A Multiplexing Immunosensor for the Quantification of Cytokine Biomarkers

by

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A Dissertation Presented in Partial Fulfillment
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ABSTRACT

Biosensors offer excellent diagnostic methods through precise quantification of bodily fluid biomarkers and could fill an important niche in diagnostic screening. The long term goal of this research is the development of an impedance immunosensor for easy-to-use, rapid, sensitive and selective simultaneously multiplexed quantification of bodily fluid disease biomarkers. To test the hypothesis that various cytokines induce empirically determinable response frequencies when captured by printed circuit board (PCB) impedance immunosensor surface, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) methods were used to test PCB biosensors versus multiple cytokine biomarkers to determine limits of detection, background interaction and response at all sweep frequencies. Results indicated that sensors for cytokine Interleukin-12 (IL-12) detected their target over three decades of concentration and were tolerant to high levels of background protein. Further, the hypothesis that cytokine analytes may be rapidly detected via constant frequency impedance immunosensing without sacrificing undue sensitivity, CV, EIS, impedance-time (Zt) methods and modeling were used to test CHI™ gold electrodes versus IL-12 over different lengths of time to determine limits of detection, detection time, frequency of response and consistent cross-platform sensor performance. Modeling and Zt studies indicate interrogation of the electrode with optimum frequency could be used for detection of different target concentrations within 90 seconds of sensor exposure and that interrogating the immunosensor with fixed, optimum frequency could be used for sensing target antigen. This informs usability of fixed-frequency impedance methods for biosensor research and
particularly for clinical biosensor use. Finally, a multiplexing impedance immunosensor prototype for quantification of biomarkers in various body fluids was designed for increased automation of sample handling and testing. This enables variability due to exogenous factors and increased rapidity of assay with eased sensor fabrication. Methods were provided for simultaneous multiplexing through multisine perturbation of a sensor, and subsequent data processing. This demonstrated ways to observe multiple types of antibody-antigen affinity binding events in real time, reducing the number of sensors and target sample used in the detection and quantification of multiple biomarkers. These features would also improve the suitability of the sensor for clinical multiplex detection of disease biomarkers.
DEDICATION

For my son, Avi, who reminded me I needed to finish my PhD so that we could go to the beach. And my wife, Mallika, who said she would drag me through this dissertation kicking and screaming if she had to. She provided the kicking and I provided the screaming. Thanks Mallika!!!
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1.1 Overview

United States health care costs associated with diagnosis almost tripled between 2000 and 2008, from $90 billion to $250 billion (Feldman 2009), far exceeding the velocity of overall health care costs, which took from 1990 to 2008 to triple from $714 billion to $2.3 trillion as shown in Fig. 1.1 (NHSG 2010). This excess expenditure is driven by the continuing transition from screening by primary physician to expensive and powerful laboratory tests (Feldman 2009). Increasing costs of diagnosis highlight the need for inexpensive equipment and operations, even when controlling for overall increased medical expenditures. Moreover, the prevention and therapeutic treatment of many diseases would be improved by early detection. Early detection can come from both improved accuracy and sensitivity of methods and from low cost and ease of use, traits which also improve the device’s suitability in a monitoring capacity. Many diseases may be diagnosed and monitored through the quantification of bodily fluid biomarkers. Biosensors offer excellent methods for the precise quantification of bodily fluid biomarkers.

Combining a biological or biomimetic element with a signal transducer, biosensors were first and remain foremost employed in blood glucose quantification for diabetes monitoring (Clark 1962; Lowe 2008A). Biosensors have also found application in: monitoring the environment (Amine et al. 2006); spread of infectious disease (Spielbauer and Stahl 2005), genetic profiling for
personalized medicine (Blouin et al. 1996); increasing drug discovery throughput by rapid secondary screening of biopharmaceutical candidates (Shamah and Cunningham 2011); explosives detection for counterterrorism purposes (Smith et al. 2008); and quantification of disease biomarkers (Rapp et al. 2010). The characteristics of the many kinds of biosensors vary, and may be selected to meet the particular demands of the diagnostics market.

![Figure 1.1 Annual Rate of Increase for US Healthcare Costs 2000-2008](image)

Fig. 1.1 Annual Rate of Increase for US Healthcare Costs 2000-2008: The annual rate of increase for diagnosis costs in the US was more than double the rate of the overall medical cost increase (Feldman 2009; NHSG 2010).

1.2 Significance

The long term goal of this research is the development of a multifrequency impedance immunosensor for easy-to-use, cheap, rapid, sensitive and selective simultaneous multiplexed quantification of bodily fluid disease
biomarkers. This will be accomplished through experimental determination of important sensor parameters.

Fig. 1.2 Diagnostic Strength vs Convenience: Symptom screening by a primary physician and other similarly basic diagnostic methods have relatively low diagnostic strength, but are comparatively easy and convenient to perform. Intensive laboratory diagnostic tests have relatively high diagnostic strength, but are comparatively less easy and convenient to perform. Point-of-Care disease biomarker biosensors have the potential to fit in the diagnostic niche between initial screening and exhaustive analysis, with ‘good enough’ diagnostic strength and convenience, ease, and speed of testing.

Diagnosis and monitoring of many diseases (and biomarker quantification in much disease research) relies on techniques that could be surpassed. Bodily fluid biomarkers are a promising avenue for disease prediction, monitoring, and diagnosis, and scientific research of disease states, and have a ready niche between primary disease screening and intensive laboratory testing, as illustrated in Fig. 1.2. In many cases, the rapidly growing library of disease-relevant biomarkers may be used to diagnose disease in earlier stages, or
diagnose the disease more accurately and precisely than current disease diagnostic methods.

1.2.1 Cytokine Biomarkers

Cytokines, which are a coherent subset of circulating biomarkers, are critical components of the immune system, and are circulating biomarkers for many disease states. Cytokine biomarkers have been found for many cancers, including those of the thyroid (Interleukin (IL)-5, IL-8 and IL-12p40) (Linkov et al. 2008), breast (IL-6 and IL-8) (Nolen et al. 2008) and prostate (Transforming Growth Factor (TGF) β1 and IL-6) (Steuber et al. 2007). The cytokines IL-6, Tumour Necrosis Factor (TNF)-α, IL-18 (Armstrong et al. 2006), and IL-10 (Vasan 2006) serve as biomarkers of acute coronary syndromes. Cytokines are predictive of multiple organ failure resulting from trauma, including IL-6 and sTNF-R (Maier et al. 2007).

Bodily fluid cytokines have functional implications in autoimmune syndromes and diseases with suspected autoimmune components, and therefore they may serve as biomarkers of those diseases. For multiple sclerosis, they include IL-1β, IL-2, IL-6, IL-10, IL-12, interferon-gamma (IFN-γ), and TNF-α (Giovannoni et al. 1997; Hollifield et al. 2003; Nicoletti et al. 1996; Woodroofe et al. 1991). For rheumatoid arthritis, cytokine biomarkers include IL-1B, TNF-α, and IL-6 (Hueber et al. 2009; Rioja et al. 2004). Cytokine biomarkers for irritable bowel syndrome include IL-6, IL-8, IL-1B and TNF-α (Scully et al. 2010), and for inflammatory bowel disease (IBD) could include IL-1β, IL-4, IL-6, IL-12, IL-17, TNF-α and INF-γ (Alex et al. 2009; Alex 2008). For systemic lupus erythematosus (SLE), they include IL-2, IL-6 and IL-10 (Chun et al. 2007). In
stages of organ rejection, they include TNF-α (Platz et al. 1996), IL-6 and IL-10 (Hassan et al. 2006).

The potential efficacy of bodily fluid biomarker evaluation should be compared to current disease diagnostic State of the Art (SOTA), with reference to not just the sensitivity and selectivity, but also assay time, expense, and convenience.

1.2.2 Diagnostic State of the Art.

Sensitivity and selectivity should not be the only factors considered in diagnosis. The earlier the stage of the disease detected, or the earlier the prediction of the disease, the more useful the diagnostic method, as mitigation or prevention efforts are best implemented as early as possible.

Current SLE referral and diagnosis requires 3 or 4 of 11 possible exhibited symptoms to have occurred, some of which require laboratory assessment, for 95% specificity and 85% selectivity. Diagnosis can be problematic and uncertain, but of course early diagnosis is important for good outcomes. After diagnosis, lifelong monitoring is required for disease management, the alternative being poorer immediate health and greater permanent organ damage. This monitoring involves a great amount and variety of laboratory diagnostics and in-person medical labor (Gladman et al. 1999). Though there have been no updates to the official SLE diagnostic criteria since 1997 (Hochberg 1997), minor revisions have been proposed (Feletar et al. 2003; Liang 2004). This leaves an opportunity for improvement via testing of diagnostic and prognostic bodily fluid biomarkers with inexpensive, easy to use biosensors.
As an example, Crohn’s disease (CD), a form of IBD, exhibits as a series of attacks that cause permanent damage to the intestines. Early detection could reduce this debilitating damage by averting and mitigating attacks (Campieri 2002). There is no specific diagnostic test for CD in the standard criteria, and no protein biomarkers in clinical use. Standard criteria include a laborious process of elimination of similarly exhibiting syndromes, and expensive radiocolonoscopy (Glickman 1994). Histological examination of colon biopsies without reference to clinical details distinguishes IBD tissue from normal tissue with 82% sensitivity and 99% specificity, and ulcerative colitis tissue from CD with 61% sensitivity and 76% specificity (Cross and Harrison 2002), though biopsy would normally only occur after manifestation of symptoms. The fact that a combination of haemoglobin and platelet count yielded 90.8% sensitivity and 80% specificity for patients already complaining of possible IBD symptoms (Cabrera-Abreu et al. 2004) reveals the potential for using bodily fluid biomarkers to ease diagnosis of this syndrome.

1.3 Rationale

Among biosensors are many promising devices that could be used for quantification of bodily fluid biomarkers. These include surface plasmon resonance (SPR) techniques, piezoelectric techniques including cantilever and quartz crystal microbalance (QCM), optical techniques and electrochemical techniques like Electrochemical Impedance Spectroscopy (EIS). All of these have their advantages and disadvantages. SPR is a very sensitive measurement technique, but often demands lengthy optimization and requires expensive, complicated instrumentation. The technique is also vulnerable to interference
signal from non-specific surface–analyte interactions, has a narrow dynamic range compared to electrochemical techniques, and can generate poor signals for small molecules (Jecklin et al. 2009; Ramirez et al. 2009). QCM biosensors offer a direct measurement of mass adhered to the device, but have lower sensitivity limits in the range of μg/ml (Marx 2003), far too insensitive for clinical ranges of many biomarkers. Optical techniques are very widely relied upon, usually utilize amplification and secondary labeling steps, increasing expense and decreasing ease of use.

As a biosensing technique, EIS has been used for biomarker detection and quantification of disease biomarkers. However, this has often involved long incubation times, or less sensitivity than required for evaluation of biomarkers at clinically relevant levels (Degefa and Kwak 2008; Du et al. 2008; Evans et al. 2008; Min et al. 2008; NavratiLOva and Skladal 2004; Rahman et al. 2007; Rickert et al. 1996; Tsai et al. 2011; Yang et al. 2007). In many instances, complicated labeling schemes were employed, which raises assay duration and cost, and make real-time monitoring impossible. The increased complication makes sensors less suitable for point-of-care applications (Rapp et al. 2010). Without any secondary labeling or signal amplification, using biosensor detection reduces time, is lower-cost and is less complicated. Simultaneous detection of different metal ions has been theorized, but the technique does not have cross-applicability to label free protein affinity detection (Lindholm-Sethson et al. 2003). EIS biosensor arrays exist (Fairchild et al. 2009; Pan et al. 2010; Sadik et al. 2009), as do multisine EIS systems utilizing convoluted waveforms to simulate an EIS frequency sweep in a reduced period of time (Blajiev et al. 2006; Creason
and Smith 1972). However, a system for mass simultaneous multiplexing using AC impedance biosensing has not yet been implemented, leaving interrogation of a single sensor with a single convoluted waveform over a period of time a potentially superior alternative to multiple sensors interrogated by multiple potentiostats.

1.4 Objectives

Ideal detection frequencies, once found, can be employed in real-time monitoring of the impedance response, as well as the use of one biosensor for multiple target concentrations via a multifrequency convoluted signal. By modeling the kinetics of antibody-antigen interaction on the biosensor surface and quantifying the impedance response over time, an acceptable minimum detection time can be determined. By empirically determining the frequency of response for multiple cytokines versus their respective antibodies, the design of a simultaneous multiplexing device may be accomplished.

In this dissertation I present three studies in impedance immunosensing that address my long term objectives. The first study presents the results of testing a printed circuit board (PCB) EIS immunosensor versus multiple cytokines, establishing critical criteria such as frequency of response. The second study presents the results of testing gold disk impedance immunosensors versus cytokine biomarkers in real time, determining affinity kinetic model of antigen-antibody association and minimum detection time of the system, and confirming robustness of results cross-platform. The third study presents the feasibility of a multiplexing impedance immunosensor for quantification of
biomarkers in bodily fluid, its method of manufacture and method of use. The specific aims are:

1.4.1 Specific Aim 1: PCB Biosensors

The first specific aim is to test the hypothesis that various cytokines induce empirically determinable frequencies of response when captured by a PCB impedance immunosensor surface. This was accomplished by determining via cyclic voltammetry (CV), and EIS methods the limits of detection, dynamic range, and response at all frequencies in the sweep. Other metrics to be determined included background interaction.

1.4.2 Specific Aim 2: Gold Disk Electrode Experiments

The second specific aim is to test the hypothesis that rapid detection of cytokine analytes via impedance immnosensing can be done without greatly sacrificing sensitivity. This was done by determining via CV, EIS and fixed frequency impedance over time (Zt) methods the limits of detection, dynamic range, detection time and frequency of response of IL-12 on CHI gold disk electrodes.

1.4.3 Specific Aim 3: Prototype Design

The third specific aim is the design of a multiplexing impedance immunosensor for quantification of biomarkers in multiple types of bodily fluid, its method manufacture and method of use. Detailed include methods and devices for sample handling, single sensor prototypes, mass production arrays, and data analysis methods thereof.
1.5 Organization of Dissertation

Chapter 1 provides an introduction to the dissertation and discusses the hypotheses, specific aims, rationale and significance of the study.

Chapter 2 is a review of the literature on biomarkers of disease found in bodily fluid, multimarker approaches to disease diagnosis, classification and variety of biosensors, immobilization methods and the electrochemical transduction methods cyclic voltammetry and electrochemical impedance spectroscopy.

Chapter 3 discusses the development and testing of PCB impedance biosensors, including empirical determination of the limit of detection, tolerance to complex solutions and optimal frequency of detection for multiple biomarkers (Specific Aim 1).

Chapter 4 discusses further experiments on CHI gold disk electrodes towards a simultaneous multiplexing system, including minimum detection time determination via Zt and confirmation of optimal frequency of detection via EIS (Specific Aim 2).

Chapter 5 discusses a bodily fluid biosensor prototype, including a model for array fabrication, a method for sample handling, and methods of data analysis (Specific Aim 3).

Chapter 6 provides a summary of the results and conclusions that can be drawn from all the chapters and suggests future avenues that can be explored based on the conclusions.
Each chapter is self-contained, with an introduction, description of the research design and methods, results, and discussion. A bibliography for the entire dissertation is presented at the end of the document.
2.1 Introduction

A biosensor is “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals,” (Nic 2010). In many diseases, early detection and monitoring results in better outcomes. Early detection results not just from sensitive diagnostic techniques, but also from affordable and convenient methods. Biosensors offer not only sensitivity and selectivity, but also convenient and inexpensive means for the detection of disease biomarkers. Convenience and low cost render them advantageous for regular monitoring of medical conditions. Coal mine canaries, by detecting dangerous subterranean gases, would qualify as whole-organism biosensors. If limited to purpose-built biosensors however, the first example would be an electrocatalytic Glucose-Oxidase (GOD) enzyme-based sensor for the detection of glucose, first publicized in 1962 (Clark 1962). This quantified glucose in diabetes patients’ blood samples through electrochemical detection of enzyme activity, in this case the reduced presence of oxygen. The sensor was amperometric- operated by measuring the current resulting from voltage applied to the electric cell- current resulting from oxygen reducing at the cathode. Since then, many different biosensor recognition and transduction methods have been developed, with different advantages and drawbacks.
Biosensors can be made selective, having high specificity for the desired target. Using biomolecules that can often discriminate between very closely related analytes, for example antibodies or enzymes, high selectivity in the sensors themselves can be achieved. However, there is often a tradeoff between selectivity and sensitivity. Reproducibility, stability over time and rapid detection are also key attributes of a biosensor. Finally, biosensors can be made to be inexpensive, durable, and simple to use. This improves their utility for clinical and home use, as best illustrated by commercial glucose sensors. Taking advantage of biomolecule specificity, biomarkers of disease are attractive targets for biosensor development (Soper et al. 2006).

2.2 Disease Biomarkers

Biomolecules, i.e. biomarkers indicative of disease states, can be found in biopsies, blood, serum, urine, sweat, tears, and saliva. Monitoring of biomarkers found in bodily fluids is especially attractive because of the relative ease, low expense, and convenience of collection. Important components of the immune system, circulating cytokine levels are important biomarkers of many disease states.
Fig. 2.1 Cytokine Biomarkers for Disease States: Yellow- Diagnostic, Blue- Prognostic, Green- Both diagnostic as well as prognostic. Abbreviations include: Multiple Sclerosis (MS), Rheumatoid Arthritis (RA), Irritable Bowel Syndrome (IBS), Inflammatory Bowel Disease (IBD), Myocardial Infarction (MI), Acute Coronary Syndromes (ACS), Interleukin (IL), Interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α), Monocyte-Induced Gamma Interferon (MIG), Hepatocyte Growth Factor (HGF), and C-C Motif Chemokine Ligand 5 (CCL5). See section 2.2 for references.

2.2.1 Multiple sclerosis

There are a large variety of individual biomolecules with different degrees of effectiveness as biomarkers for Multiple Sclerosis (MS), and cytokines are among the most effective (Bielekova and Martin 2004; Giovannoni 2006). Serum cytokines relevant as MS biomarkers include interleukin (IL) -1β, IL-2, IL-6, IL-10, IL-12, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) in remission stages (Giovannoni et al. 1997; Hollifield et al. 2003; Nicoletti et al. 1996; Woodroffe et al. 1991).

For example, the 75kDa cytokine IL-12 is a proinflammatory marker which ranges between 0-5 pg/ml in normal serum, while rising to 5.5-18.9 pg/ml during MS (Hafler and Weiner 1995; Leonard et al. 1995; Nicoletti et al. 1996). During remission step testing, IL-12 levels decrease back to normal levels. Because of the cyclic nature of the cytokine pathway in MS, and the fact that other ailments
or diseases could interfere with a single marker sensor, a multimarker sensor would be of benefit. Studies have shown that changes in cytokine IL-12 level are observed in stroke, brain inflammation, Lyme disease infections (Garcia-Monco et al. 1990; Hansen et al. 1990; Kohler et al. 1988; Schluesener et al. 1989), tumors, and lupus (Steinman 2001). Serum IL-12 is elevated in Secondary Progressive MS. Elevated expression of the IL-12p40 subunit is seen in the cerebrospinal fluid of relapsing/remitting MS patients with active plaques demonstrated by magnetic resonance imaging (Galboiz and Miller 2002). Levels of another cytokine, IL-10, have been shown to drop in MS patients and in other diseases such as Crohn’s (Detkova et al. 2003), and also in pregnant women (Holmes et al. 2003).

2.2.2 Cancer

Serum biomarkers have been tested for efficacy in distinguishing malignant thyroid tumors from benign thyroid tumors and undiseased thyroid. IL-5 was found to be the best univariate thyroid cancer marker. In a multimarker panel, Interleukin-8 (IL-8), epithelial growth factor, hepatocyte growth factor (HGF) and Interleukin-12 P40 were the most efficacious four factors separating neoplastic and normal thyroids (Linkov et al. 2008). Multimarker panels have been found to be more efficacious than their individual constituents for prediction and detection of breast cancer metastases (Molina et al. 2005).

2.2.3 Rhumatoid arthritis (RA)

Joint biopsies of rodent models of RA showed elevated IL-1B, TNF-α, and IL-6. These biomarkers are also found elevated in the synovial fluid of human RA patients. Successful prednisolone treatment of the RA rodent models
corresponded with a reduction in IL-1B and IL-6 levels (Rioja et al. 2004). IL-6 and IL-1a are serum biomarkers of response to treatment of RA by etanercept (Hueber et al. 2009).

2.2.4 Irritable Bowel Syndrome (IBS)

Female IBS patients were found to have elevated serum IL-6 and IL-8, tested via an electrochemiluminescent immunoassay, compared to age-matched controls. Those patients with extra-intestinal symptoms also had elevated IL-1B and TNF-α (Scully et al. 2010).

2.2.5 Inflammatory Bowel Disease (IBD)

In a murine model of IBD, serum IL-4, IL-6, IL-12, IL-17 and INF-γ were found to best discriminate between healthy and sick animals, and between various types of disease (Alex et al. 2009). In serum taken from IBD patients, IL-1β, IL-4, IL-6 and TNF-α were found to discriminate between different disease types (Alex 2008).

2.2.6 Trauma

Late-onset multiple organ failure could be predicted among trauma patients using a combination of serum IL-6 and sTNF-R levels (Maier et al. 2007).

2.2.7 Sepsis

Septic febrile neutropenic patients were found to have higher levels of serum IL-8 compared to nonseptic febrile neutropenic patients (El-Maghraby et al. 2007). IL-10 was found to be higher in septic shock patients than cardiogenic shock patients (Marchant et al. 1995). Elevated serum IL-12 was found to be diagnostic of sepsis in neonatal patients. Serum IL-10 levels were also
diagnostic, though at lower sensitivity (Sherwin et al. 2008). Trauma patients with sepsis were found to have higher IL-18 than those without (Oberholzer et al. 2001).

### 2.2.8 Lupus

Serum IL-10 and IL-6 are significantly elevated in Systemic Lupus Erythematosus (SLE). Depending on other factors, IL-2 is either depressed or elevated among SLE patients (Chun et al. 2007). IL-12 levels have also been found to be significantly elevated in SLE patients (Manukyan et al. 2010).

### 2.2.9 Acute Coronary syndromes (incl. heart attacks)

In unstable angina cases, an increase in serum IL-6 levels that occurs within 8 hours after admission is associated with increased incidence of myocardial infarction (MI), refractory angina and death. High IL-6 levels are indicative of angina cases that benefit most from invasive procedures. Among recovering patients, elevated TNF-α is associated with recurrent Myocardial Infarction (MI) and death. TNF-α in 95th percentile indicated 2.7x risk of death/MI. Mouse experiments show elevated IL-18 are indicative of arterial plaque formation (Armstrong et al. 2006), while elevated circulating IL-6, IL-10, IL-18 and TNF levels in humans are linked to the existence of arterial plaques (Vasan 2006).

### 2.2.10 Organ rejection

Serum TNF-alpha has been found to be elevated in liver transplant patients with acute transplant rejection (Platz et al. 1996). Circulating IL-6 was found to be four times higher in patients with postoperative complications compared to patients without. IL-10 plasma concentrations also may serve as a
useful biomarker of postoperative complications in liver transplant patients (Hassan et al. 2006).

2.2.11 Multimarker detection for disease state evaluation

Using multiple factors has been found to be much more effective than using one for detecting trisomy 21, in terms of sensitivity, specificity, and cost effectiveness (Schneider and Mizejewski 2007), while markers such as serum Transforming growth factor (TGF) -β1 and IL-6 add specificity and sensitivity when staging prostate tumors (Steuber et al. 2007). Combining information from evaluating four prostate cancer markers found in urine exceeded the sensitivity and specificity of those markers alone, and far outperforms serum prostate-specific antigen (PSA) for diagnosis (Laxman et al. 2008).

Multimarker with empirically selected serum biomarkers exceeded sensitivity and specificity of ovarian cancer detection with its signature biomarker, CA 125 (Kozak et al. 2003). Analysis of gene expression from ovarian cancer biopsies revealed many potential biomarkers. A multimarker approach with groups of responsive, covarying markers proved, when compared with groups of markers with the best receiving operator characteristic (ROC) curves, ie. more sensitive and selective (Tchagang et al. 2008).

Cytokeratin-19 (CK-19), mammaglobin-2 (MGB-2) and NY-BR-1 mRNA were quantified in sentinel lymph node biopsies of breast cancer patients via reverse-transcription polymerase chain reaction (RT-PCR). This three-marker method was found to have a superior ROC curve when compared to two-marker histochemistry analysis of sentinel lymph nodes biopsies for metastases detection (Nissan et al. 2006).
Disease states where use of multiple biomarkers is confirmed to improve diagnostic sensitivity and selectivity serve to illustrate the advantage of using multiple biomarkers for that purpose. By definition, adding factors such as biomarkers to a model will increase the predictive value of the model. However, these additional factors must be examined to evaluate if they contribute more than a theoretical factor which varies randomly in relation to the response variable (eg. the disease state) (Montgomery 2005). The variety of cytokine biomarkers diagnostic to many disease states would serve as impetus for exploration of systems to detect and quantify these biomolecules. One such category of systems would be biosensors.

2.3 Biosensors

Biosensors detect or quantify analytes using biological material or a biomimetic recognition element associated with a transducer to achieve detection. Biosensors can be classified by both their recognition element and their transducer, device elements held in close physical proximity by an interface method. Recognition elements can be enzymatic, immunological, nucleic acid based, or based on other selective biological molecules. These physically interact with the target. Signal from enzymes consists of the reaction products. Transduction methods for enzymatic signaling are usually electrical, optical or fluorescent. Signal from most immunological, nucleic acid based, or other affinity biomolecule based recognition elements is the close physical association with the target. Transduction methods for affinity biosensors, if enzymatic labeling is not involved, are usually electrical, optical or piezoelectric (Kissing 2005; Nicu and Leïchlé 2008).
Fig. 2.2 Schematic of a Biosensor: Biosensors consist of a biological or biomimetic recognition element held in close physical proximity to a signal transducer. Recognition elements physically interact with the target to be detected. These can be any kind of selective biological molecule, but usually are enzymatic, immunological, or nucleic acid based. Transducing the biological signal into an electrical signal is usually done by electrical, optical, fluorescent, or piezoelectric means (Kissinger 2005; Nicu and Leichlé 2008).

2.3.1 Biosensor Recognition Elements

Biosensor recognition elements are usually classified as one of two types—affinity biosensors, which physically associate with the target analyte, and enzymatic biosensors, which react with the target analyte. Affinity biosensors take advantage of the selective binding properties of certain biomolecules, most often antibodies, receptors or nucleic acids. Biomolecules with high affinity and specificity for the given target are desirable. Most often observed in this category are immuno-sensors, taking advantage of versatile antibody libraries. A target analyte is used to produce polyclonal antibodies in a host organism, or to create a line of monoclonal antibodies. Antibodies can exhibit cross-reactivity, or nonspecific binding, reducing the specificity of the immunosensor and its tolerance for complex solutions. If antibodies can be raised against something, then an immunosensor can be created for the target—including bacteria, viruses, drugs and other chemicals. Applications of immunosensors include food safety, environmental monitoring, biowarfare monitoring (Eggins 2002), and disease diagnosis (Tang et al. 2007). Less commonly, nucleic acids have been used as recognition elements, either as straight strands to detect complementary nucleic
acids, or as aptamers, which are biomolecules with tertiary structure selected for affinity against a specific target. Nucleic acid sensors for detection of specific nucleic acid sequences find application in detection of cancers, viruses and alleles. Aptamers find applications more like those of immunosensors (Ronkainen et al. 2010).

Catalytic or biocatalytic or enzymatic biosensors are a major division of biosensing devices that use enzyme recognition elements for their specificity and their catalytic activity using purified enzymes, whole cells or tissue sections in the biosensor. Enzymes have very selective catalytic activity versus a specific target, they are usually very tolerant of complex mixtures. Enzymatic biosensors are set up to recognize the enzyme product. Enzymatic sensors are less versatile than affinity biosensors, relying on a limited library of enzymes (Ronkainen et al. 2010). The blood glucose monitor, the most commercially successful biosensor, employs the enzyme glucose oxidase (Nicu and Leïchlé 2008).

2.3.2 Biosensor Transducers

Transducers convert a biological recognition event into an electrical signal. Transducers used include optical, electrical (more properly electroanalytical), thermal, piezoelectric (Song et al. 2006) and acoustic (Lowe 2008) means of signal production. Piezoelectric transducers have included quartz crystal microbalance (Janshoff et al. 1997; Tedeschi et al. 2005) and cantilever (Campbell and Mutharasan 2008). Optical transducers include absorbance (Aoyagi and Kudo 2005); chemiluminescence (Mathew and Alocilja 2005); a hand-held laser induced fluorescence detector (Fruetel et al. 2005); and surface plasmon resonance (Leonard et al. 2004; Rasooly 2001). Less catagorizable transducers employed include surface acoustic wave devices.
(Berkenpas et al. 2004) and semiconductor based field effect transistors (Schoning and Poghossian 2002). Electrical transducers include potentiometric, amperometric, and impedimetric/conductimetric devices (Nicu and Leïchlé 2008; Wang 2006), which can be enhanced with magnetic nanoparticles (Fredj et al. 2008).

Electrochemical transduction techniques can operate with both affinity and enzymatic recognition elements, though not all will be equally optimal. These techniques can be organized by the method of measurement, which includes current, voltage, or impedance measurement.

For voltammetric and amperometric techniques, the current of an electrochemical circuit is measured. Typically, a voltage is applied to a working electrode versus a reference electrode, and current is measured at the working electrode. This current comes from electrochemical redox reactions at the working electrode surface, limited by mass transport. In amperometric techniques, a constant potential is applied and current is measured over time. In voltammetric techniques, current is measured in response to alteration of potential with time. Amperometric techniques include single-potential amperometry and pulsed amperometry (Trojanowicz et al. 2003). Voltammetric techniques include linear sweep voltammetry, cyclic voltammetry, hydrodynamic voltammetry, differential pulse voltammetry, square-wave voltammetry, ac voltammetry, polarography, and stripping voltammetry (Ramirez et al. 2009; Ronkainen et al. 2010). The key to using these techniques for biosensor transduction is to have an electroactive chemical as the biological measurand or as a proxy for the biomarker.
Cyclic Voltammetry (CV) is a very commonly used voltammetric technique. CV is a useful method for the studying electroactive species and electrode surfaces (Demirok 2010). In this electrolytic method, the solution is unstirred such that the measured current is limited by target diffusion at the working electrode surface. The input voltage is a series of linear sweeps back and forth between two voltages. The forward scan produces a current peak for oxidation of any analyte through the range of the potential scan. The current increases as the oxidation potential of the target is reached, and falls off as the target is depleted close to the electrode surface. Following the reverse linear voltage sweep, it reaches a potential where product formed during forward scan starts reducing and produces a current of reverse polarity from the forward scan. This produces a reduction current peak, usually mirroring the oxidation peak in shape. Larger differences or nonsymmetric reduction and oxidation peaks can indicate a nonreversible reaction. The parameters of CV make the technique useful for studies of redox reactions at electrode surfaces.

The parameters that can be derived from CV include the magnitude of peak current ($I_p$), peak potential ($E_p$), number of electrons transferred per reactant molecule ($n$), rate constant, diffusion coefficient ($D$), and electrochemical reversibility. In reversible systems, $I_p$ is described by the Randles–Sevcik equation.

$$I_p = 2.69 \times 10^5 A n^{3/2} D^{1/2} V^{1/2} C$$

Where $A$ is electrode area, $C$ is target concentration and $v$ is the scan rate ($V \cdot s^{-1}$) (Bard and Faulkner 2001). In a theoretically reversible system at STP, peak separation is 59 mV divided by the number of electrons transferred
per reactant molecule (Arya 2008). Cyclic voltammetry can be used to quantify
the concentration of redox species in solution.

Current resulting from reversible electrochemical systems controlled by
charge transfer at the electrode can also be evaluated by the Butler-Volmer
equation (Eq. 2.2), where $i$ is current density, $\alpha_c$ and $\alpha_a$ are the charge transfer
coefficients for the cathode and anode respectively, $n$ is the number of electrons
involved in the reaction, $F$ is the Faraday constant, $R$ is the universal gas
constant, and $\eta$ is the activation overpotential (Ozarem and Tribollet 2008).

$$i = i_0 \left\{ \exp \left( \frac{\alpha_c n F}{RT} \eta \right) - \exp \left( - \frac{\alpha_a n F}{RT} \eta \right) \right\}$$  (2.2)

In potentiometric methods, the potential of an electrochemical system is
measured at negligible current. Examples include glass pH electrodes and ion-
selective electrodes. By coating these sensors with an enzyme that produces an
ion specific to the electrode type, these sensors can be employed as biosensor
transducers (Ronkainen et al. 2010).

In impedimetric/conductimetric methods, conductivity is measured at a
series of frequencies (Arya 2008). Impedance spectroscopy biosensors operate
by input of a small signal and by comparison with the output signal determining
the real and imaginary impedance (Bard and Faulkner 2001). The most
prominent method is Electrochemical Impedance Spectroscopy (EIS).

Impedance biosensors measure the electrical impedance of an interface
in AC steady state by imposing a small sinusoidal voltage at a particular
frequency, with a DC offset, and comparing the resulting current with the input.
The impedance is given by the resulting current-voltage ratio. When repeated at
different frequencies, the technique is called EIS. EIS has been used to study
electrochemical phenomena, mostly affinity, of many different biological interactions (Daniels and Pourmand 2007). If the impedance of the electrode solution interface changes when the target analyte is captured by the probe, EIS can detect that impedance change. Alternatively, the impedance or capacitance of the interface may be measured at a single frequency. Impedance measurement can be performed label-free (Daniels and Pourmand 2007; Davis et al. 2005; Katz and Willner 2003; Muhammad-Tahir and Alocilja 2003; Tili 2006). EIS is a rapid, sensitive technique that has been heavily employed for biosensor signal transduction. EIS biosensors detect their target analytes at concentrations of pg/ml and lower (La Belle et al. 2007). Unlabeled EIS sensing in other laboratories has been shown to detect proteins to a lower limit of 1pg/ml concentration (Tsai et al. 2011).

2.3.3 Biosensor Immobilization Techniques

It is also important to get the affinity recognition element in close proximity to its transducer. This is done with direct adsorption to a sensor surface, physical entrapment in or onto a polymer layer, direct covalent attachment, or by use of a linker molecule. The immobilization method used depends on the recognition element, the transducer and the stability requirements for the biosensor. There are advantages and disadvantages to each technique. A selection of immobilization techniques are illustrated in Fig. 2.3.

Direct adsorption, relying on a hydrophobic surface, is simple, fast and inexpensive, but only really optimal for single-use sensors. The immobilization is relatively unstable, depending on local pH, temperature, and the nature of the adsorbed biomolecule itself. Many biomolecules, especially proteins, can denature and lose function because of the hydrophobic interaction. If the
hydrophobic surface is not blocked most proteins will be able to denature and adhere to it, producing a nonspecific signal for many sensor types.

Entrapment of the recognition element behind a permeable membrane is simple and can be done with practically anything. This is one of the only approaches for affixing tissue to a sensor. The recognition element is well-insulated, making the sensor more durable and opening up possibilities for long-term use. Mass production is difficult with this method, and diffusion across the membrane slows response time. Related to entrapment behind a permeable membrane is entrapment of the recognition element inside a polymer gel. This method is good for mass production, but leaves biomolecules vulnerable to denaturation by free radicals in the polymer.

Covalent bonding of the biomolecule to the transducer surface results in intimate, stable association between them. Due to the close coupling, there is little barrier to target diffusion, producing a rapid-response device. The chemistry for this immobilization can be complex and costly. This method can often reduce the activity of the biomolecule by hindering its active sites. It also reduces the working lifetime of the biomolecule.

Utilizing both covalent bonding of recognition elements and a surface polymer layer, recognition biomolecules can be covalently bonded to a ‘polymer’ layer, including molecule monolayers. Advantages of this technique include, potentially, a controlled number of immobilization sites, lower steric hinderance of biomolecule active sites and increased signal size. It can be a relatively complex procedure, and on the output side yield more complicated kinetics and diffusion.

Capture systems like biotin-streptavidin yield surfaces where specificity may be freely switched because that they can easily be regenerated after use.
These are usually very expensive and involve complicated derivitisation. The multi-layer structure can reduce signal, and the components of the system may bind to interferents in complex solution (Mayes 2002).

A widely-used method of associating the biological recognition element of an affinity biosensor with the transducer is use of a self-assembled monolayer (SAM). SAMs are organic assemblies formed by absorbing molecules from solution or the gas phase onto the surface of solids or in regular arrays on the surface of liquids. Metal or metal oxides tend to adsorb organic materials because the interactions between these two components low the free energy of the interface of metal or metal oxide and the surrounding environment (Adamson 1997). The adsorbates organize spontaneously into crystalline structures, which can alter interfacial properties and have a significant influence on the stability of nanostructures of metals and metal oxides.

Two distinct approaches can be used to fabricate SAMs. The Langmuir-Blodgett technique involves the transfer of a pre-assembled film at an air-water interface to a solid substrate, whereas the spontaneous adsorption of molecules

![Fig. 2.3 Biosensor Immobilization Methods: Recognition elements maintained in close physical proximity with transduction elements through examples of (a) physisorption, (b) covalent bonding, (c) entrapment, (d) capture systems, (e) phospholipid insertion and (f) self-assembled monolayers.](image)
onto a substrate by immersing the substrate into an appropriate amphiphile (Ulman 1996).

Although there are a large number of molecules able to form SAMs on a solid substrate, the organosulfur (alkylthiols, di-n-alkyl disulfides etc.) /noble metal system is the most investigated to date. It is the best characterized system in terms of stability and physico-chemical properties. The mechanism of chemisorption of alkylthiols on gold is considered to be an oxidative addition of S-H bond followed by reductive elimination of hydrogen (Eq. 2.3).

\[
\begin{align*}
R-S-H+Au_0^n & \rightarrow R-S-Au^+Au_0^n+1/2H_2 \\
R-S-S-R+Au_0^n & \rightarrow 2R-S-Au^+Au_0^n+H_2
\end{align*}
\] (2.3) (2.4)

In the case of disulfides, the mechanism is thought to follow Eq. 3 (Ulman 1996). The alkyl chains of thiols are in the all trans-conformation, tilted at an angle of ~20-30° from normal to gold surface (Bain et al. 1989).

SAMs are easy to prepare, and no special instruments or harsh conditions are required to construct such a system. They are critical in stabilizing and functionalizing nanomaterials, and can link the external environment to the properties of substrates (e.g. electric and optical properties of metallic structures) (Phoenix and Drexler 2004). It is simple to link biomolecules to electrode surfaces using SAMs (see Chapter 3, Fig. 3.4). The incorporation of multiple molecular components within a monolayer offers the opportunity to control the microenvironment at molecular level and makes such system even more versatile. All these features make SAMs play an important role in biosensing, drug delivery and molecular imagine and many other fields.

The well organized molecular architecture on the sensing surface is resistant to specific adsorption of undesired entities and is able to bind selected
biological species (i.e. proteins, nucleic acids and whole cells). For this particular purpose, mixed alkylthiol SAMs can be prepared by coadsorption of alkylthiols with different length and functional end groups on the substrates. One thiol molecule carries a functional group (e.g. –carboxyl) that can be used to covalently bind biological reporters and the other thiol molecule has a functional group (e.g. –hydroxyl) that shows resistance to nonspecific adsorption (Uchida et al. 2005).

2.4 Electrochemical Impedance Spectroscopy (EIS)

EIS was introduced in chapter 2, section 2.3 (transducers). In order to model a system using the technique, a frequency sweep is taken over a frequency range (for instance, 1Hz-100,000Hz) under appropriate run conditions, $V_{\text{applied}}$, amplitude, redox probe concentration, etc. Electronic impedance measurements of total impedance ($Z$), the real component of impedance ($Z'$), and the imaginary component of impedance ($Z''$) are done at each frequency. A Nyquist plot will be made using the values for the real component ($Z'$) on the x-axis and the values for the imaginary component ($Z''$) on the y-axis. This process is illustrated in Fig. 2.4.
Fig. 2.4 Electrochemical Impedance Spectroscopy (EIS) Data and Analysis: (a) Initial processing of EIS data requires comparing (input) a sinusoidal voltage which is applied to the sensor and the (output) resultant current reconverted to voltage to determine the ($\theta$) phase shift and ($|Z|$) absolute impedance of the system, the latter of which is proportional to the reduction in amplitude. The resulting information can be used in (b) Bode plots, which can compare the absolute impedance or the phase shift versus frequency, or in (c) Nyquist plots, which compare ($Z'$) real, and ($Z''$) imaginary components of the resulting impedance, which may both be determined from the phase shift and the absolute impedance.

After electrical data has been received, it must be interpreted appropriately. The most general form of modeling of the data from EIS comes from an equivalent circuit model approach using a Warburg coefficient. Other complex models for EIS have also been developed. In many impedance spectroscopy biosensors, models besides Randles or Warburg are used to fit the data (Lasseter et al. 2004; Navratilova and Skladal 2004; Peng et al. 2007; Rickert et al. 1996). Constant phase elements are often used to interpret data that otherwise would be cumbersome using only standard circuit elements (McMillion et al. 2005). These basic circuits for Nyquist modeling along with their associated plots are shown in Fig. 2.5.
Fig. 2.5 Nyquist EIS Nyquist Plots and Equivalent Circuit Models: Data plots corresponding with equivalent circuit models of impedance, including (a) Randles, incorporating Rs, representing solution resistance, Cdl, representing the double-layer capacitance of the electrode surface, and Ret, representing the Resistance of the electrode surface, (b) Warburg, additionally incorporating W a warburg element representing diffusion-limited transfer of electrons and (c) Constant Phase Element models, incorporating a constant phase element indicative of an uneven or rough electrode surface.

The general form of a Nyquist plot for these experiments will be a partial semicircle followed by a linear region as is seen in Fig. 2.5b. If a very fast electron transfer process occurs, the radius semicircle will be very small, and the curve will primarily consist of the linear region. If a very slow electron transfer process occurs, the semicircle region will be present with no linear area (Fig. 2.5a). The Nyquist plot can be plotted using the $Z'$ and $Z''$ values in ZsimpWin™, Microsoft Excel™ or an equivalent program. Once the Nyquist plot has been created, a 2nd order polynomial equation can be fit to the values in the semicircle region of the curve. Using this equation the maximum value of the plot, as well as each of the two intercepts with the real impedance axis (x-axis), is calculated. The solution resistance ($R_s$) and Warburg Impedance ($Z_w$) are properties of the bulk electrolyte solution and diffusion properties of the redox probe. The solution resistance can be calculated as the intercept of the semicircle (for instance as
seen in Fig. 2.4 and Fig. 2.5a) with the Z' axis in the high frequency (low Z' value) ranges (Katz and Willner 2003).

The electron transfer resistance (R_{et}) controls the electron transfer kinetics of the redox probe at the electrode interface. The immobilized IgG and bound IL-12 will serve as a varying electron transfer resistance (R_{imm} and R_{bound}) connected in series with the constant electron resistance of the unmodified electrode (R_{elec}) resulting in Eq. 2.5:

$$R_{et} = R_{elec} + R_{imm} + R_{bound} \quad (2.5)$$

The electron transfer resistance is a function of the heterogeneous electron transfer rate constant (k_{et}). This is shown in Eq. 5 below, where R= 8.31 J mol^{-1} K^{-1} (gas constant), T is the temperature (K), F=9.65 x 10^4 C equiv^{-1} (Faraday constant), A is electrode area (cm^2), [S] is the concentration of redox probe (mol cm^{-3}), and n is the number of molecules transferred per molecule of redox probe (Katz and Willner 2003).

$$R_{et} = \frac{RT}{nF^2A k_{et}[S]} \quad (2.6)$$

The value for electron transfer resistance can be easily calculated using the Nyquist plot. The difference between the two values of the semicircle intercept with the Z' axis is equal to the electron transfer resistance.

The double layer capacitance depends on the electrical double layer, and is a function of the dielectric permittivity introduced into the double charged layer molecules (C_{dl}). The equation below can be used to determine the capacitance. In this equation A is the area of the electrode (cm^2), δ is the thickness of the separating layer, $\varepsilon_0$ is the dielectric constant of a vacuum, and $\varepsilon_p$ is the dielectric
constant of the separating layer (Katz and Willner 2003), as can be seen in Eq. 2.7.

\[ C_{dl} = \frac{\varepsilon_0 \varepsilon R A}{d} \]  

(2.7)

Just as the electron transfer resistance can be calculated as resistors in series, the double layer can be calculated as capacitors in series. The result is that the double layer capacitance is controlled by the smallest capacitance, as shown in Eq. 2.8.

\[ C_{dl}^{-1} = C_{\text{elec}}^{-1} + C_{\text{imm}}^{-1} + C_{\text{bound}}^{-1} \]  

(2.8)

In order to calculate the double layer capacitance, the characteristic frequency of the system must be found. The characteristic frequency \((f_0)\) is calculated by determining the frequency in Hz (as measured during the EIS testing) that corresponds to the maximum value on the polynomial curve. This frequency is multiplied by \(2\pi\) to convert it to units of radians/second, and is equal to \(\omega_o\) (Katz and Willner 2003):

\[ \omega_o = 2\pi f_0 \]  

(2.9)

This characteristic frequency is computed as in Eq. 2.10.

\[ f_0 = (C_{dl} R_{el})^{-1} \]  

(2.10)

The double layer capacitance can be calculated using the characteristic frequency and the electron transfer resistance, and thus from the Nyquist plot, and is calculated as seen in Eq. 2.11.

\[ C_{dl} = (f_0 R_{el})^{-1} \]  

(2.11)

Both \(C_{dl}\) and \(R_{el}\) are important parameters for evaluation of an electrochemical system, and for these studies, evaluation of a biosensor surface, though other parameters of interest were determined, most importantly the
optimal frequency of detection and the impedance at the optimal frequency of detection (see chapters 3 and 4). Briefly, the raw data and plots of frequency versus impedance at different target concentrations were used to determine the frequency of maximum response over the concentration range tested. The impedances determined at this optimal frequency were plotted versus target concentration to obtain a relationship with target concentration.

2.5 Conclusions

The remainder of this dissertation provides the experimental protocol, results, analysis, and proposals for future work to be done. Chapter 3 discusses the development and testing of printed circuit board EIS biosensors, including the determination of optimal frequency of detection for individual biomarkers. Chapter 4 discusses studies performed on commercial gold electrodes, including fixed frequency experiments towards making use of the optimal frequency of detection and determining minimum detection time. Chapter 5 discusses the design of and protocol of use for a bodily fluid biosensor prototype utilizing multiple fixed frequencies for simultaneous quantification of multiple analytes on one sensor. Chapter 6 provides a summary of results and conclusions, and suggestions for future work based on these studies.
3.1 Introduction

Chapter 2 discussed disease biomarkers and methods of biosensing, including Electrochemical Impedance Spectroscopy (EIS). An immunosensor utilizing unlabeled EIS makes sense as a technology with the goal of creating a sensitive, inexpensive, rapid biosensor to fill the gap between primary disease screening and intensive laboratory testing. Development requires the creation of an initial prototype system, as detailed in this chapter. Criteria for assessing the system should be obtained, and extant unlabeled EIS state of the art forms a benchmark for evaluation of the system. The system must be physically created, so the electrode must be built from something, and a method of immobilization and a target molecule and corresponding affinity molecule must be chosen. Printed circuit boards (PCB) form an excellent basis for flexible, rapid construction of electrodes. As well, IL-12 is a biomarker with low physiological and disease-state concentrations, requiring sensitivity in detection systems, thus making a good choice for a test molecule for detection in the development of an ostensibly sensitive system.

3.1.1 EIS Biosensors

Unlabeled biosensing with EIS has a significant record in literature, some of which is summarized in Fig. 3.1. It has been shown to detect salmonella from $10^0$ - $10^6$ colony-forming-units/ml (CFU/ml), with an incubation time ranging from
2-16 hours (Yang et al. 2004; Yang et al. 2003). Unlabeled EIS immunosensors have detected hemoglobin at 20ng/ml (Hleli 2006), 2,4-dichlorophenoxyacetic acid at 45nM (10ng/ml) (Navratilova and Skladal 2004), and dust mite antigen Der p2 at 1pg/ml (Tsai et al. 2011). Unlabeled EIS immunosensors have obtained lower limits of detection of 300pg/ml for 4,4-bis(4-hydroxyphenyl) valeric acid (Rahman et al. 2007). Using EIS, antibodies at 0.167mg/ml were detected using secondary antibodies in an unlabeled fashion (He et al. 2005). In other studies, EIS in conjunction with secondary antibody recognition elements detected antibodies with a limit of detection (LOD) of 67 μg /ml, unlabeled (Yang et al. 2007). Synthetic peptide recognition elements, used with EIS, have detected antibodies at 8.7μg/ml (Rickert et al. 1996). Synthetic peptide EIS-systems have also been used for detection of biomarkers (CDK2) at a limit of 50pM, or 1.7ng/ml (Evans et al. 2008). Aptamers have been used as recognition elements for EIS sensors to detect proteins at 100nM with platelet-derived growth factor (PDGF) (Degefa and Kwak 2008), 10pM (360pg/ml) thrombin and 10nM (5ng/ml) ATP (Du et al. 2008) and 10pM (170pg/ml) IFN-γ (Min et al. 2008), while oligonucleotides have been used as EIS recognition elements for the detection of nucleic acids (Cai et al. 2004; Davis et al. 2005; Lucarelli et al. 2005; Patolsky et al. 2001).
Fig. 3.1 Unlabeled Detection Limits for Various Targets: Targets (x-axis labels), detected with EIS biosensors using recognition elements like secondary antibodies (a, b) (He et al. 2005) (Yang et al. 2007), synthetic peptides (c, d) (Rickert et al. 1996) (Evans et al. 2008), aptamers (e-g) (Degefa and Kwak 2008) (Du et al. 2008) (Min et al. 2008), and antibodies (h-j) (Tsai et al. 2011) (Hleli 2006) (Rahman et al. 2007). Full target name for (j) is 4,4-bis(4-hydroxyphenyl) valeric acid.

3.1.2 Interleukin-12

Not included in the above survey of unlabeled EIS biosensing is previous work done in the LaBelle lab detecting Interleukin-12 (IL-12) concentrations using gold sputtered silver wire working electrodes down to 5pg/ml (La Belle et al. 2007). A number of factors influenced choosing IL-12 as a test biomarker for impedance immunosensor development. IL-12 is a cytokine biomarker with clinically relevant levels in very low ranges. At a molecular weight of 75 kDa, IL-12 is a heterodimer composed of disulfide-bonded 40 kDa and 35 kDa subunits, which themselves have little intrinsic biological activity (Engel and Neurath 2010). It is part of a larger family of cytokines that also comprises IL-23 and IL-27 (Trinchieri 2003). IL-12 is produced by antigen presenting cells including macrophages and dendritic cells, and induces protective immunity through its
effects on other cytokines and lymphocytes. It is a growth factor for T cells and NK cells (Gately et al. 1998), activates Th1 cells, induces cytolysis in T cells, NK cells, and macrophages, and induces production of other cytokines (Metzger 2010). The IL-12 receptor is expressed on T cells and NK cells. Binding of IL-12 to its receptor can lead to activation of the STAT4 intracellular pathway, though multiple different STAT pathways can be activated (Engel and Neurath 2010). STAT4 itself is a transcription factor promoting production of proteins that affect the cytokine activity of the cell (Wurster et al. 2000).

IL-12 is normally found at less than 5.0 pg/ml in healthy adults (Nicoletti et al. 1996). It is found at 5.5-19 pg/ml in the serum of MS patients (Hafler and Weiner 1995). It is found at elevated serum levels in mice with toxic shock syndrome (Gately et al. 1998), children with acute invasive streptococcus infections (Wang et al. 2008), patients with thyroid metastases (Linkov et al. 2008), Systemic Lupus Erythematosus (SLE) (Manukyan et al. 2010) and Irritable Bowel Disease (IBD) (Alex et al. 2009). Compared to other bodily fluid biomarkers, the healthy (approximately 5pg/ml) and disease state concentrations of IL-12 are very low, requiring a very discriminating technique for useful quantification and making IL-12 an excellent choice for development of a sensitive label-free EIS immunosensor.

3.1.3 Printed Circuit Board Biosensors

With those performance targets in mind for IL-12 detection and unlabeled EIS in general, the physical substrate from which the biosensor is constructed becomes a present and pertinent choice. In-lab experience, particularly, indicated the importance of precise and reproducible positioning of electrodes when
performing EIS. The types of biosensor recognition elements and transduction methods were discussed in Chapter 2, but the further advantages offered by various physical substrates for the transduction element present a subsequent choice.

Producing the first Printed Circuit Board (PCB), photolithography was first applied to pattern conductors on an insulator in 1936 by Paul Eisler (Strong and Eisler 1944). In biosensors, printed circuit boards offer the advantages of fast, inexpensive, precise and reproducible fabrication. Printed circuit boards are used in biosensors as the base of the biosensing device assembly (Beach et al. 2005; Dempsey et al. 1997; Jobst et al. 1997; Petrou et al. 2002). More recently, they have been employed as the base of the sensing surface itself. Copper is easily reactive, potentially leading to noise in an electrochemical assay. Consequently, electrochemical biosensors directly incorporating printed circuit boards have involved the deposition of material onto the electrode surface, such as carbon nanotubes (Yun et al. 2008) or gold (Bhavsar et al. 2009; Fairchild et al. 2009).

3.2 Methods

3.2.1 Fabrication of PCB sensor arrays

In the current study, the PCB electrodes were a standard three-electrode pattern designed to interface with a potentiostat, including an Ag/AgCl reference electrode, a gold counter and a gold working electrode. The arrays were designed using computer aided design software (AutoCAD, Autodesk) allowing for easy modification, and grouped into eight arrays of five electrodes. For production, this layout was printed onto a transparency and carefully aligned to one, 15.2cm x 15.2cm, pre-sensitized copper clad PCB board (MG Chemicals).
The transparency and the board were held in place in a UV light box, where unmasked portions of the board were exposed to 423nm UV light for 110 seconds. The PCB board was removed and promptly immersed in 0.25M, pH 14 sodium hydroxide (MG Chemicals), for approximately 2 minutes with mild agitation and brushing to remove the photoresist. After a thorough rinse with distilled water, the board was suspended in a tank with 1.1M ammonium persulfate at pH 1 (MG Chemicals) at 40°C and agitated with air bubbled throughout until all exposed copper was removed (8-10 minutes), and then rinsed thoroughly with distilled water. The board was then cut into arrays of 5 sensors each and stored. This fabrication procedure is summarized in Fig. 3.2.

Fig. 3.2 Photoetching and Development of the PCB Array: First, mask aligned with photoresist-coated copper-clad board placed into light box (a), to be removed after 2 minutes of UV exposure (b). Exposed photoresist removed by immersion and scrubbing with sodium hydroxide developer solution (c) followed by rinsing with water to neutralize (d). Developed board etched in ammonium persulfate until all exposed copper is removed (e) and then washed with water again to stop etchant (f).
3.2.2 Electroplating

In the electroplating process, a stored array was first rinsed with acetone to remove the photoresist, then immersed in sodium perborate (Caswell) at 98°C for 2.5 minutes and slight agitation for degreasing, followed by a distilled water rinse and stored in hot distilled water. Allowing only the leads to dry, the array was inserted into an edge connector attached to a power supply. The array was immersed in nickel sulfate solution (Caswell) pH 4.5 at 43.3°C, along with a nickel anode at a distance of 7cm from the array, and a 10mA/cm² current applied until a total charge transfer of approximately 6.3°C. The array was rinsed with hot distilled water, then degreaser, then with hot distilled water again, without removal from the edge connector. The array was then immersed in a gold sulfate (Caswell) solution at pH 9.6 and 60°C, opposite a gold anode 7cm distant. 0.8mA/cm² was applied until a total charge transfer of approximately 0.15C. Silver nitrate solution was used to brush plate silver onto each reference electrode using a fixed potential of 1.7V for 30 seconds. Each silver reference was chlorinated with a bleach solution for 2 minutes. 100μl+ volume wells were affixed to the array, one well per 3-electrode set. To reduce surface contamination, the array was stored in pH 7.4, 1x phosphate buffer saline (PBS) at room temperature until use. Fig. 3.3 shows a representative schema of an electroplating cell and a set of electroplated sensors.
Fig. 3.3 Electroplating: A representative schema of the nickel electroplating cell with the PCB electrodes acting as cathode, accepting Nickel ions, while a Nickel anode donates ions to solution (left), and a set of freshly gold-plated PCB electrodes attached to a edge connector (right).

3.2.3 Immobilization of antibody

The electrodes were immersed in reagent grade ethanol with 1 mM 16-mercaptophexadecanoic acid (16-MHDA) (Aldrich) in solution, and allowed to set at room temperature for 1h. Next, the sensor surfaces were carefully rinsed with ethanol and then distilled water. A rubber sheet with well holes (Intl Equip Components) was adhered to the board to create wells on the sensor surface capable of holding a sample volume of 100μl. A 100μl droplet of a 10mM N-hydroxysulfosuccinimide (sulfo-NHS) (VWR International), 40 mM N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) (Pierce biotechnology) solution was placed in each well, and allowed to sit for 1h at room temperature. After rinsing with PBS buffer, a 100 μl droplet of a 50 μg/ml solution of anti-cytokine IgG (R&D systems) in PBS buffer was placed in each well and left at room temperature for 1h. After rinsing with PBS, 100 μl droplets of 1% ethanolamine were added to the wells for 30 min at room temperature to block unreacted carboxyl groups of 16-MHDA and EDC/NHS. The blocked sensors were rinsed
and stored in PBS at 4 °C until use. Fig. 3.4 is a summary of steps for immobilization of antibody after MHDA deposition.

![Diagram](image)

Fig. 3.4: EDC/NHS Chemistry for Functionalization of the Electrode: Addition of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) to electrode-immobilized 16-mercaptohexadecanoic acid (MHDA) (a), and the subsequent reaction with N-hydroxysulfo succinimide (sulfo-NHS) (b). Addition of antibody (c) and immobilized antibody upon substitution for the leaving group (d).

### 3.2.4 Methods for Verification of Electrode Functionalization

To ensure that the antibodies were immobilized onto gold working electrode surface, a secondary labeled antibody was used. A 2 µl drop of the 2.5 µg/ml of an Alexa680 infrared dye labeled secondary antibody was placed in the wells of the anti-IL-12 antibody functionalized electrode for one hour at room temperature and without any light exposure. Next, the electrodes were washed with PBS and immediately imaged using a LI-COR Odyssey Imaging System.
An IL-12 ELISA kit was utilized to test antibody specificity (R&D Systems). Dilution sets of recombinant human IL-12, Interleukin-2 (IL-2), Interleukin-10 (IL-10), Tumor Necrosis Factor alpha (TNF-α), and Interferon gamma (IFN-γ) (R&D Systems) were made. The manufacturer protocol was followed in detecting the IL-12 (and cross-reactivity verification studies). In summary, 200 μl of sample or control was added to each well, incubated for 2 hrs at room temperature then washed. Each well was then incubated for 2 hrs at room temperature with μl horseradish peroxidase-labeled anti-IL-12, then washed, then incubated for 20 min in the dark with 200 μl of hydrogen peroxide and tetramethylbenzidine. Finally, 50 μl of a 2 N sulfuric acid was added to each well without washing. A spectrophotometer (UV2501-Shimzdu) was used to test the absorbances at 450 nm. A background absorbance at 540 nm was subtracted to account for interference from the well material. The other targets were detected by similar protocol, the only difference being the substitution of the each target for IL-12.

3.2.5 Electrochemical Methods (CHI 660c)

All electrochemical methods were performed with a CHI 660C potentiostat (CH Instruments) in series with a CHI 684 64-channel multiplexer (CH Instruments). 3-electrode sensor surfaces were immersed in a redox probe solution containing 5mM potassium ferrocyanide and 5mM potassium ferricyanide in PBS. To employ a sensor array, it was removed from PBS, dried, connected to an edge connector in electrical contact with instrument leads, and the wells filled with 100μl of redox probe with appropriate concentration of antigen or other biomolecules when required. After measurement was done, it
could be put into the next stage of immobilization, exposed to appropriate experimental conditions, or simply immersed in PBS again.

For cyclic voltammetry (CV) measurements, potential started at -0.1V, was swept to 0.4 V at the rate of 0.1V per second, then back down to -0.1V. This was repeated for a total of 3 cycles. CV was employed to determine the formal potential, which was obtained from the average of the cathodic and anodic voltage peaks, and also peak separation, which was obtained from the comparison of the cathodic and anodic voltage peaks. Peak separation was used as a crude measure of surface cleanliness of the sensor or of adsorption to the sensor surface.

For EIS measurements of the bare electrode, the AC amplitude was set to 5mV and swept from 100khz to 1hz, with the static DC offset for the EIS of each individual electrode obtained from the bare electrode formal potential as shown by CV. The same was done for EIS measurement of the electrode at various stages of immobilization.

To measure impedance from antigen-sensor interactions the same EIS instrument settings were used as described above. Test solutions were also prepared using the same redox probe as described above. All cytokine sample solutions were diluted in the redox probe and stored at 4 °C until use. Each electrode of the connected PCB electrode array was tested versus each target concentration in turn, with no incubation time.

To measure sensor-antigen interaction with background interference, PCB electrodes were studied with an array of target concentrations in 0%, 1%, and 100% fetal bovine serum.
3.2.6 Analytical Methods

EIS data was analyzed in order to determine equivalent circuit parameters and the optimal frequency. In order to evaluate the equivalent circuit parameters of the sensors, the Nyquist plots were modeled using software (ZSimpWin, Electrochemical software). Circuit parameter fittings via least squares iteration were made until an optimal model was determined. EIS circuits including but not limited to Randles and Warburg were tested and compared. Once the optimal fit was achieved, parameters were tabulated and coefficient of determination was calculated over the target concentration range.

The raw data and plots of frequency versus impedance at different target concentrations were used to develop the frequency of maximum response for data interpretation. In order to determine the frequency at which maximum changes in impedance are observed, a plot was made of percent change in impedance over all the cytokine concentrations tested versus frequency. This was also compared to a plot of frequency versus the coefficient of determination as determined from linear fits of impedance versus concentration at every employed frequency. Thus, an optimal frequency was identified for detection of antibody-antigen binding events.

3.4.2 Determination of Limit of Detection

According to the IUPAC Gold Book, the lower Limit of Detection (LOD) is the quantity or concentration \( c_L \) that corresponds with the smallest measure \( x_L \) that can be detected with reasonable certainty to be different from a blank in a given analytical procedure. \( x_L \) is determined by:

\[
 x_L = \bar{x}_{bi} + k s_{bi}
\]

(3.1)
where \( \bar{x}_{bi} \) is the mean of the blank measures, \( s_{bi} \) is the standard deviation of the blank measures. The numerical confidence level, \( k \), is usually best chosen to be 3 (Long and Winefordner 1983; McNaught and Wilkinson 2006). In this particular case, repeated blanks were run on separate disposable sensors as to avoid the possibility of degradation. The relative standard deviations (RSD) of these blank runs were averaged, then the average RSD multiplied by the blank run impedance of the sensors which were run versus target (also standing in for \( \bar{x}_{bi} \)) to obtain \( s_{bi} \). The relationship between concentration and impedance obtained from a regression of the concentration curve was used to calculate \( c_L \) from the resulting \( x_L \), as per the IUPAC recommendation. Independent sensors could be used in this way to obtain the relative variability of blank runs.

**3.3 Results**

**3.3.1 Impedance and Antibody Immobilization**

Fig. 3.5 shows Nyquist plots taken from the AC sweeps of the bare, antibody immobilized (Fig. 3.5b) and biomarker (IL-12) bound electrodes (Fig. 3.5c, Fig. 3.6c). Fig. 3.6 shows AC sweep experiments on one electrode. Fig. 3.7 shows Bode plots comparing data from PCB electrodes that, when exposed to 1000pg/ml IL-12, were interrogated with the EIS transduction technique (Fig. 3.6c), to an equivalent circuit model incorporating constant phase elements (CPE) (Fig. 3.7a insert). Fig. 3.8 shows the slopes of impedance vs log(concentration) at each frequency as well as the RSQ values of those slopes from the same experiment. Fig. 3.9 shows a plot of concentration versus impedance at 5Hz as taken from the AC sweep experiments. Bare electrodes (Fig. 3.5a, Fig. 3.6a) displayed a Nyquist plot dominated by the Warburg
element. After MHDA was immobilized (Fig. 3.6b), impedance increased to its highest point in the experiments, with Nyquist plots relatively dominated by CPE double-layer capacitance and the resistance of the electrode. After antibody was immobilized (Fig. 3.5b, Fig. 3.6c), the Nyquist plots displayed a decrease, or downward shift, with absolute impedance decreasing as well as $R_{et}$ in some models (data not shown). As the target molecules bound to the immobilized antibody in a concentration gradient, impedance (and $R_{et}$) changed in an increasing direction (Fig 3.6c,d, Fig. 3.9). In addition, as the concentration gradient proceeded, the CPE elements became proportionally more resistive (data not shown). Models incorporating CPEs were the best fit lower-order models for data taken after immobilization. As shown (Fig. 3.7), at lower frequencies, impedance was dominated by the constant phase element resembling a resistor with minor capacitive effects (CPE 2), in series with a relatively weak resistor ($R_{et}$) (Fig. 3.7 bar scales). Mid-range frequencies were dominated by a CPE element (CPE 1) resembling an imperfect capacitor. High-end frequencies were again dominated by $R_s$. 
Fig. 3.5 Immobilization and Modeling of the PCB Electrode: (a) Bare electrode conforming to Warburg model (displayed on insert). (b) Anti-IL-12 immobilized electrode and (c) IL-12 detecting electrode both conforming to an equivalent circuit model with constant phase elements (displayed on insert).
Fig. 3.6 Nyquist Spectra on one PCB Electrode: (a) bare, clean electrode (Fig. 3.5a) (b) antibody-immobilized electrode versus various IL-12 concentrations (including Fig. 3.5b, c) and (c) MHDA-only on the electrode surface. Insert (d) shows 5 Hz impedance vs log (concentration) for the sensor.
Fig. 3.7 Equivalent Circuit Modeling: Bode plots comparing EIS PCB immunosensing data (points) at 1000pg/ml IL-12 exposure (Fig. 3.6c) against an equivalent circuit model (line). (a) Frequency versus Absolute Impedance (|Z|), the dotted lines representing the values of the equivalent circuit elements. Insert in (a) is the equivalent circuit model, with a constant phase element (CPE 2) in series with a resistor (Ret), both of which are in turn parallel with a constant phase element CPE 1. This is in series with another resistor (Ret). The bar scales at the top of (a) and (b) identify the circuit elements that dominate |Z| in each frequency range.
3.3.2 IL-12 Detection

Electrodes were tested against a concentration gradient of IL-12 from 0 to 1000pg/ml, and the results plotted, as seen in Fig. 3.6c. Increasing the target concentration resulted in an increasing absolute impedance at most frequencies (Fig. 3.8). Equivalent circuit elements (data not shown) from best fit models also displayed significant trends over the concentration gradient.

![Graph showing optimal frequency determination](image)

**Fig. 3.8** Optimal Frequency Determination: At each individual frequency, taken from the data shown in Fig. 3.6c, the slopes of impedance vs log(concentration) (a), as well as the RSQ value of those slopes(b). Below 10,000 Hz, the RSQ values of the log/linear fits were above 95%. The greatest slope of impedance vs log(concentration) occurred at 1.18Hz. The peak at 4.54 Hz corresponds to the typically resulting optimal frequency for IL-12.

Optimal frequencies were determined by first finding the slope of a fit of impedance versus concentration, over the entire concentration gradient, for every frequency tested. This result, along with corresponding R² values, was plotted against frequency (Fig. 3.8) to observe patterns in frequency versus response. For this experiment, the greatest slope of impedance vs log(concentration)
occurred at 1.18Hz. There was also a local maximum at 4.54 Hz with a nearly equivalent slope.

Impedance at 5Hz (4.54 Hz) was plotted against log[IL-12] (Fig. 3.9) and a best-fit model was obtained. This indicated a log-linear relationship between the initial test concentration of IL-12 and the impedance resulting from EIS interrogation of the exposed immunosensor.

![Graph showing impedance at 5Hz (4.54 Hz) against log IL-12 concentration.](image)

Fig. 3.9 Impedances (datapoints) at 5Hz (4.54Hz): for the concentration gradient previously shown (Fig. 3.6c). From the linear regression (line), impedance was found to equal $y = 26.316 \ln(x) + 1420.8$ (ohms).

In this set (3 sensors, 5 blank experiments per sensor), the average blank RSD was found to be 2.72%, or a blank standard deviation of 36.7Ω for this sensor. From the commonly followed IUPAC guidelines (Long and Winefordner 1983; McNaught and Wilkinson 2006), the smallest measure which can reasonably be considered distinguishable from no concentration at all would be
at 3 standard deviations above the blank measure, or in this case 1462.2Ω. From linear regression, the concentration corresponding to the Limit of Detection (LOD) in pg/ml is found to be:

\[
C = e^{\frac{x-14208}{28.3}}
\]  

(3.2)

where \(x\) is the impedance(Ω), resulting in a LOD of 4.3pg/ml.

3.3.3 Multimarker detection

PCB sensors were immobilized with various antibodies, and then tested against concentration gradients their respective cytokines, IL-2, IL-10, IL-12, IFN-λ and TNF-α, with the EIS transduction technique. As initial solution concentration of antigen increased, resultant Nyquist plots shifted towards an increase in impedance. Generally, a log-linear relationship was obtained between concentration and impedance, whether from equivalent circuit elements (not shown) or from optimal frequency data. The frequency where occurred maximal response (optimal frequency), assumed to be the greatest slope of impedance change over concentration change, was found for each marker (Fig. 3.10f). For each marker at physiological levels, optimal frequency impedances were found to be statistically significant over control. For IL-12, optimal frequency was confirmed at 5Hz. For IL-2, IL-10, IFN-λ and TNF-α, optimal frequency was found at 31.5Hz, 117.2Hz, 17.44Hz and 9.77Hz.
Fig. 3.10 Sensor Impedances over Concentration Range of 1 to 10,000 pg/ml: Impedance for each MS biomarker on its respective sensor over a wide range of concentration was determined for (a) IL-2, (b) IL-10, (c) IL-12, (d) TNF-α, and (e) IFN-γ at optimal frequencies (f). Data adapted from (Fairchild et al. 2009). Not shown is the optimal frequency found for IFN-γ, 9.77 Hz.

3.3.4. Background interaction

EIS was used to interrogate PCB electrodes exposed to concentration gradients of IL-12 in solution with 0%, 1%, and 100% Fetal Bovine Serum (FBS). Impedance results were normalized versus the blanks, or the experiments where IL-12 concentration equaled 0. Solutions with 0% FBS, simulating purified patient samples, was detected with a log/linear concentration-impedance relationship between 0.1-1000pg/ml concentration. As interferant was added to create a
dilute (1% FBS) complex solution simulating partially purified patient samples, the upper concentration range of the log/linear relationship contracted to 100pg/ml (Fig. 3.11c), with a nonlinear relationship thereafter. Increasing the FBS concentration from 1% to 100% (Fig. 3.11a, b) to simulate unprocessed patient serum samples, the log/linear range shortened to 0.1-10pg/ml, concentration-impedance relationship following a nonlinear trend at higher IL-12 concentrations.

![Fig. 3.11 Background Interaction: Impedance vs concentration and best fit lines for IL-12 in (a,b) redox probe with 100% Fetal Bovine Serum (FBS) (c,d) redox probe with 1% FBS (e) redox probe. Data adapted from (Bhavsar et al. 2009).](image)

**3.3.5 Estimation of IgG Coverage and IL-12 Maximum Binding Concentration**

The surface area of an electrode, using the working area as 12.192x12.192 mm² and a thickness of 25.4 μm was calculated as:

\[
SA_{elec} = 1 \times (12.192 \times 12.192 \text{ mm}) + 4 \times (0.0254 \times 12.192 \text{ mm})
\]

\[
= 1.4988 \times 10^{-4} \text{ m}^2
\]
The surface area taken up by 1 molecule of IgG (assuming dimensions of 2x7x10 nm, Wrigley, 1983; Valentine, 1967) would be found from these dimensions and how the molecules are attached to the surface. Typical assumptions of immobilization of IgG using a cross-linker such as MHDA is that one of the antigen binding sites is immobilized so that only one of the variable regions is presentable to an antigen.

\[ \text{SA}_{\text{IgG}} = \pi \times (1 \text{ nm}) \times (5 \text{ nm}) = 1.5708 \times 10^{-17} \text{ m}^2 \]

Assuming an ellipsoidal arrangement for a protein, an estimated 0.74 packing can be made (Donev, 2004). Therefore, the maximum amount of surface loading the IgG molecules can make on one side of the gold coated silver electrode would be:

\[
\text{Max Coverage} = 0.74 \times (0.987 \times 10^{-6} / (1.5708 \times 10^{-17})) \\
= 4.65 \times 10^{11} \text{ IgG molecules on a single electrode}
\]

Verification of adequate amount of IgG in solution comes from the protocol for fabrication. Therein, a 1 ml of a 50 \( \mu \text{g/ml} \) solution of IgG in phosphate buffer saline, pH 7.4, was used. First, we must assume IgG has MW = 150 kDa

\[
6.022 \times 10^{23} \text{ molecules}/150,000 \text{ gm} = 4.0115 \times 10^{18} \text{ molecules/gm}^{-1} \\
= 0.1 \text{ ml} \times 50 \mu \text{g/ml} \times 1 \text{ gm/(}10^6 \mu \text{g}) \times 4.0115 \times 10^{18} \text{ molecules/gm}^{-1} \\
= 2.0056 \times 10^{13} \text{ molecules of IgG in solution}
\]

If coat 1 electrode, overnight, this volume, a maximum of:

\[
= 2.0056 \times 10^{13} / (4.65 \times 10^{11} \text{ IgG}) \\
= >100\% \text{ of an electrode can be coated.}
\]
3.4 Discussion

3.4.1 Impedance and Antibody Immobilization

Warburg dominance of the bare electrode (Fig. 3.5a, Fig. 3.6a) equivalent circuit model indicated diffusion-limited electron movement and low surface impedance compared to solution impedance. The prominence of CPE double-layer capacitance after MHDA immobilization is indicative of an uneven or irregular electrode surface, increasing in relative strength (data not shown) through antibody immobilization and exposure to IL-12.

As surface accumulation increased, changes occurred in the impedance spectra. Most notably, impedance was highest when the electrodes were immobilized only with MHDA. The impedance then decreased upon functionalization with antibody, only to increase again upon binding with target. The consistently-observed (other data not shown) decrease in electron transfer resistance and surface capacitance is most likely explainable as the effect of a charged molecule (IgG) reducing the surface capacitance effect of a relatively nonpolar molecule (MHDA). Coupled with the increased CPE significance and the decrease of the capacitive proportion of the CPE, this is possibly indicative of mixed immobilization on the surface, with not all available MHDA paired with antibody, and of yet more increased roughness as antigen associates with some of the available antibodies. The CPE element parallel with a CPE-and-resistor (Fig. 3.7a, insert) could indicate a mixed surface wherein one component displays pure CPE behavior with emphasis on capacitance, and the other component displays CPE behavior with emphasis on resistance, in addition to a purely resistive component.
3.4.2 IL-12 Detection

As expected, impedances increased overall with increasing initial concentrations of IL-12. In some equivalent circuit models, $R_{et}$ increased, possibility indicating increased electrode resistance as antigen associated, while $R_s$, indicative of solution resistance, remained relatively constant. The increasingly resistive proportion of the CPE was possibly indicative of antigen association, in that antigen association could be increasing surface roughness.

In addition, a local frequency response maxima was found at 4.54 Hz, corresponding with the value found elsewhere (Tlili 2006) and in other experiments (not shown). Using this optimal frequency, comparison with blank data was used to obtain a lower LOD for the sensor. The resulting lower LOD of 4.3pg/ml is significantly lower than that reached by most other label free impedance biosensors (Fig. 3.1).

A clear contrast with state of the art label-free EIS biosensors is the lower detection limit and the reduced incubation time. Additionally, the optimal frequency of detection is another metric rarely found in literature. In our study, the sensor was able to detect IL-12 at physiological ranges, not achieved by other unlabeled EIS sensors. Undemonstrated, however, is the ability to distinguish between very close levels of IL-12 that would be necessary to distinguish between physiological and disease-state levels of IL-12.

3.4.3 Multimarker Detection
Five separate cytokine biomarkers were detected in solution by the PCB EIS immunosensor. The 5Hz optimal frequency of IL-12 was confirmed, and optimal frequencies for four other cytokines were obtained for the first time in the discipline. This demonstrates the versatility of the PCB immunosensing system when immobilized with different antibodies and tested against different antigen types.

3.4.4 Background Interaction

The detection of IL-12 at low levels, even when combined in complex solution with FBS, is indicative of the robustness of the immobilization and blocking against nonspecific interferants. However, the slope change in the 1% FBS and 100% samples was most likely due to background interactions. This also decreased the range of log-linear relationship between concentration and impedance. The extreme case, 100% FBS, showed an increase in slope, and a decrease of detection limit to 10pg/ml. This could be attributed to diffusion issues more than nonspecific surface interactions as the controls for 0 pg/ml (not shown) showed no significant increase between different FBS concentrations.

3.4.5 Estimation of IL-12 coverage

With an estimate of anti-IL-12 surface coverage, it becomes apparent that the IL-12 ranges used may not even approach ranges required for saturation of the sensor. This indicates the relationship of impedance to initial target concentration is probably not based on saturation of the sensor at any point. A linear relationship between initial target concentration and antibody-antigen association may be occurring, and being measured by the system.
3.5 Conclusions

The results of this study showed that the unlabeled PCB EIS immunosensor had a demonstrable lower detection limit of 4.3pg/ml, lower than almost all extant unlabeled EIS sensors. This indicates that PCB biosensors are capable of rapid detection of biomarkers in low in a label-free manner, indicating their suitability for further development. The results also showed that the system was tolerant (ie. still sensitive to target when exposed) to background interference vis a vis high concentrations of fetal bovine serum. This indicates the suitability of the system for testing patient samples with minimal pretreatment. The PCB platform testing yielded, for the first time, five optimal frequencies of detection for the five biomarkers tested. These frequencies allow the freedom of using fixed-frequency impedance analysis on these PCB electrodes over longer periods of time. These frequencies could also find use in simultaneous detection of multiple targets with one sensor. With the optimal detection frequencies known, a frequency sweep would become unnecessary, making instrumentally and post-analytically simpler impedance versus time experiments the interrogation mode of choice. By immobilizing a sensor with multiple antibody types and interrogating it with a multi-frequency convoluted signal, a sample could be tested for more than one target in a simultaneous fashion.
CHAPTER 4

GOLD DISK ELECTRODE EXPERIMENTS TOWARDS SIMULTANEOUS MULTIPLEXING OF MULTIPLE CYTOKINE BIOMARKERS

4.1 Introduction

Chapter 3 covered studies demonstrating the optimal frequencies of detection for several biomarkers. The existence of such optimal frequencies implies that, instead of using an EIS frequency sweep, interrogating a biosensor at innumerable different AC perturbations, it may be preferable to utilize a single frequency for detection, saving time and analysis effort. Furthermore, it should be possible to immobilize multiple antibody types on a sensor, and then utilize multiple optimal frequencies in a single convoluted signal to interrogate the sensor for the presence of several antigens simultaneously. However, to create a simultaneously multiplexing impedance biosensor, parameters besides the optimal detection frequencies must be determined. One way to do this, and take a step towards creation of such a biosensor, is with Impedance-time measurements. Where Electrochemical Impedance Spectroscopy (EIS) feeds many frequencies through the sensor at a given offset, a fixed-frequency impedance-time (Zt) technique feeds one frequency into the system at a given DC offset. This is illustrated in Fig. 4.1.
Fixed frequency methods are not unknown to impedance biosensing. A fixed 20kHz signal has been used to interrogate a polyaniline-coated sensor for detection of antibody to 500ng/ml (Sergeyeva et al. 1996). Similarly, a fixed 200kHz signal was used to detect anti-IgG binding on diamond and silicon electrodes (Yang et al. 2007). A fixed 20kHz, 10mV signal was used in conjunction with a collagen-coated gold electrode interrogating a sample for the presence/activity of collagenase down to 0.2 mg/ml (Saum et al. 1998). A fixed 2954Hz, 10mV signal was used for interrogation of an immunosensor detecting 2,4-dichlorophenoxyacetic acid, at 100μg/ml (Navratilova and Skladal 2004). A 1.28GHz, 40mV fixed signal was used to interrogate an affinity nanogap sensor for detection of thrombin to determine affinity molecule vs antigen kinetics (Schlecht et al. 2006). A 10kHz, 5mV signal was found to be optimal for monitoring the growth of Salmonella typhimurium on an interdigitated electrode (Yang et al. 2004). Likewise a fixed 1kHz, 50mV signal was found to be optimal
for interrogating an impedance sensor detecting *Salmonella typhimurum* in bulk solution (Yang 2008).

**4.2 Methods**

**4.2.1 Immobilization**

Wells (affixed as seen in Fig. 4.5a) (Eppendorf) were cut from 1m micropipette tips and hung in an empty tip box. Electrodes (CH Instruments) were polished on 3um, 1um and 0.05um alumina in deionized water on polishing pads (Buehler) before sonication in deionized water. CV and EIS were determined, and interpreted to evaluate gold surface quality (see 3.2.4, Methods of Verification for Electrode Functionalization).

Immobilization was generally performed as described in chapter 3 with some variations. Briefly, for immobilizing anti-IL-12 IgG, 100μl of a 1mM 16-Mercaptohexadecanoic acid (16-MHDA) (Sigma) solution in reagent grade ethanol was placed into a newly affixed well, and the well covered with parafilm to prevent evaporation. The sensors were allowed to set at room temperature (RT) for 1h. The surface was carefully rinsed with ethanol and then distilled water. EIS was performed to evaluate MHDA immobilization. 100μl of 40mM 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Sigma), 10mM N-Hydroxysulfosuccinimide (NHS) (Sigma) solution was placed in a newly affixed well. After 1h at RT, the sensor was rinsed in phosphate-buffered saline (PBS) buffer. Next, a 100μl droplet of a 50μg/ml solution of anti-IL-12 IgG (R&D Systems) in PBS buffer was placed in a newly affixed well on the electrode and left at RT for 1h. The sensors were carefully rinsed with PBS and then 100μl of 1mM ethanolamine (Sigma) was added for 30m at RT to block unreacted
carboxyl groups of 16-MHDA activated with EDC/NHS. The blocked sensors were washed with PBS and stored in PBS at 4 °C until future use.

4.2.2 Electrochemical Methods

As described in chapter 3, all electrochemical methods were performed with a CHI 660C potentiostat (CH Instruments) in series with a CHI 684 64-channel multiplexer (CH Instruments). However, instead of printed circuit board (PCB) chips with the reference, working and counter electrodes affixed to the same surface, an apparatus was used with a separate gold disk working electrode (CH Instruments), platinum wire counter electrode (CH Instruments) and Silver/Silver Chloride wire reference electrode.

To determine the response of CHI electrodes versus a concentration gradient, first fully immobilized electrodes were rinsed and new wells for testing affixed. 100μl of each of a gradient of antigen (R&D Systems) concentrations in 1x PBS were successively placed in the well for one hour incubation. After each incubation, the electrode was rinsed and the evaluation well affixed for EIS testing in 1x redox probe. After the EIS frequency sweep, the electrode was rinsed and the incubation well affixed for the next successive concentration, the procedure repeated until the concentration series was complete.

For long term fixed frequency over time measurements, fully immobilized CHI electrodes were rinsed and new wells affixed. 100μl of 1x redox probe was placed in the wells and Zt mode of the CHI 660c potentiostat was initiated at one of various frequencies, at DC offset as determined previously by CV. 1μl injections of PBS, redox probe, and cytokine solvated in redox probe were done at intervals to achieve desired antigen concentration.
For short term fixed frequency over time measurements, fully immobilized CHI electrodes were rinsed and new wells affixed. 100μl of each of a gradient of antigen (R&D Systems) concentrations in 1x redox probe were successively placed in the well. Upon placement of test solution, Zt mode of the CHI 660c potentiostat was immediately initiated for a 90-second duration at one of various frequencies at a DC offset as previously determined by CV. After the Zt interrogation, the electrode was rinsed and the well replaced for the next successive concentration, the procedure repeated until the concentration series was complete.

4.2.3 Analysis

Evaluation of CVs, Nyquist modeling of EIS data, and determination of optimal frequency was done as per the procedure specified in chapter 3. Briefly, ZsimpWin software was used to find the best-fitting Nyquist model, and parameters with the most significance were plotted against concentration. In order to determine the ideal fixed frequency for detection, the raw data and plots of frequency versus impedance at different target concentrations were used. A plot was made of percent change in impedance over all the cytokine concentrations tested versus frequency, as well as a plot of frequency vs the coefficient of determination as determined from linear fits of impedance vs concentration at every employed frequency. Once optimal frequency was determined, plots of impedance versus concentration at said optimal frequency were made and evaluated.

To evaluate fixed frequency versus time data, models were created in Simulink to determine kinetic parameters and modes of action within the electrochemical cell.
4.2.4 Kinetics models

The system to be studied is a non-stirred 100ul cylindrical volume 0.5 cm in diameter and 0.51cm depth. Antigen is solvated in aqueous solution, reacting with antibody immobilized on an electrode at the bottom of the cylinder. This can be approached in one of two ways. First, it could be assumed that the association-dissociation reaction at the surface of the electrode is limited by diffusion. That is, the potential rate of uptake of antigen by the immobilized antibody exceeds the rate at which the antigen reaches the surface. This would be called a diffusion-limited model. Alternatively, it could be assumed that the rate at which antigen reaches the surface exceeds the potential rate of uptake of antigen by that surface. In this model, termed kinetic-limited, the reaction at the surface is only limited by the antigen-antibody affinity.

At higher surface affinities, whether due to antibody affinity or a higher surface concentration of antibody, and where convection effects are negligible, a diffusion-limited model would be favored. However, if the sensor system is diffusion-dominated, then it should not be assumed that over long periods of time and in the relatively large space, that convective effects will not also be important. At lower surface affinities or where convection effects are important, a kinetic-limited model would be favored. As the patterns present in the data are consistent whether at short or long durations of antigen solution exposure to the electrodes, as will be shown in the discussion of the binding model in 4.4.1, the more likely model is kinetic-limited.

Basing a model off of reaction kinetics, a basic dissociation equation, where complex (product) $A_xB_y$ breaks down into $x$ subunits of reactant $A$ and $y$ subunits of reactant $B$.
\[ A_xB_y \rightleftharpoons xA + yB \]  \hspace{1cm} (4.1)

X has a dissociation constant \( K_d \) which is defined as the following (Eq. 4.2),
where, with brackets denoting concentration, \([A]\) is the concentration of reactant A, \([B]\) is the concentration of reactant B, and \([A_xB_y]\) is the concentration of the complex.

\[
K_d = \frac{[A]^x[B]^y}{[A_xB_y]} \hspace{1cm} (4.2)
\]

In simple situations, where \(x=y=1\), this formula is simplified. One case would be a reaction involving antibodies (Eq. 4.3), where antibody \(Ab\) and antigen \(Ag\) are the reactants, and antibody-antigen complex \(AbAg\) is the product.

\[ Ab + Ag \rightleftharpoons AbAg \]  \hspace{1cm} (4.3)

This yields a simpler equilibrium dissociation constant (Eq. 4.4), where \(K_{\text{reverse}} (k_r)\) is the reverse reaction rate constant, and \(K_{\text{forward}} (k_f)\) is the forward reaction rate constant (Eq. 4.4).

\[
K_d = \frac{[Ab][Ag]}{[AbAg]} = \frac{k_{\text{reverse}}}{k_{\text{forward}}} = \frac{k_r}{k_f} \hspace{1cm} (4.4)
\]

By setting the antibody-antigen complex \([AbAg]\) equal to the surface concentration \(\Gamma\), the surface being where the antibody-antigen complex would be located, and removing the equilibrium condition equation rate equation for the formation of antibody-antigen complex may be obtained (Eq. 4.5) (Karlsson et al. 1991).

\[
\frac{d\Gamma}{dt} = k_f[Ab][Ag] - k_r[\Gamma] \hspace{1cm} (4.5)
\]

To simplify the model, it is useful to assume that the available anti-IL-12 is not significantly diminished by binding, that the antigen mixture is homogenous, and that the surface is fully covered with anti-IL-12 (\(\Gamma_{Ab}=1\)). A simulink model
using these assumptions was created (Fig. 4.2), then resultant proportions of occupied antibodies plotted at various initial antigen concentrations and sensor exposure times for illustrative purposes.

\[
\frac{dl}{dt} = k_f[Ab][Ag] - k_r[AbAg]
\]

Fig. 4.2 Rate-limited Simulink Model: Utilizing Eq. 4.5 (inserted equation), this model assumes full surface coverage of the sensor with anti-IL-12, that the available anti-IL-12 is not significantly diminished by binding, and that the antigen mixture is homogenous.
4.3 Results

4.3.1 Model Output

Fig. 4.3 Theoretical Binding to the Surface Over Time (Rate-Limited Model): Plots of (Γ) antibody-antigen complex divided by total antibody versus time as calculated by the Simulink model (Fig. 4.2), where (a,b) the results for the first 100 seconds are shown, (c,d) the results for the first hour are shown, (a,c) all four concentrations are shown, or (b,d) the lowest two concentrations used are shown. Initial concentrations include (e) 2.5ng/ml, (f), 250pg/ml, (g) 25pg/ml and (h) 2.5pg/ml.

To explore the model, sample data was plotted. First, the proportion of the surface-immobilized antibodies occupied with antigen as a function of time was plotted at initial antigen (IL-12) concentrations of 2.5pg/ml, 25pg/ml, 250pg/ml, and 2.5pg/ml. This was done over periods of both 100 seconds (Fig. 4.3 a,b) and 1 hour (Fig. 4.3 c,d). Then, the proportion of antibodies occupied was plotted as a function of said concentrations at both 100 seconds and 1 hour exposure (Fig. 4.4). Over time, as in-solution antigen was depleted, the rate of antibody-antigen complex formation slowed. However, the proportion of antibodies occupied at a given time had a linear, first-order relationship with respect to initial IL-12 concentration. There was also a linear, first-order
relationship between initial binding rate and initial IL-12 concentration. In an alternative higher-order model (not shown) where the concentration of available surface antibody decreased as it was occupied by antigen, results did not diverge by more than 1% at 1 hour exposure until target concentration had exceeded 50ng/ml.

![Graph showing theoretical binding to the surface over concentration](image)

Fig. 4.4 Theoretical Binding to the Surface Over Concentration (Rate-Limited Model): Plots of antibody-antigen complex divided by total antibody (Γ/Ab) (Proportion occupied) versus test solution initial antigen concentration (Concentration IL-12) as calculated by the Simulink model (Fig. 4.2), concentrations as shown earlier (Fig. 4.3) showing (a) the results after the first 100 seconds of sensor exposure and (b) the results after the first hour of sensor exposure. Best-fit regressions indicated that, for both exposure times, the proportion of antibodies occupied is modeled to be directly proportional to the initial concentration of target in solution.

### 4.3.2 EIS Experiments

Nyquist plots taken from the AC sweeps of the bare, antibody-immobilized and biomarker bound (IL-12) CHI electrodes were made (Fig. 4.5). Also plotted was the Nyquist of the MHDA immobilization (Fig. 4.6b). Upon MHDA immobilization, there was an increase, or shifting of the Nyquist plot to be observed. As antibody was immobilized, the Nyquist plot shifted toward an intermediate level of impedance in between that displayed by bare electrodes.
and the MHDA-immobilized electrodes. As the antigen molecules bound to the immobilized antibody the system reversed, with an increase in the overall signal becoming evident. Various concentrations of IL-12 were examined and the results plotted (Fig. 4.6c-g). As expected, increasing the target concentration resulted in an increasing signal. Over the concentration range used (0-2500pg/ml) increasing the initial concentration of antigen resulted in increasing impedance.

![Graph showing impedance (Z') and reactance (Z'') against concentration](image)

**Fig. 4.5 Immobilization and Modeling of the CHI Electrode:** Immobilization state and corresponding data for (a) Bare electrode with data (d) conforming to Warburg model, (b) Anti-IL-12 immobilized electrode and (c) IL-12 detecting electrode with respective data sets (e, f) conforming to an equivalent circuit model incorporating constant phase elements.
Fig. 4.6 Nyquist Spectra on One Gold Disk Electrode: Real ($Z'$) versus imaginary ($Z''$) impedance for EIS spectra run on one gold disk electrode. Data was taken from the sensor in various states, (a) the electrode bare and clean, (b) MHDA Monolayer immobilized on the electrode (c) fully immobilized sensor immersed in redox probe with no protein, fully immobilized electrode immersed in test concentrations of IL-12 of (d) 2.5pg/ml, (e) 25pg/ml, (f) 250pg/ml and (g) 2500pg/ml.
Fig. 4.7 Impedance at 5 Hz: The impedance at 5Hz for the concentration gradient displayed above (Fig. 4.6) plotted against (a) concentration of IL-12 and (b) proportion of antibodies occupied by antigen (IL-12) as determined by modeling binding relative to concentration and time used (Fig. 4.4b). Best-fit linear regressions with R2 values above 95% found the impedance(Ω) was equal to 758.67ln(IL-12) + 4213.2 ohms or equal to 696.43ln(proportion bound IL-12) +13906.
Fig. 4.8 Modeled Minimum Detection Time: (a) Modeled 5Hz impedance vs time shown for (c) 2,500pg/ml, (d) 250pg/ml, (e) 25pg/ml, (f) 2.5pg/ml, (g) 0pg/ml, determined by adapting a model parameter-data relationship similar to that in Fig. 4.7b to convert the concentration gradient shown in Fig. 4.6. The (b) lower limit of detection (LLD) plotted against time, LLD determined using the relative standard deviation for the blank experiments determined in Chapter 3, as well as the procedure for determining LLD outlined in 3.3.2.

Minimum detection times were determined by combining the model with data. Model impedance at various time points and concentrations was determined by converting the model parameter of $[\text{AgAb}]/[\text{Ab}]$ via a relationship between impedance and the model parameter (similar to that shown in Fig. 4.7b)
determined from the concentration gradient shown in Fig. 4.6. This was plotted as shown in Fig. 4.8a. It was assumed that the relative standard deviation of the blank measures as determined in 3.3.2 could be employed for modeling purposes with the CHI electrodes. This RSD was employed with the IUPAC method as detailed in 3.3.2 and the modeled 5Hz impedances shown in 4.8a to determine theoretical lower limits of detection (LLD) across time points from 1 second to 1 hour incubation with target IL-12. These results are shown in Fig. 4.8b.

Absolute impedance $|Z|$ at 5Hz was taken from the EIS IL-12 concentration gradient data and used as a metric for evaluation. Plots were made comparing impedance at 5Hz to the concentration gradient and to the corresponding proportion of antibodies occupied by antigen as determined by the model (Fig. 4.2). Least squares regression was used to determine that target antigen concentration and the resulting 5Hz impedance have a positive log-linear relationship (Fig. 4.7a), as do the proportion of antibodies occupied by antigen and the associated 5Hz impedance (Fig. 4.7b).

The maximum impedance response was found to occur at 5Hz (Fig. 4.9). This was determined by finding the greatest slope of impedance change over log[concentration] change where the linear fit of said slope was still significant.
Fig. 4.9 Determination of Optimal Frequency: The greatest slope in impedance vs log concentration plots occurs at 5 Hz (a) while remaining in the range of frequencies where there is a good log-linear fit to said plots (b).
4.3.3 Short Term Zt Experiments

Fig. 4.10 Short-term Impedance-vs-Time: Concentration gradients (0-2500pg/ml) of IL-12 as detected by CHI gold sensors interrogated by the Zt transduction technique at (a) 4.63Hz, (b) 1.18Hz and (c) 4630Hz, approximately 100 seconds incubation time, output metric absolute impedance |Z| (ohms). Impedance obtained from 4.63Hz experiment plotted (d) the proportion of antibodies occupied by antigen (IL-12) as determined by modeling (Fig. 4.2) binding relative to concentration and time exposed. Best-fit linear regression found the impedance was equal to $420.09\ln[\text{IL-12}] + 15956$ ohms. Also included in are overlays of the data and best fit line of the (a) 5Hz (4.54 Hz) EIS data presented in Fig. 4.7a, as well as the (f) 1.18Hz and (g) 4590Hz data.

Concentration gradients of IL-12 were run against CHI gold working electrodes immobilized with anti-IL-12 interrogated with various frequencies using the Zt transduction technique with no incubation time. The absolute impedance |Z| at 90 seconds was used as a metric for evaluation. Plots were made comparing impedance at chosen frequencies to the concentration gradient. At 5Hz (4.63 Hz) (Fig. 4.10a), there was a log-linear relationship present between initial IL-12 concentrations and impedance. This is also the case at the other frequencies (Fig. 4.10b, c), however with higher baselines compared to signal.
Comparison (Fig. 4.10d) was also made to the corresponding proportion of antibodies occupied by antigen as determined by the model (Fig. 4.4a). Least squares regression was used to determine that the proportion of antibodies occupied by antigen and the associated 5Hz (4.63Hz) impedance have a log-linear relationship (Fig. 4.10d). Comparing the Zt data and the fixed-frequency data resulting from EIS interrogation (Fig. 4.7a), the 5Hz (4.54 Hz) data (Fig. 4.10e) is similar, with Zt having higher baseline impedance than EIS. At 1Hz (1.18 Hz), the EIS data (Fig. 4.10f) had both a greater slope (signal) and lower baseline impedance. At 5000 Hz (4590Hz), the log-linear best fit has both a higher baseline and a higher slope.

4.3.4 Long Term Zt Experiments

CHI gold electrodes immobilized with anti-IL-12 were run against various concentrations of IL-12 achieved via injection of small aliquots, while the system was continuously evaluated with the Zt transduction technique at 10Hz. After diffusing to the surface, antigen created changes in impedance significantly greater than background noise or peaks produced by injections of redox probe without IL-12 (Fig. 4.11a). The maximum change in impedance per unit time (velocity) resulting from injections of antigen was used as a metric, as was the total change in impedance resulting from injections of antigen (peak height) and the amount of time passing between injection of antigen and achievement of a maximum impedance before the return of the system to equilibrium (time to peak). A similar metric to velocity, the change proportion of antibody immobilized on the surface which are occupied with antigen, as calculated by the model (Fig. 4.2) (model velocity) was found to have a linear, first order relationship with initial antigen concentration. The average of maximum velocities at various
concentrations as obtained by the long-term Zt experiments was compared to the model velocity corresponding to the test concentrations used, yielding a relationship characterizable as linear and first order. A similar metric to peak impedance, the maximum proportion of immobilized antibodies occupied by antigen after exposure to a concentration of antigen, as determined by the model (maximum binding), was also found to have a linear, first order relationship with antigen concentration. The average peak impedance as determined by long-term Zt experiments at the various concentrations used was compared to maximum binding, also yielding a relationship characterizable as linear and first order. Finally, comparing time to peak directly to antigen concentration yielded no significant linear relationship over the concentrations used.
Fig. 4.10 Long-term Zt Experiments: An example of (a) a CHI gold working electrode immobilized with anti-IL-12 and exposed to various concentrations of IL-12 antigen via injection, interrogated by a fixed frequency (10Hz) at 5mV perturbation, resultant impedance plotted vs time. Labeled on-chart are injections into the system, which in-system are diluted 100-fold, an example of a peak height (impedance increase subsequent to an antigen addition) measurement, and impedance velocity (maximum change in impedance/time subsequent to an antigen addition) measurement. Contrasting the (b) average maximum measured velocity with the modeled (Fig. 4.2) maximum velocities corresponding to tested concentrations of (e) 25pg/ml, (f) 250pg/ml and 2,500pg/ml, yields a loosely linear relationship. Contrasting the (c) average peak heights resulting from various test concentrations with the modeled maximum proportion of immobilized antibodies occupied by antigen corresponding to the test concentrations, a loosely linear relationship is also obtained. In comparison of (d) time from injection of antigen to peak impedance directly to the concentrations of antigen tested, there is a high baseline to signal ratio, and the relationship is best modeled in a nonlinear fashion.

4.4 Discussion

4.4.1 Binding Model

In both chapters 3 and 4, when interrogating the sensor with the EIS technique, it has been found that the initial concentration of antigen in solution and the resultant impedance have a log-linear relationship. In this model, as the
surface concentration of antigen at any given time is proportional to the initial concentration of IL-12 in a linear, 1st order fashion, the relationship between the surface concentration of antigen at impedance is likewise predicted by way of comparison to be log-linear. This is further confirmed by the chapter 4 EIS results.

According to the plotted model, a difference between a blank sample and a solution with target would manifest itself in immediate change in the surface concentration of target antigen (Fig. 4.3), directly proportional to the initial concentration of antigen in solution (Fig. 4.4). Assuming that corresponding induced impedances can be distinguished, the model indicates that Zt experiments with the shorter incubation time periods could be useful for distinguishing between different antigen concentrations.

Log-linear concentration-impedance relationships are well documented in EIS affinity sensors (Du et al. 2008; Hleli 2006; Lasseter et al. 2004; Tsai et al. 2011; Xu et al. 2012). In this iteration, the relationship between initial antigen concentration and sensor impedance has repeatedly been found to be log-linear, as shown in both chapter 3 and 4 data. This indicates that according to this affinity model, the relationship between antigen binding and impedance will also be log-linear: that is, impedance will be a logarithmic function of antigen surface concentration, as seen in Fig. 4.7b and Fig. 4.10d. A direct comparison of model and experimental results is seen in Fig. 4.12.
Fig. 4.12 Comparison of Experimental and Model Results: Data from (a) EIS experiments showing 5Hz impedance versus initial IL-12 concentration at one hour incubation, with best-fit equation (Fig. 4.7a), (b) Model showing proportion of antibodies occupied versus initial IL-12 concentration at 1 hour incubation, with best-fit equation.

One alternate model for explaining surface concentration of antigen is a steady-state diffusion limited model, as defined by Fick’s law in one dimension (Eq. 4.6), where $J$ is the diffusion flux (mol*m$^{-2}$*s$^{-1}$), $D$ is the diffusion coefficient (m$^2$s$^{-1}$), $C$ is the concentration (mol*m$^{-3}$), and $x$ is the relative position (m). This could model the situation where steady-state diffusion occurs within a diffusion boundary layer near the electrode, beyond which convection effects dominate.

$$J = -D \frac{\partial C}{\partial x}$$ (4.6)

By setting boundary conditions of concentration at the surface of the electrode equal to 0, and at distance L from the electrode equal to the initial
concentration $C_0$, Eq. 4.7 is obtained, where $C(x)$ is concentration as a function of distance from the electrode.

$$C(x) = C_0 \left( \frac{x}{L} \right)$$  \hspace{1cm} (4.7)

And the corresponding flux is found to be as per Eq. 4.8.

$$J = -\frac{DC_0}{L}$$  \hspace{1cm} (4.8)

According to this 1-D diffusion model, flux towards the surface will be a linear function of the initial antigen concentration, indicating that at any arbitrary incubation time, surface concentration of antigen will also be a linear function of initial concentration. This is similar to the result obtained by the kinetic model, and thus presenting the same conundrum of surface impedance increasing with the log of the surface concentration of antigen.

In three dimensions, and modeling the electrode surface as a hemisphere, a steady-state diffusion results in the flux as seen in Eq. 4.9, adapted from Crank (Crank 1975), where $Q_t$ is the amount of solute (antigen) reaching the electrode in a given amount of time $t$, $a$ is the radius of the electrode, $b$ is the distance from the electrode (a radius) at which bulk concentration $C_0$ is reached, and $D$ is the diffusion coefficient.

$$Q_t = 4\pi D t \frac{ab}{b-a} C_0$$  \hspace{1cm} (4.9)

Here, the amount of antigen at the surface is also a linear function of the surface concentration. Non-steady-state diffusion effects are deemed unlikely as an explanation for this relationship as well, as the relationship is observed at both
brief intervals (90-100 seconds) (Fig. 4.9a) and longer durations (1 hour) (Fig. 4.7a) of incubation.

Otherwise, although the Nyquist plots do not conform to an equivalent circuit indicating the presence of Nernst impedance, a Nernst impedance is one possible mechanism creating the log-linear relationship between initial solution concentration of antigen and resultant impedance. This would be possible if the reference is off of formal potential and, by depleting one of the redox probe species, resultant current is limited by diffusion through the biomolecule-immobilized surface. By increasing surface concentration (and hence physical blocking), the limited redox species would be decreased linearly, and impedance increased logarithmically. This type of mechanism could be detected by using fluorescently labeled antigen in conjunction with impedance sensing, or by measuring the concentration of antigen in solution after removal from the sensor.

Another possible mechanism explaining the relationship would be saturation of surface antibody limiting the surface concentration, thereby indicating a linear bound-antigen impedance relationship. With an antibody population of sufficiently heterogenous affinity, saturation could create a surface-binding-to-initial concentration proportion resembling a logarithmic relationship over several decades of concentration. One way to determine if this is occurring would be by changing the antibody source, or deliberately hobbling affinity. Again, this could be detected through fluorescent labeling of target antigen, or measurement of test solution concentration after removal from the sensor.

4.4.2 EIS Experiments

The pattern of changes in Nyquist spectra during immobilization and experimentation confirmed patterns observed in chapter 3. Most notably,
impedance was higher when the sensor was immobilized only with 16-MHDA than when the sensor was also immobilized with antibody. This consistently-observed decrease in electron transfer resistance and surface capacitance is most likely explainable as the effect of a charged molecule (antibody, in this case) reducing the surface capacitance effect of a relatively nonpolar molecule (MHDA).

In contrast to state of the art label free EIS biosensors (see 3.1.1), label-free EIS detection on chi electrodes demonstrated generally lower levels of antigen detected, and the potential for a shorter incubation period. The log-linear relationship between antigen concentration and electrode impedance confirms the results of chapter 3, and here it is further explained in light of the model, with a possible empirical relationship between binding to the sensor surface and observed impedance. Overall, these EIS results indicate the cross-platform viability of the techniques used.

The optimal frequency results confirm published results (Bhavsar et al. 2009; Fairchild et al. 2009) and the IL-12 optimal frequency results presented in chapter 3. The optimal frequency results also the indicate cross-platform viability of the optimal frequency statistic. Modeling of the minimum detection time utilizing this data indicates how the lower limit of detection is affected by alteration of antigen-sensor exposure time. This analysis indicates lowering incubation time to very short durations may be feasible without compromising lower LOD goals.
4.4.3 Short Term Zt Experiments

Fixed frequency impedance experiments of 90-seconds duration further confirmed the optimal frequency concept (Fig. 4.9). Below optimal frequency, increases in impedance over target concentration are observable, but there is what could be a marked saturation effect. Above the optimal frequency, impedance does not correlate strongly with concentration, and the baseline impedance makes up a larger and larger proportion of the signal. Using Zt at the optimal frequency, the log-linear relationship between antigen concentration and impedance strengthens, confirming this association. This was further investigated by comparison with the model, indicating a possible empirical relationship between bound antigen and impedance as measured by Zt. The results also demonstrated Zt detection below the levels observed by current fixed-frequency impedance systems (see 4.1). Comparison between Zt, with no incubation time, and EIS performed after 1 hour incubation revealed that similar detection capability was achieved.

4.4.4 Long Term Zt Experiments

Unlike other experiments, long-term measurement of impedance at a fixed frequency showed a linear relationship between concentration and impedance, demonstrated both with the maximum rate of impedance increase and the total impedance increase provoked by the introduction of antigen, and similarly holding a linear relationship with corresponding model metrics. However, perturbing the system with a DC offset and low frequency AC signal continuously for hours could have had unforeseen effects. Using a wider range of concentrations would have been helpful in understanding this further. However,
utilizing this technique, the effects of injection with lower concentrations could not successfully be separated from background noise. Additionally, with manual introduction of antigen using small aliquots as not to perturb the fluid, significantly higher concentrations could not be achieved with commercially available concentrations of IL-12. Automated sample handling would assist in employing higher concentrations with this technique.

The time between introduction of sample and resulting peak impedance did not vary greatly by concentration used. This is in accordance with the model, which predicts that the amount of antigen bound at any given time relative to the initial antigen concentration introduced should be invariant. However, if equilibrium binding is indicated by peak impedance, then the model is inaccurate in that it predicts a greater amount of time until a near-equilibrium condition is reached. Additional factors present could be investigated by utilizing labeled antigen in simultaneous conjunction with this technique.

4.5 Conclusions

This study has presented impedance biosensing experiments using modeling, frequency sweep and fixed-frequency methods. Rapid detection of IL-12 was suggested with the kinetic sensor model, and confirmed with fixed frequency experiments, and confirmed again by modeling of minimum detection time. This informs the goal of utilizing fixed frequency methods for rapid, simultaneously multiplexed biosensor transduction. The frequency sweep experiments on the alternate platform demonstrated the cross-platform robustness of the experimental procedure. The frequency sweep results also confirm an optimal frequency of 5Hz for IL-12, as determined in chapter 3.
Optimal detection frequencies for biomarkers are necessary for simultaneous detection of multiple biomarkers with one sensor immobilized with multiple antibodies. When interrogated with multiple (optimal) frequencies over time, such sensors would respond with greater change in impedance at those optimal frequencies corresponding to the biomarkers with greater concentration in test solution.
CHAPTER 5

DESIGN OF PROTOTYPE SIMULTANEOUS MULTIPLEXING DEVICE

5.1 Introduction

One of the pervasive challenges in drug therapeutic efficacy studies, proteomic or genomic biomarker discovery, personalized medicine, etc., is in multiplexing the assay to allow for the detection of a set of biomarkers that identify a disease signature (Sluss 2008). These kinds of studies require the use of high throughput assays where tens of thousands of targets can be investigated simultaneously and as rapidly as possible (Borrebaeck 2007). Also, single marker studies have led to the conclusion that a multiplexed system looking at more than one marker can improve specificity and sensitivity over a single marker alone in cancers (Havrilesky et al. 2008; Molina et al. 2005; Tchagang et al. 2008). Many limitations must be overcome to enable a multi-marker, rapid, label-free, simple-to-use Point-of-Care (POC) device that works with complex solutions like blood samples. There have been some creative solutions to these issues including multiplexing and labeling of the target (Pregibon et al. 2007), downstream processing (Bensch 2006), etc. However, much of the multiplexing and instrumentation issues have not yet been resolved and the future looks even more demanding. In the early stages of personalized medicine, a push has been made for high throughput screening of patient samples with respect to drug safety (Katsanis et al. 2008) and to therapeutic and diagnostic evaluation (Bonnefoy 2008). Limitations in these methods also typically include: instrumentation, display, sample preparation, size, costs, complexity, and/or a
requirement of labeling (Borrebaeck 2007; Pregibon et al. 2007). Many limitations must be overcome to enable a multi-marker, rapid, label-free, simple-to-use POC device that works with complex solutions like blood samples. These limitations include a lack of simplified operation, multiplexing targets, ambient temperature reagents, and rapid assay, among others (Lewandrowski 2008; Nichols 2008).

Multiplexing sensors by innovative means would reduce instrumentation demands, thereby lowering costs, speeding up the assays, and directly impacting health. This could be accomplished by a multisine impedance immunosensor with ‘tuned’ antibodies coupled with an appropriate potentiostat and software to not only multiplex and encode a perturbation signal but also to deconvolute the output. This would be done so that multiple molecular recognition elements and targets could be interrogated on a single sensor using a rapid and label-free approach.

Building upon impedance immunosensing experiments with printed circuit board (PCB) electrodes presented earlier, Chapter 4 discussed impedance immunosensing experiments on CH instruments (CHI) gold disk electrodes, including electrochemical impedance spectroscopy (EIS), impedance vs time measurement using a fixed frequency (Zt) in the short and long term. Additionally, Chapter 4 discussed equivalent circuit modeling of the electrode surface and kinetic modeling of electrode affinity. Towards the project’s its ultimate goal, that of a rapid, simultaneously multiplexing impedance immunosensing system, earlier experiments established that PCB biosensors were capable of rapid detection of biomarkers in physiological and disease-state concentrations in a label-free manner and with minimal sample preparation. Chapter 4 experiments also brought the project towards this resolution. The
immunosensing EIS experiments on CHI electrodes versus concentration gradient demonstrated cross-platform robustness of the sensor preparation and detection technique, and confirmed optimal frequency, the former useful for the overall goal by demonstrating that alterations in sensor composition and alterations in sensor and solution well geometry were tolerable, while the latter was useful for the overall goal by demonstrating the frequency-dependant nature of the change of impedance due to binding of target antigen. The kinetic model of affinity biosensing for the system was useful for the overall goal by showing that under at least one interpretation it should be possible to distinguish between different concentrations of target in a short time, and over a wide range of concentrations. The short term Zt experiments were useful for the overall goal by demonstrating detection of target antigen was possible within 90 seconds at optimal frequency, in conformation with the model. The long term Z-t experiments were useful for the overall goal by demonstrating that an impedance response above baseline could be provoked by introduction of target antigen to the solution well, also in conformation with the model.

Chapter 5 discusses the design of an impedance immunosensing system that includes new features for additional utility, such as provisions for automated sample handling and testing included in the redesign of the PCB sensor wells and holder. However, this design also utilizes the aforementioned results in specific ways. Having shown the Zt method (introduced in chapter 4 section 4.1) can be utilized at specific frequencies for favorable affinity detection within a short period of time, this impedance sensing technique is elaborated upon to be able to interrogate a sensor for multiple kinds of affinity interactions. This end
requires alterations of both the data handling and the potentiostat itself, and an introduction to various ways that it could be accomplished.

5.1.1 Analog and digital convolution/deconvolution

Fig. 5.1 Schema of Optimal Detection Frequencies

![Graph showing impedance vs frequency with labels for IL-12, IFN-γ, TNF-α, IL-2, and IL-10 at different frequencies (5 Hz, 9.77 Hz, 17.44 Hz, 31.5 Hz, and 117.2 Hz)].

Fig. 5.1 Schema of Optimal Detection Frequencies: IL-12 at 5Hz, IFN-g at 9.77Hz, TNF-a at 17.44Hz, IL-2 at 31.5Hz, and IL-10 at 117.2Hz. It has been demonstrated that these frequencies can be ‘tuned’ farther apart by coupling nanoparticles to the antibodies prior to sensor immobilization (Demirok 2010).

The optimal frequencies, determined in the results presented in chapter 3 and confirmed in the results presented in chapter 4, crudely amount to the stop band (i.e. frequency band of increased impedance) of their respective antigen-antibody interaction at the sensor surface when interrogated by an ac voltage sweep. A representation of these optimal frequencies can be seen in Fig. 5.1. Several antigen-antibody pairings with (or modified to have) distinct optimal frequencies could be immobilized together on the sensor surface to create a sensor with multiple optimal frequencies, each stop-band corresponding to the presence of a particular antigen target in solution. This single electrode could be interrogated for multiple targets with EIS, each frequency targeted for a brief time and at different points in time, with much time and data wasted on irrelevant
frequencies. Alternatively, it could also be interrogated sequentially at the optimal frequencies, an AC voltage for each passed through the sensor one after the other, however foregoing the opportunity for continuous impedance data over time. Some additional signal processing presents the opportunity of a further alternative. The multiple-antibody-immobilized working electrode could be interrogated with simultaneously at all optimal frequencies, the AC voltages multiplexed into one convoluted signal, as illustrated in Fig. 5.2. The output AC signal would then be deconvoluted, as illustrated in Fig. 5.3, before comparison with the individual input signals for evaluation, as illustrated in Fig. 5.4. The impedance increases at frequencies of concern, corresponding with different antigen-antibody pairings, could then be monitored continuously over the span of time of the experiment.

Fig. 5.2 Convolution and Input into the Sensor: a (a) low frequency, high amplitude waveform (a) is combined with a (b) higher frequency, lower amplitude waveform to form the input voltage (c) Vin for multisine Zt measurement to be input to the sensor immobilized with different antibody types.
Fig. 5.3 Output Signal Deconvolution: The (a) sensor immobilized with multiple antibody types outputs, through the potentiostat, a voltage signal (b) $V_{out}$ which is then filtered into its (c) low frequency and (d) high frequency components.

Fig. 5.4 Comparing $V_{out}$ with $V_{in}$: Comparison of output waveforms at (a) high and (c) low frequencies with their respective (b) high and (d) low frequency inputs (see Fig. 4.1 for method) resulting in data revealing (e) impedance of the sensor over time for multisine $Z_{t}$ at (f) high and (g) low frequencies.

To accomplish this signal processing, various approaches may be taken. Convoluting AC voltages into a single multiplexed, multisine input signal can be
accomplished post-generation by analog methods, or pre-generation by digital control. Deconvolution of the output signal likewise could be performed prior to analog-to-digital conversion using analog circuit components, or after conversion by digital processing. For example, in order to simulate an EIS frequency sweep in a shorter amount of time, multisine EIS systems generally interrogate a system with complex AC signals which are then interpreted via digital signal processing (Blajiev et al. 2006; Creason and Smith 1972), though these devices do not posses multisine Zt capability.

Crudely, analog signal processing could be anything that affects the signal- for example a stiff wall acts as a low pass filter for sound waves, leaving low frequency sound waves with higher gain than those at high frequency. However, for electrical signals, analog signal processing uses passive and active circuit elements, like as resistors, capacitors, inductors, operational amplifiers and non-linear devices. Output is obtained in real time, and is continuous rather than sampled. They can be easy to assemble and inexpensive, but, especially with higher order filters, difficult to maintain, calibrate and modify (Skolnick and Levine 1997). Furthermore, an ideal first order filter only has fourfold power loss every octave of frequency off the target frequency.

A digital approach has some distinct advantages over analog approaches. With analog signal processing, the system has to be redesigned every time the specifications are changed. Design components may not be available and the processor must use components with parameters within some tolerance. Components may suffer from parameter variations due to room temperature, humidity, supply voltages, and many other aspects, such as aging,
component failure. In contrast, digital signal processing can be used to implement different versions of a system by changing the software on the processor, and will vary less due to environmental circumstances, thus exhibiting flexibility and repeatability. However, digital signal processing consists of numerical computations and there is no guarantee that the processing can be done in real time, i.e. it can lag. In addition, if the input and the output signals are analog, then an analog-to-digital converter (ADC) and a digital-to-analog converter (DAC), which can be expensive, are additionally needed to implement analog processing by digital means (Yarlagadda 2010).

5.1.2 Potentiostats

The potentiostat has two tasks: To measure the potential difference between working electrode and reference electrode without polarizing the reference electrode, and to compare the potential difference to a preset voltage and force a current through the counter electrode towards the working electrode in order to counteract the difference between preset voltage and existing working electrode potential.

This is done using an operational amplifier (OPA). When the working electrode is connected to the non-inverting input (+), the reference electrode to the inverting input (-), and the counter electrode to the output, the difference between working electrode and the reference electrode will be amplified and inverted by the OPA. By feeding a matching current to the counter electrode, current passes through the electrolyte to the working electrode. This polarizes the working electrode so that the difference between the reference electrode input and the working electrode input is zero. This can keep the potential of the working electrode on the potential of the reference electrode. A voltage source
could be inserted in series between the reference electrode input and the reference electrode to shift the potential of the working electrode to a different value referring to reference electrode. Measuring the voltage across a resistor in the counter electrode wiring, proportional to the current flowing, could be used to measure the current through the counter electrode. Alternatively, the same could be done in the working electrode wiring (Doelling 2000).

![Fig. 5.5 Schematic of a Potentiostat](image)

Fig. 5.5 Schematic of a Potentiostat: Device settings are input by the user, controlling the processor to output a digital signal which is converted into analog signals. The analog signals have their high-frequency range filtered out before forming the voltage offset between the counter and working electrodes. Current passing between the counter and working electrodes is converted to voltage, which then has high-frequency components filtered out before being reconverted to digital and passed to the processor. Data output is returned to the user.

5.2 Device

The sensor and holder designs build off of existing PCB array designs. The individual multiplexed, multisine sensor for POC and laboratory use, as seen in Fig. 5.6a, is dimensioned to fit a plate with a well as seen in Fig. 5.6b-c. With an elastic polymer on the bottom layer, the well plate seals to the top of the PCB array, closing inflow and outflow channels milled into the bottom of the well plate. Inflow and outflow ports at the edges of the well plate connect with respective ports on the individual sensor holder, and thereby to peristaltic or equivalent...
pumps for the purpose of carrying sample to and from the wells. The holder is similar to the array holder pictured in Fig. 5.9, but scaled for a single sensor.

But the single-sensor is not the only useful configuration for this device. The PCB array configuration, for the purpose of testing larger numbers of biomarkers, for the most part for experimental uses, may be seen in Fig. 5.6d and 5.7. This array is dimensioned to fit a plate with an array of wells, as seen in Fig. 5.6e-f and Fig. 5.8. Similarly to the single sensor, the array well plate seals to the array, also completing inflow and outflow channels for each of the individual wells, which can connect with fluid channels in the electrode holder seen in Fig. 5.9.

Manufacturing of this device could be accomplished by means available at Arizona State University. To manufacture the single and array multiplex sensors, first the layouts, for example as seen in Fig. 5.8, would be printed onto a transparency and carefully aligned to a pre-sensitized copper-clad board, which
would then be exposed to UV light. After development and removal of exposed photoresist and copper, nickel and gold would be electroplated onto the electrode and lead portions of the device, but not onto the contact terminals. Subsequently, Ag/AgCl would be plated onto the reference electrode. Finally, the electrodes would be immobilized with biomolecules, first with a thiol monolayer, then linking the desired antibodies through EDC/NHS chemistry. These manufacturing methods are detailed in chapter 3. To manufacture the well or well array as seen in Figs. 5.6 and 5.8, two thin sheets of plexiglass or equivalent plastic would first be obtained, of thickness 0.025" and 0.05". These would be milled with the well and fluid channel features, then the two layers adhered with a permanent weld. At that point, a thin elastic polymer would be adhered to or created on the bottom of the well layer.
Fig. 5.7 Dimensioned Array for use in Multisine Zt: Etched on copper clad board, (bottom) contact leads for attachment to an edge connector are connected through leads of 0.01" width to (top) 5 sets of 3 electrodes, which are plated in nickel and then gold. Each set has a (left) counter electrode (center) working electrode and (right) reference electrode, the reference electrode further coated with Ag/AgCl.

Fig. 5.8 Dimensioned Well Plate Array with Flow Channels: Wells of 100ul volume, centered over each 3-electrode set, are connected to individual flow channels of 0.02"x0.025" cross section.
Adaption of the apparatus for data processing can be handled several ways. Generation of the convoluted signal can be handled by a digital waveform generator. By placing band-pass filters in series with the output line, as seen in Fig. 5.10, the $V_{out}$ signal can be split into its component parts prior to comparison with the input $V_{in}$ component by digital signal processing methods. Alternately, the $V_{out}$ signal could be split after analog-to-digital conversion using digital signal processing methods. Both approaches necessitate the construction of a potentiostat device and the creation of a data analysis program. As a third option, some custom-built potentiostats for performing multisine EIS could theoretically also perform multisine $Z_t$ experiments with minimal modification (Pettit et al. 2006; Polonschii et al. 2007). These could be acquired by purchase. The single electrode or electrode array, with holders, may also be used with a commercial potentiostat such as a CHI-660c for interrogation by other electrochemical transduction methods.
Fig. 5.10 Modified Potentiostat for Multisine-2 Waveforms: The potentiostat as described in Fig. 5.5, excepting that the output signal $V_{out}$ is split before band filtering to obtain AC voltages at particular frequencies. To take advantage of this feature, the processor would output a multisine signal through the potentiostat to the electrodes. This particular device would be capable of handling a sensor with two different optimal frequencies.

For use after preparation of all components, the sensor (or array), the well plate (or well array plate), edge connector and holder would be assembled as seen in Fig. 5.9. In this configuration, the contact terminals of the sensor(s) form electrical connection with contacts in the edge connector, linking the sensor surfaces and the solder tails of the edge connector. Also, seating of the well plate on the sensor(s) would form wells connected to channels in the plate, which in turn would be connected to inflow and outflow fluid leads in the holder.

The inflow fluid leads would then be connected to a peristaltic pump for liquid inflow without direct contact without the pump machinery. At their termini, the inflow fluid leads could be attached to any fluid required, or to y-connector to rapidly and efficiently select between different fluid sources. The outflow fluid leads would connect to a disposal container. The edge connector solder leads would be connected to the appropriate respective terminals of a potentiostat, whether for multisine $Z_t$ or for other techniques. The potentiostat, in turn, would
be connected to a personal computer with controlling software and data analysis software. For POC use, the potentiostat and holder and edge connector could be made as one piece, simplifying the use of the device when specialized for one task.

5.3 Conclusions

Creating a device with increased automation of sample handling and testing has the advantages of reducing variability due to exogenous factors and increasing the rapidity of the assay, and the side-benefit of easing sensor or sensor array fabrication. With easier fabrication, the device will have the further advantage of low cost. Furthermore, by employing simultaneous multiplexing through multisine perturbation, multiple types of antibody-antigen affinity binding events may be observed in real time, reducing the number of sensors, and consequently the amount of test sample used, in the detection and quantification of multiple biomarkers. This also reduces the amount of time consumed and/or peripheral equipment such as potentiostats and computer terminals required, easing convenience and making practical POC use. Besides POC, this system would have application in biomarker research as well as for basic biosensor development, being capable of observing impedance over time associated with recognition events.
CHAPTER 6

SUMMARY AND FUTURE WORK

6.1 Introduction

Costs associated with diagnosis have been growing at a faster pace than overall US healthcare costs (Feldman 2009; NHSG 2010). This trend results from the increasing employment of complex, powerful laboratory testing in medical diagnostics versus primary physician screening (Feldman 2009). This highlights a possible opportunity for systems which could bridge the gap between low and high end diagnostic methods, being both sufficiently easy to use and of sufficient diagnostic utility to act as an intermediate diagnostic step between point of care primary physician screening and intensive testing requiring specialist attention. Biosensors for the detection of bodily fluid biomarkers are one technology which may be used to exploit this opportunity (Rapp et al. 2010), to both speed diagnosis and to reduce the effort involved. Medical conditions for which quantification of multiple bodily fluid cytokine biomarkers would be diagnostic or prognostic include Multiple Sclerosis (Giovannoni et al. 1997; Hollifield et al. 2003; Nicoletti et al. 1996; Woodroffe et al. 1991), cancers (Linkov et al. 2008; Molina et al. 2005), Rhumatoid arthritis (Hueber et al. 2009; Rioja et al. 2004), Irritable Bowel Syndrome (Scully et al. 2010), Inflammatory Bowel Disease (Alex et al. 2009; Alex 2008), Lupus (Chun et al. 2007; Manukyan et al. 2010), trauma (Maier et al. 2007), sepsis (Marchant et al. 1995; Oberholzer et al. 2001; Sherwin et al. 2008), and organ rejection (Hassan et al. 2006; Platz et al. 1996).
A biosensor is “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals,” (Nic 2010). If limited to purpose-built examples, the first example, in 1962, would be a glucose detection biosensor utilizing Glucose-Oxidase enzyme-based detection (Clark 1962). Biosensors have proliferated widely since then, and count among their variety systems which employ various kinds of recognition elements (Nicu and Leîchlé 2008; Ronkainen et al. 2010), immobilization methods (Mayes 2002; Ulman 1996), and transduction methods (Aoyagi and Kudo 2005; Campbell and Mutharasan 2008; Janshoff et al. 1997; Leonard et al. 2004; Lowe 2008; Mathew and Alocilja 2005; Nicu and Leîchlé 2008; Song et al. 2006). One particular category of transduction method, electrical, includes impedimetric techniques (Daniels and Pourmand 2007), which may be used in conjunction with affinity recognition elements and self-assembled monolayer immobilization for rapid, sensitive detection of target analytes (La Belle et al. 2007). In impedimetric techniques a system is interrogated with an AC voltage in combination with a DC offset, and the comparison of the output with the input obtaining impedance. Electrochemical Impedance Spectroscopy (EIS) is an impedimetric transduction technique wherein this comparison is repeated at different frequencies, and has been utilized in biosensing for detection of target antigen down to concentrations of 1pg/ml (La Belle et al. 2007; Tsai et al. 2011). The impedimetric impedance-time (Zt) technique instead interrogates a system with one frequency for a given amount of time. Using impedimetric techniques, specific frequencies have been found where the change in target concentration
affects the impedance of an affinity biosensor most strongly (Lasseter et al. 2004; Yang et al. 2004; Yang 2008).

Diagnostic and research biomarker sensing systems have value added when multiplexing, for identifying a disease signature clinically or for research purposes in a laboratory setting, investigating more markers simultaneously is useful (Borrebaeck 2007; Sluss 2008). For multiplexing in an impedimetric affinity biosensor, a system could be interrogated with multiple frequencies at the same time, or simultaneous multiplexing.

With the long term goal of the design of a simultaneously multiplexing impedance affinity biosensor, in the current work three studies were performed in regard to impedance immunosensors. These studies were done to determine the parameters required to develop a simultaneous multiplexing impedance affinity sensor, in short, to find the optimal frequencies for a selection of biomarkers, to demonstrate the effectiveness of EIS and Zt techniques for affinity sensing, to determine the incubation time required for effective sensing and, finally, to design a system to be used as a simultaneously multiplexing impedance affinity biosensor. The conclusions from the three studies presented in this dissertation are summarized below and future avenues of research discussed.

6.2 Summary

Specific aim 1 was to test the hypothesis that various cytokines induce experimentally determinable frequencies of response when captured by affinity molecules immobilized to the surface of a biosensor. In chapter 3, this hypothesis was tested by developing printed circuit board (PCB) 3-electrode immunosensors and interrogating them with EIS in the presence of concentration gradients of
antigen corresponding to the immobilized antibody. This study showed that five different cytokine biomarkers had distinct ‘optimal frequencies’ in relation to impedimentric sensing. The data also showed that the PCB EIS immunosensor had a demonstrable lower detection limit of 4.3pg/ml when studying the cytokine Interleukin-12 (IL-12), and that the system was also tolerant (ie. still sensitive to target when exposed) to background interference vis a vis high concentrations of fetal bovine serum. In addition, equivalent circuit models of the data revealed that

Specific aim 2 was to test the hypothesis that rapid detection of cytokine analytes via impedance immunosensing could be done without greatly sacrificing sensitivity. The second study demonstrated that distinct detection of different concentrations of IL-12 could be accomplished within 90 seconds of exposure. This was shown via kinetic modeling and confirmatory Zt studies. The data showed that the immobilization method and electrochemical techniques were robust across platforms. This was demonstrated via EIS immunosensing vs concentration gradients of antigen, and subsequent equivalent circuit modeling. The data also showed that interrogating the immunosensor with a fixed, optimum frequency could be used for sensing target antigen. This was demonstrated via Zt immunosensing at various frequencies vs concentration gradients of antigen.

Specific aim 3 was the design of a multiplexing impedance immunosensor for the simultaneous quantification of biomarkers of multiple types, its method of manufacture and its method of use. The second study presented a device that reduced variability due to exogenous factors and increased rapidity of assay with eased sensor fabrication. This was shown via its increased automation of sample handling and testing. The second study also presented a way to observe multiple types of antibody-antigen affinity binding events in real time, reducing the number
of sensors and target sample used in the detection and quantification of multiple biomarkers. This was achieved by presenting methods for simultaneous multiplexing through multisine perturbation of a sensor. This would also improve the suitability of the sensor for POC multiplex detection of disease biomarkers.

6.3 Conclusions

In this dissertation, the overall goal was experiments toward the creation of a multiplexing immunosensor for the quantification of cytokine biomarkers. Through utilization of rapid, simple and relatively inexpensive impedance biosensing, the creation of a ‘good enough, easy enough’ device was a promising target. The first study focused on determination of perturbation frequencies at which impedance changes the most over the concentration gradient, i.e. optimal frequencies. This study demonstrated, for apparently the first time, the optimal frequencies of the cytokine biomarkers Interleukin-10 (IL-10), Interferon gamma (IFN-y), Tumor Necrosis Factor alpha (TNF-a), and Interleukin-2 (IL-2). The first study also demonstrated label-free EIS biosensing of biomarkers, in this case IL-12, to a lower LOD than in almost all current literature, in addition to showing a log-linear relationship between impedance and concentration over five decades of concentration. The first study also demonstrated detection of target to low levels even in the presence of complex solution. Utilizing the optimal frequencies determined and sensing techniques practiced in the first study, the second study was focused on determining the efficacy of rapid, label-free fixed-frequency impedance biosensing. The second study demonstrated that rapid quantification of target antigen with fixed-frequency impedance biosensing was theoretically possible and achievable in
reality. In the course of the second study, it was also demonstrated that the results of the EIS technique were consistent across transition to a different sensing platform, across transition to different exposure times, and even across transition from frequency sweep impedance sensing to fixed frequency impedance sensing. Utilizing the knowledge of distinct optimal frequencies and tolerance of impedance immunosensing to interferants determined in the first study, and the demonstration of the efficacy of short-term fixed frequency impedance sensing for detection, the third study was focused on designing a multiplexing impedance immunosensor for the quantification of biomarkers in bodily fluid. The third study demonstrated designs for simultaneous, multisine detection of multiple biomarkers on one sensor utilizing one fixed, complexed perturbation signal. and the third study also demonstrated an array scaleup thereof for detection of even greater numbers of different antigens, and for replication or other experimental purposes. Successful development of this system would realize the creation of an invaluable tool for biomedical study and medical diagnosis.

6.4 Future Experiments

Studies utilizing a device for simultaneous multiplexing invite the investigation of a disease for which multiple biomarkers may be measured for improved diagnosis or prognosis. For example, in one study patients with malignant thyroid tumors have been shown to be best distinguishable from normal controls with a model utilizing a concentrations determined for a group of 4 among 19 cytokines surveyed in serum. These four were Interleukin-8 (IL-8), IL-12, epithelial growth factor (EGF), and hepatocyte growth factor (HGF).
Median quartiles of serum concentration as detected by fluorescent immunoassay were, for normal subjects compared to patients with malignant thyroid tumors; for HGF, 148.9-248pg/ml versus 67-178pg/ml, for IL-8, 3.49-4.19pg/ml versus 2.71-4.51pg/ml, for IL-12p40, 35-66.4pg/ml versus 34.5-84.1pg/ml, and for EGF, 28.04-56.7pg/ml versus 0.02-5.01pg/ml. These markers were only found to be efficacious for diagnosis in combination (Linkov et al. 2008).

With detection limit goals indicated by these findings, initial experiments will utilize the well and sensor array in conjunction with a standard CHI 660C potentiostat. The biomarkers chosen for initial study will be those with the lowest detection limit requirements in physiological or disease states, in this case IL-8 and EGF. Like in the previous studies in this dissertation, EIS impedance will initially be used to analyze the systems under study. A concentration gradient of purified target samples will be introduced onto sensors immobilized with their respective antibodies to determine concentration effects, responsivity and sensitivity (Table 6.1a). Controls will include blanks to test for sensor stability. Equivalent circuit parameters will be calculated and quantified against the concentration of the target (or control). Finally, optimal frequencies will be calculated, and the impedances at the respective optimal frequencies of the biomarkers over their concentration gradients will be plotted. This study could then be repeated for the other two markers (Table 6.1b), and then repeated again for all markers with serum components added to test for nonspecific binding (Table 6.1c). If optimal frequencies of some antigen-antibody pairs are found to be too close to one another, the frequencies could be 'tuned' by
addition of conductive nanoparticles to the antibody prior to immobilization on the sensor surface as demonstrated by Ugur Demirok (Demirok 2010). Repetition of concentration gradients would be performed (Table 6.1d) to determine and refine the ‘tuned’ frequencies as necessary.

With optimal frequencies for biomarker quantification established, experiments could be performed utilizing the Zt technique with the constant frequency set at said optimal frequencies. First, concentration gradients of the first two targets (IL-8 and EGF) will be performed versus their respective antibodies (Table 6.1e), with a control of the targets being switched at single concentrations, the AC frequency used being held constant on each sensor. This experiment will be performed to confirm that a relationship between a concentration gradient and resultant impedance will be obtained using Zt, and also for the purpose of comparison with impedances obtained previously via EIS. Next, at single concentrations to avoid undue experimentation, the biomarkers will be tested against their respective antibody at their respective optimal frequencies (Table 6.1f), against their nonrespective antibodies at their respective optimal frequencies (Table 6.1g), and against their nonrespective antibodies at their nonrespective optimal frequencies (Table 6.1h). This will be done to assure that undue cross-reactivity is not occurring at the test frequencies, i.e. at the nonoptimal frequencies or when appropriate antigen-antibody combinations are not present, no detection should occur.

Next will come simulated multisine experiments. Since no mass-produced commercial instrument can perform true multisine Zt, it will be done by continuing to use the single frequency Zt transduction technique as done in the previous experiments. At a single concentration, a mixture of both antigens will be tested
versus sensors with each antibody separately immobilized at the appropriate frequencies for the antibodies (Table 6.1i), with control experiments at switched frequencies (Table 6.1j). Mirroring this, single sensors immobilized with both antibody types will be tested versus single antigen types at a single concentration and at the appropriate frequencies for the antigen (Table 6.1k), with control experiments at the switched frequencies (Table 6.1l). Then in addition, single sensors with both antibody types immobilized will be tested versus a mixture of both antigens at a single concentration, at either frequency (Table 6.1m). These experiments will show that the antigens can be detected independently in the same solution with the same sensor simply by changing the frequency with which the sensor is interrogated.

Then a concentration gradient of both antigens, with test concentrations chosen with the aid of experiment design software (Design-Expert, Stat-Ease) to avoid unnecessary experimentation effort, will be run against sensors immobilized with both antibodies on one surface, with optimal frequencies utilized at any given point also chosen with the aid of experiment design software (Table 6.1n). This will be done to demonstrate that a relationship between concentration gradients for two antigens in mixed solution and the resultant impedances can be obtained by altering the frequency with which the sensor is interrogated, crudely simulating a multisine sensor run versus a concentration gradient.

In yet another simulated multisine experiment, one concentration of mixed-target solutions with serum components added will again be tested versus both antibodies immobilized on the sensor. Allowing for sufficient antigen-antibody association time, the respective optimal frequencies will be used to probe the sensor in a time-separated manner (Table 6.1o). Again, interactions
from complex solution, nonspecific binding and other factors could possibly interfere with the signal, and will specifically be looked for in data analysis. This experiment will be repeated with the order of the frequencies switched. Combined, this data could be gathered and used to simulate a combined, true multisine signal analysis problem utilizing patient samples.

Finally, utilizing custom software and hardware, a true multisine, simultaneously multiplexing experiment will be performed using both antibodies and antigens (Table 6.1p), and the results compared with a concurrent simulated multisine experiment (Table 6.1q) for the purpose of ensuring the proper operation and efficacy of the multisine potentiostat and software. After this point, concentration gradients of biomarkers could be run, the other two thyroid cancer biomarkers could be brought back into the study, and further testing using serum components to help simulate patient samples could be done. Then the system will be ready for testing versus patient samples directly. For example, 'blind' solutions (patient or mock-up) could be tested against the sensors to determine antigen concentrations based on only the impedances obtained at optimal frequencies versus blank solution and the blind solution, and concentrations verified via conventional immunofluorescent techniques. The system will also be ready for development with other biomarker systems for other diseases and disorders.

There are many hurdles to pass in the development of a multisine, simultaneously multiplexing impedance immunosensor for detecting cytokine biomarkers, including extensive experimentation and development of signal processing methods. However, with the information provided in the studies presented in this work, it is brought that much closer to realization.
REFERENCES


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APPENDIX A

INSTRUMENT METHODS
Odyssey Imaging System (Li-Cor), instructions for use

Turn on the Odyssey Imaging System by pressing the power button on the front panel: ![power button]. Allow to warm up for at least one minute.

Start the Odyssey software in a linked computer. Select the default settings and select ‘OK’. If creating a new project folder, choose ‘New’ from the ‘File’ drop-down menu. Otherwise, open an existing project folder by selecting ‘Open’ from the ‘File’ drop-down menu. In either case, a new window will open where the save location may be changed and the title for the project entered. In this window, select ‘Scan’. A new window will pop up that indicates where the project is stored. Select ‘OK’ in this window.

At this point, a window will pop up requesting a login and password. Once entered, press ‘OK’.

The ‘Scanner Console’ window will open. Here, select the scan area, scan resolution, focus offset (the focus depth), and the intensity.

Place the item to be scanned in the lower left hand corner of the scan surface, before closing the lid of the Odyssey Imaging System.

Select ‘Start Scan’, and the scan will start, displaying in real time in the ‘Scanner Console’ window. When it is finished, select the ‘Save’ button to save the scan, or ‘Close’ if not saving the scan. If saving, a new window will pop up prompting for a scan title. Press ‘OK’ once this information is entered, closing the window and reverting the program to its main screen.

Select the appropriate scan in the ‘Scan’ pane of the main program screen. This will reveal an ‘original analysis’ icon. Double click this to display the
700nm and 800nm images from the scan. The scan components may be viewed superimposed or adjacently, and can also have their brightness adjusted.

Images may be exported directly by using the ‘print screen’ keyboard button, or by selecting ‘Export Image’ and then ‘Export Image View’ from the ‘File’ drop-down menu. This will open a new window titled ‘Export View’. There, the image quality and file format may be selected. Select ‘Save’. The window will close and the new image saved in the scan folder with its parent.
CHI 660c Electrochemical Workstation with CHI 684 Multiplexer,
instructions for basic experiments (Cyclic Voltammetry, AC Impedance, and Impedance – Time)

First, the electrochemical system to be examined should be prepared to a point where it can be readied for testing quickly. Connect the electrode leads from the CHI 684 multiplexer to the electrochemical system. The electrode leads should be pre-labeled. Match the working lead with the intended working electrode, the reference lead with the intended reference electrode, and the counter lead with the intended counter electrode. Note which channel(s) of the multiplexer is/are being used. Next, the instruments should be activated, before starting the CHI 660c software.

Press the ‘Technique’ button: ionic. A new window will open for technique selection, with a list of available techniques. Among the more used are ‘Cyclic Voltammetry’, ‘A.C. Impedance’, and ‘Impedance – Time’. After selecting the preferred technique, hit ‘OK’. The technique selection window will close. If a new technique was selected, a parameters window will automatically open. If not; press the ‘Parameters’ button: ionic. A new window will pop up with settings pertaining to the technique which was selected. If selecting Cyclic Voltammetry, relevant options include the initial voltage (Init E (V)), the maximum voltage (High E (V)), the minimum voltage (Low E (V)), the starting scan direction (Initial Scan Polarity), the Scan Rate (Scan Rate (V/s)), the number of 1-way sweeps (Sweep Segments) and the maximum sensitivity (Sensitivity (A/V). If selecting A.C. Impedance, relevant parameters include the DC offset (Init E (V)), the AC frequency range (High Frequency and Low Frequency), and the AC amplitude
(Amplitude (V)). If selecting Impedance –Time, relevant parameters include the DC offset (Init E (V)), the AC amplitude (Amplitude (V)), the fixed AC frequency (Frequency (Hz)), the sample interval (Sample Interval (sec)), and the running time (Run time (sec)). After selecting parameters, press the ‘OK’ button, and the window will close.

On the ‘Control’ drop-down menu, select ‘Multiplexer’. A new window labeled ‘Multiplexer’ will open where multiplexer channels can be selected. Place (a) checkmark(s) next to the channel(s) to be used. They will be run in numerical order. Press the ‘Browse’ button. In the ‘Save As’ window that opens, select a folder where the data file(s) will be saved and create a base file name. Press the save button and the ‘Save As’ window will close. On the data files, the channel number will be appended to the end of the base filename which was created. Finish readying the electrochemical system for testing and press the ‘Run Multiplexer’ button, which will close the ‘Multiplexer’ window and start the experiment.

A data file with a .bin extension will be created with the data from each experiment as each experiment comes to an end. Opening any such file will display the data from the experiment in the lower window. To overlay more data sets in this graph, in the ‘Graphics’ drop-down menu select ‘Overlay Plots.’ This graph may be copied using the ‘print screen’ keyboard button, or by selecting the ‘Graphics’ drop-down menu and then selecting ‘Copy to Clipboard.’ To convert the data files to text for further analysis, in the ‘File’ drop-down menu select ‘Convert to Text.’
New experiments may be run with the same parameters simply by re-entering the ‘Multiplexer’ window, changing the filename and channels appropriately, and pressing ‘Run Multiplexer.’
ZSimpWin (EChem Software) 3.21, instructions for data processing.

Start by converting the raw CHI .bin data files to be processed into text files. Note the folder location of these text files.

Open the ZSimpWin program. Once ready, hit the ‘Open’ button:  

Next, navigate to the appropriate folder and open a text data file to be analyzed. By default, a Nyquist graph of the data will appear in the lower section of the program window.

By selecting among the chart type buttons, various types of charts portraying the data may be chosen to appear in the lower section of the program window, including, respectively, Nyquist (the default), Bode plots, Real and Imaginary impedance vs Frequency, Real vs Imaginary admittance, Capacitance, and Model Error. After modeling, these plots will include modeled data points with the raw data points. These plots may be copied to the clipboard by selecting the Output drop-down menu, then selecting the ‘copy plot’ option, or simply by hitting the print screen button.

By selecting the ‘Select model and run mode’ button:  , a pop-up window will appear prompting the selection of an equivalent circuit model. By default, within the window, the ‘Initial values of the parameters’ should be selected as ‘Auto Setup’, and the ‘Run mode’ should be ‘Iterate’. Select the model to be tried, and hit the ‘OK’ button. The program will then create a best fit to the data using the selected model, which will appear in the graph area with the
original data. When fitting is done, the prompt, ‘Want to save results?’ will appear. Answer the prompt by selecting no.

Next, select the ‘Edit parameters and run’ button: 🎨. A new window will open.

Inside, the numbers in the ‘end’ column correspond to the parameters from the best fit equivalent circuit model last run. Under the ‘Rel. std. error in percents’ column is the relative standard error for each term. Also available is the chi-squared term, and the % that the modeled data at each frequency is off from the actual data Z values. This data should be copied manually, as the automatic results presentation available within Zsimpwin is not in a format useful for analysis software such as Matlab or Excel, and is less time-efficient to use.

After copying desired data, hit ‘Cancel’. Then open the next text file to be analyzed, or change the analysis model as needed.
APPENDIX B

RAW DATA
Fig. B.1 Blank Data: (a, b, c) Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the blank solution sweeps which form the basis for the blank relative standard deviation used to calculate the lower LOD (limit of detection) in section 3.3.2.
Fig. B.2 IL-2 Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-2 EIS-sweep which is shown analyzed in Fig. 3.10, antigen concentrations including (a) 0pg/ml (blank solution), (b) 1pg/ml, (c) 5pg/ml, (d) 10pg/ml, (e) 50pg/ml (f) 100pg/ml, (g) 500pg/ml, (h) 1000pg/ml, (i) 5000pg/ml, and (j) 10000pg/ml
Fig. B.3 IL-10 Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-10 EIS-sweep which is shown analyzed in Fig. 3.10, antigen concentrations including (a) 0pg/ml (blank solution), (b) 1pg/ml, (c) 5pg/ml, (d) 10pg/ml, (e) 50pg/ml, (f) 100pg/ml, (g) 500pg/ml, (h) 1000pg/ml, (i) 5000pg/ml, and (j) 10000pg/ml
Fig. B.4 IL-12 Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-12 EIS-sweep which is shown analyzed in Fig. 3.10, antigen concentrations including (a) 0pg/ml (blank solution), (b) 1pg/ml, (c) 5pg/ml, (d) 10pg/ml, (e) 50pg/ml, (f) 100pg/ml, (g) 500pg/ml, (h) 1000pg/ml, (i) 5000pg/ml, and (j) 10000pg/ml
Fig. B.5 IFN-γ Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IFN-γ EIS-sweep which is shown analyzed in Fig. 3.10, antigen concentrations including (a) 0pg/ml (blank solution), (b) 1pg/ml, (c) 5pg/ml, (d) 10pg/ml, (e) 50pg/ml (f) 100pg/ml, (g) 500pg/ml, (h) 1000pg/ml, (i) 5000pg/ml, and (j) 10000pg/ml.
Fig. B.6 TNF-α Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the TNF-α EIS-sweep which is shown analyzed in Fig. 3.10, antigen concentrations including (a) 0pg/ml (blank solution), (b) 1pg/ml, (c) 5pg/ml, (d) 10pg/ml, (e) 50pg/ml (f) 100pg/ml, (g) 500pg/ml, (h) 1000pg/ml, (i) 5000pg/ml, and (j) 10000pg/ml
Fig. B.7 0% FBS Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-12 in 0% FBS EIS-sweep which is shown analyzed in Fig. 3.11, concentrations of IL-12 from 0 to 10000pg/ml
Fig. B.8 1% FBS Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-12 in 1% FBS EIS-sweep which is shown analyzed in Fig. 3.11, concentrations of IL-12 from 0 to 10000pg/ml
Fig. B.9 100% FBS Raw Data: Nyquist plots (real versus imaginary impedance over a frequency sweep) of raw data for the IL-12 in 100% FBS EIS-sweep which is shown analyzed in Fig. 3.11, concentrations of IL-12 from 0 to 1000 pg/ml
Fig. B.10 Impedance-Time Raw Data: Z-t (Impedance versus time) plots of raw data from IL-12 tested versus various frequencies, some of which is shown analyzed in Fig. 4.10, concentrations of IL-12 from 0 to 2500pg/ml.
APPENDIX C

ANTIBODY FREQUENCY EFFECT
Fig. C.1 Antibody Frequency Effect: Nyquist plots are shown for (a) bare electrodes and (b) antibody immobilized electrodes including electrodes used for (c) IL-2, (d) IL-10, (e) IL-12, (f) IFN-γ and (g) TNF-α. The greatest shift in the frequency spectrum due to antibody immobilization was to be found in the range of 5-10Hz, with no significant difference between the different antibodies used.