective treatment to patients.

5.2 Detection of adrenocorticotropic hormone

Chapter 4.2 has demonstrated a proof-of-concept study for the highly sensitive detection of ACTH using enzymatic amplification and EIS at disposable miniaturized SPGEs. The observed detection limit was 100 fg/mL in PBS, and we have also demonstrated high selectivity of our immunoassay to detect for 100 fg/mL ACTH in blood and plasma samples. The dynamic range determined was 100 fg/mL to 100 pg/mL, the upper limit of the dynamic range could be increased to a higher concentration by using a higher concentration of streptavidin conjugated ALP. Due to our limited resources for antibodies and ALPs, we used 10% (v/v) for antibody and 10 µg/mL for ALP, but higher concentrations of these expensive reagents could result in a better coverage of the surface. Both chapter 4.1 and chapter 4.2 used...
SPGE, but the linker film DTTSP was less soluble in ethanol compare to mercaptohexanoic acid used in Chapter 4.1. Further comparisons between the disulfides and thiols based linkers are discussed in Chapter 5.4 “Self-assembled film from organosulfur precursors”. DTSSP already has an amine reactive NHS-ester that could form covalent bond with antibodies, which was much easier to handle than using EDC/NHS with mercaptohexanoic acid. Avoiding the use of EDC could also minimize side-reactions such as hydrolysis reactions and the formation of N-acylurea.

### 5.3 Detection of growth hormone

In Chapter 4.3, we used screen-printed carbon electrodes for the detection of rat growth hormone. The miniaturized carbon electrodes have different surface chemistry compare to gold electrodes. In this study we modified the carbon surface with a film of aryl diazonium, carbon electrodes allow higher anodic working potential for reductive adsorption of the aryl diazonium film, and further electrochemical pretreatment of 4-methoxy group. Our XPS and contact angle studies have shown the successful attachment of aryl diazonium and antibodies on the surface. SPR study suggested that the antibody had a small dissociation constant 1.44 (±0.15) nM for rat GH. Impedimetric detection of rat GH exhibited high analytical performance demonstrating selectivity, sensitivity, and wide dynamic range. Results demonstrated the immunosensor has a calculated detection limit of 4.8 pg/mL GH in buffer, with a dynamic range between 10 pg/mL and 1000 pg/mL GH. Detection of rat GH in the presence of increasing concentrations of complex protein mixtures present in blood demonstrated the applicability of the technique to clinical samples. The immunosensor obtained comparable results with commercial GH kits for the detection of GH in blood samples collected from male rats with ages from two weeks to nineteen weeks; the results demonstrated that the immunosensor provided a promising platform for detections in real samples. This method could be further developed for detection of hGH, or for
detection of synthetic GH doping in athletes. To detect synthetic GH doping, two sets of electrodes would be needed, with one set targeting 22 kDa isoform hGH using mono-clonal antibodies, and another set using polyclonal antibodies to detect both 22 kDa and 20 kDa isoforms of hGH. Since the synthetic GH represent the 22 kDa isoform hGH and causes reduction of 20 kDa isoform hGH, the charge transfer resistance ratio between the two sets of electrodes could be compared for to determine whether athletes have been doping GH or not. One potential disadvantage of using charge transfer resistance to detect hGH might be the interference of growth hormone binding protein (GHBP), a protein that is sometimes tightly bound with 22 kDa hGH isoform. The non-specifically adsorbed GHBP could lead to false positive results due to its size and blocking of electrode surface. Thus, each assay would need to be individually validated. While our proof-of-principle studies have demonstrated application potential in clinically relevant samples, more detailed surface characterizations, quantification studies using real samples would have to be performed.

5.4 Self-assembled film from organosulfur precursors

In this thesis, the SPGEs were modified with modified with a thiol (RSH) mercaptohexanoic acid for the detection of L. pneumophila in Chapter 4.1, and modified with disulfide (RSSR) DTSSP for the detection of ACTH in Chapter 4.2. Another commonly applied organosulfur is dialkylsulfide (RSR). Spectroscopic results have shown that the affinity between metal and dialkylsulfides are weaker than the affinity between metal and thiols or disulfides. However it is less prone to oxidation compare to thiols and has a higher solubility than disulfides. It would be useful for protein binding to compare the dialkylsulfide with thiols and disulfides in a mixed self-assembled film. Studies have shown that mixed self-assembled films offer more surface chemical functionalities, or enhance binding capacity of ligands by reducing steric hindrance between large molecules. For thiols, this could be done with coadsorption
of mixture of thiols RSH and R’SH in similar length, solubility, but with different functional end-
groups.\textsuperscript{12, 13} Studies has shown that the surface and solvents would bias one form of thiol over the other, thus; the mole fraction of the solution mixture doesn’t represent the mole fraction of the self-assembled film. For disulfides, this could be done with adsorption of asymmetric disulfides (RSSR’), again the ratio of RS and R’S could be different in the mixture versus surface bond RS and R’S, just like thiols.\textsuperscript{14} For dialkylsulfides, it could be done with asymmetric dialkylsulfides (RSR’), even though the interaction with gold surface is weaker compare to thiol and disulfides, but the molecule will remain intact and have the ratio of R and R’ on a self-assembled film.\textsuperscript{15} Self-assembled film should not only allow modification to immobilize ligands, but also minimize the non-specific adsorption of proteins or other biomolecules on the electrode surface. Another experiment that can be proposed is to compare a different chemical composition of self-assembled film to see which one demonstrates better anti-fouling properties in blood and plasma samples. For example, oligo- (OEG) or poly (ethylene glycol, PEG) based self-assembled films have been successfully utilized to supress non-specific adsorption issues on surfaces.\textsuperscript{16} Although studies have suggested that PEG and OEG demonstrated better non-fouling properties due to “hydration barrier”, it is important to compare the anti-fouling properties in different settings such as over a wide range of pH and ionic strength conditions. The immobilization procedures and the stability of self-assembled films should also be tested over time.\textsuperscript{17}

5.5 Improving on aryl-diazonium salt modified electrodes

Compare to organosulfur modified gold electrodes, aryl-diazonium salts modified carbon electrodes could withstand 700 K temperature without being detached from the electrode surface.\textsuperscript{18} However, there are several disadvantages limiting its applications in biosensors. The diazonium salts are less controllable to film thickness compare to organosulfurs. Due to side reactions, the radicals could form
bond at the ortho-positions of a surface bond aryl group,\textsuperscript{19} or attach an hydrazine group to the electrode surface.\textsuperscript{20} More efforts need to be done to optimize the reduction adsorption of aryl-diazonium to our SPCEs to control the film thickness. The multi-layer formation could be minimized by blocking 3- and 5- position of the diazonium salt. Like organosulfur linkers, diazonium salts could also be synthesized with different chemical compositions, such as PEG or OEG. For the detection of GH using SPCE, using a mixed aryl-diazonium salt modified electrode could improve the binding of biomolecules. Recently, advances in interesting applications of diazonium salts in biosensors have demonstrated that patterning of the sensing substrates could be performed on metal surfaces such as Co, Ni, Cu, Zn, Pt and Au.\textsuperscript{21-26}

5.6 Site-directed antibody immobilization

The antibody immobilization technique described in this thesis was based on the covalent bond formed with amine groups randomly scattered on the antibody surface. Such random site immobilization technique has two major drawbacks, the random antibody orientation and decreased antibody active sites.\textsuperscript{27, 28} We could improve on these major draw backs by using site-directed antibody immobilization techniques to minimize the random orientation of antibodies and increase the antibody active sites. This could alternatively lead to an increase in the sensitivity and better reproducibility of the immunosensor.\textsuperscript{29, 30} There are three widely applied site-directed antibody immobilization techniques for biosensors.\textsuperscript{31-34} The first technique relies on the affinity interactions between Fc region of the antibody and Fc binding proteins such as protein A and protein G. Both protein A and protein G could bind to a range of antibody subclasses IgG, IgM, IgD, IgA in species human, rabbit, mouse, goat, etc., but each has its range of binding affinity towards these subclasses, for example protein A has a much weaker binding affinity towards human IgG3 compared to protein G.\textsuperscript{35-37} The binding mechanisms are also different in both proteins. Protein A has five binding domains, each recognizing amino acid residues in
CH2 and CH3 domains of antibody Fc region.\textsuperscript{38} Protein G only has three binding domains, recognizing the amino acids residues at the CH2 and CH3 interface. Studies also found that native protein G had affinity towards albumin, but this issue was resolved by using recombinant protein G from \textit{E.coli}.\textsuperscript{39} Studies has shown that protein A and protein G improved the orientation of immobilized antibodies, thus leading to higher sensitivity in comparison to random immobilization techniques.\textsuperscript{40} To improve orientation of the immobilized antibodies, it is also important to immobilize these Fc binding proteins with proper orientation. Several orientation-based immobilization techniques have been reported. Ahmed et al.,\textsuperscript{41} reported a method using tyrosinase to convert tyrosines on protein A to o-quinones, and then immobilized through the reaction between primary amine groups on polyallylamine. Protein A has only 5-8 tyrosine residues with only 1 tyrosine in the Fc binding domains, whereas 67-69 lysine, arginine, and histidine residues with 8 residues on the Fc binding domains (for amine coupling with EDC/NHS technique). Although not all of these amino acids are accessible, immobilization through tyrosine residues would lead to fewer orientations and higher activity of protein A. Gal et al.,\textsuperscript{42} reported a technique using elastin fused protein A and protein G, where antibodies were first immobilized with fusion protein A or protein G, and then the complex was immobilized to a hydrophobic surface with temperature-triggered hydrophobic interaction by elastin component of the fusion protein A and protein G.\textsuperscript{42}

The second type of site-directed immobilization technique uses antibody fragments. Fab fragments are most frequently used, the Fab region of an antibody binds to antigens, and it is composed of a constant domain and a variable domain. This fragment is connected with the Fc region by disulfide bridges, and could be digested using pepsin, bromelain, or ficin.\textsuperscript{43}
This Fab fragments is particular useful for gold sensor surfaces, because the sulfhydryl group on the Fab fragment has strong affinity for gold surfaces. However, this technique has a disadvantage due to the ambiguous mechanism when Fab region being fragmented, and reduced. The fragment could denature and lose chemical activity. Other recombinant antibody fragments such as scFv fragments have also been reported, but studies have shown that it was less stable than Fab fragments.\(^{44}\)

The third site-directed immobilization technique is based on the oligosaccharide moieties of the antibody. For example, IgG class antibodies usually have two oligosaccharide chains in each of the CH2 domain. These oligosaccharide moieties are distant from the antigen binding domains; thus, immobilization through these oligosaccharide moieties has minimal effects on the binding activity compared to the previous site-directed techniques. This oligosaccharide moiety could be oxidized and then coupled to functional groups such as amines or hydrazines.\(^{45, 46}\)

It is crucial to control the extent of antibody oxidation under specified reaction conditions, extensive oxidation could also oxidize other amino acids, which can lead to the denaturation of binding sites. Other studies have also reported to use boronic acid to form cyclic covalent complexes with the oligosaccharide moiety.

All of these site-directed immobilization techniques have their unique advantages and disadvantages, but overall studies have shown they improve efficacy of the immunosensors.

In conclusion, our preliminary results suggested that we were able to develop sensitive and selective electrochemical sensors for the detection of \textit{L. pneumophila}, ACTH, and rat GH. According to our hypothesis, with the current advances in miniaturized electrodes and potentiostats, we can state that this thesis can be considered as the groundwork for the development of portable and inexpensive...
electrochemical immuno sensors for medical and environmental diagnostics. The electrochemical biosensors have great potential for commercialization following careful optimization studies.

5.7 References


# Chapter 6

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