A PHOTONIC CRYSTAL BIOSENSOR APPLICATION FOR ASSESSMENT OF IRON DEFICIENCY

BY

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DISSERTATION

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ABSTRACT

Iron deficiency anemia afflicts 1 in 3 individuals, mostly women and children worldwide. A novel application using iron-oxide nanoparticles (IONs) and a photonic crystal (PC) optical biosensor as an immunodiagnostic platform for detection of serum ferritin and soluble transferrin receptor (sTfR), biomarkers for iron deficiency, is presented. Two assay formats were explored in this research: 1) standard sandwich assay (SA) and 2) inverse sandwich assay (IA). For both of these formats, total analytical error was quantified. Commercialized enzyme-linked immunosorbent assays (ELISAs) served as the reference methods to quantify the analytical error of the PC biosensor measuring both biomarkers in the IA and SA format. In the SA human liver ferritin (450 kDa), clinical serum controls, and three commercially available ferritin ELISA tests were used to evaluate the PC biosensor assay in terms of inter- and intra-assay variability, spike-recovery (%), and limit of detection (LOD). In the IA format, functionalized iron-oxide nanoparticles (fAb-IONs) were used as magnetic immuno-probes to bind sTfR (85 kDa) and minimize non-specific signals, while enhancing detection on the PC biosensor. Studies were conducted examining the binding ratios of fAb-IONs and sTfR, inherent imprecision, LOD, inter- and intra-assay replication, and nanoparticle aggregation. In both assay formats, a linear dose-response curve was elicited using the fAb-IONs. The final study was a comparison of studies experiment comparing the analytical performance of the IA on the PC biosensor to FDA-certified ELISAs measuring ferritin and sTfR. Samples from chronic kidney disease patients on hemodialysis were tested on both platforms. Total analytical error was quantified for the PC biosensor measuring ferritin and sTfR. The calculated total analytical error exceeded the quality specification for both biomarkers. Future optimization studies should aim to decrease the errors to be within the quality specifications.
The studies contained within this dissertation would not have been possible without the contributions of many individuals. Dr. Juan Andrade has been the primary source of discussion concerning all bottlenecks that were encountered with the photonic crystal biosensor. I want to thank Dr. Brian Cunningham for fielding technical questions regarding his technology and serving on my committee. Thank you to Dr. Nicki Engeseth and Dr. Kenneth Wilund for serving as members on my committee. I am grateful to Sherine George and Weili Chen from Dr. Cunningham’s lab for assisting me and explaining to me more technical aspects of the PC biosensor and the physics of nanoparticles, respectively. Also, I am grateful to the members of Dr. Andrade’s lab; they have been a source of social and mental support. Finally, I want to thank my wife, Alicia, and other family members for encouraging me and giving me support throughout my doctoral research.
# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................... vi
LIST OF FIGURES ......................................................................................... vii

CHAPTER 1. OVERVIEW ............................................................................... 1
  1.1. Introduction .......................................................................................... 1
  1.2. Long-term goal, Research Objective and Hypothesis ............................ 4
  1.3. Rationale and Significance .................................................................. 5
  1.4. Thesis Structure .................................................................................. 6
  1.5. References .......................................................................................... 8

CHAPTER 2. LITERATURE REVIEW ............................................................ 10
  2.1. Biomarkers of iron deficiency Anemia .................................................. 10
  2.2. Magnetic Nanoparticles ....................................................................... 13
  2.3. Diagnostic Platforms for Iron Deficiency Anemia ................................. 17
  2.4. Photonic Crystal Biosensor .................................................................. 24
  2.5. Validation of Diagnostic Methods ....................................................... 26
  2.6. References .......................................................................................... 34

CHAPTER 3. A PHOTONIC CRYSTAL BIOSENSOR ASSAY FOR FERRITIN
  UTILIZING IRON-OXIDE NANOPARTICLES ........................................... 38
  3.1. Introduction ........................................................................................ 38
  3.2. Materials and Methods ...................................................................... 40
  3.3. Results ............................................................................................... 46
  3.4. Discussion .......................................................................................... 52
  3.5. Limitations ........................................................................................ 58
  3.6. Conclusion ........................................................................................ 58
  3.7. References ......................................................................................... 60

CHAPTER 4. ENHANCED SANDWICH IMMUNOASSAY USING ANTIBODY-
  FUNCTIONALIZED MAGNETIC IRON-OXIDE NANOPARTICLES FOR
  EXTRACTION AND DETECTION OF SOLUBLE TRANSFERRIN
  RECEPTOR ON A PHOTONIC CRYSTAL BIOSENSOR ............................ 62
  4.1. Introduction ....................................................................................... 62
  4.2. Materials and Methods ...................................................................... 65
LIST OF TABLES

Table 1. Advantages and limitations of biomarkers of iron deficiency anemia ..................11
Table 2. Serum ferritin and sTfR cut-off values .................................................................13
Table 3. Experiments to quantify analytical error .................................................................27
Table 4. Intra-assay and Inter-assay variability of different immunoassay platforms ........48
Table 5. Detection of ferritin in control sera on PC biosensor and commercial ELISAs ....49
Table 6. Recovery of ferritin from control sera in several immunoassay platforms ........51
Table 7. Precision of various methods measuring sTfR in Liquichek™ sera ....................80
Table 8. Means, Standard Deviations, Coefficient of Variations and Range of Measurements .................................................................................................................................98
Table 9. Total Analytical Error (TE_{calc}) for IDA biomarkers using the Inverse Sandwich Assay on the PC biosensor .................................................................105
# LIST OF FIGURES

**Figure 1.** World map of anemia distribution and prevalence in pre-school children .................................................................2  
**Figure 2.** Photonic crystal biosensors incorporated into a microplate ........................................4  
**Figure 3.** Steps for selection and separation of IDA biomarkers in various matrixes .................................................................................................5  
**Figure 4.** Magnetic separation using TurboBeads® ......................................................................................15  
**Figure 5.** Size distribution of 30-nm iron-oxide nanoparticles .................................................................16  
**Figure 6.** HemoCue® handheld device ..................................................................................................................18  
**Figure 7.** Sandwich ELISA format ..........................................................................................................................21  
**Figure 8.** Sensorgram and schematic of gold surface plasmon resonance ..............................................................23  
**Figure 9.** Photonic crystal biosensor dielectric surface materials ........................................................................25  
**Figure 10.** Difference Plot for S-creatinine .........................................................................................................30  
**Figure 11.** Comparison Plot for soluble transferrin receptor ............................................................................32  
**Figure 12.** A schematic of the three immunoassay types on the PC biosensor and dynamic reading graph .............................................................................................................47  
**Figure 13.** Comparison of responses from three immunoassay types on the PC biosensor .................................................................47  
**Figure 14.** Standard responses and measurement agreement/bias of four immunoassay platforms .................................................................................................................................50  
**Figure 15.** Schematic of standard sandwich assay and inverse sandwich assay .................................................................63  
**Figure 16.** Recovery of soluble transferrin receptor using inverse sandwich assay .........................................................................72  
**Figure 17.** Optimization of fAb-IONs concentration to maximize dose-response measurement of sTfR in the inverse sandwich assay .........................................................................................73  
**Figure 18.** Transmission electron microscope image of fAb-IONs pre- and post-magnetic separation ........................................................................................................................................76  
**Figure 19.** Inverse sandwich assay response on PC biosensor platform .....................................................................................77  
**Figure 20.** Difference plots comparing concentrations of sTfR in the IA and in the SA on the PC biosensor to those from commercial ELISAs .........................................................................................79  
**Figure 21.** Difference plots comparing measured concentrations of sTfR and Ferritin .................................................................................................................................100  
**Figure 22.** Calibration curves on the PC biosensor using the IA and on the ELISAs measuring sTfR and ferritin ..................................................................................................................101  
**Figure 23.** Comparison plots showing the analytical range and correlation coefficient of sTfR and ferritin ...........................................................................................................................103
CHAPTER 1

OVERVIEW

1.1. Introduction

Iron deficiency anemia is a world health problem. Iron deficiency anemia (IDA) is the most prevalent micronutrient deficiency in the world. According to the World Health Organization (WHO), approximately 1 billion people are anemic worldwide due to iron deficiency, especially women and children in resource-poor areas of developing countries (Stoltzfus, 2011; WHO, 2004). Factors that may lead to iron deficiency include increased iron requirements, limited external supply, and increased blood loss (Gisbert et al., 2009). Iron has a fundamental role in all body cells; thus, IDA results in deleterious effects impacting individuals, societies and economies. IDA during childhood leads to reduced cognition and academic performance (Pollitt et al., 1989; Webb and Oski, 1973), with effects leading into adulthood. Among adults, IDA compromises immune system function (Joynson et al., 1972), work capacity and pregnancy outcomes (Black et al., 2008). The Harvard Step Test study with Guatemalan labor workers showed work productivity was positively associated with hemoglobin levels (Horton and Ross, 2003). Since these landmark studies in the 1970’s and 1980’s, more recent studies have verified these findings (Sachdev et al., 2005; Horton and Ross, 2003). Although incorporation of iron into diets through fortification or supplementation strategies has led to significant improvements in most developed economies, the problem is still prevalent in low-income countries, where the identification of people suffering from or at risk for IDA remains a critical hurdle. Figure 1 show a world map from the WHO with regions where anemia is most
prevalent in preschool children. It is estimated by the WHO that half of the anemia cases are due to ID.

![Figure 1. World map of anemia distribution and prevalence in pre-school children. Anemia distribution and prevalence is similar in pregnant women throughout the world (WHO, 2008)](image)

**Identification of micronutrient deficiency is a roadblock.** Identification of populations suffering from IDA is one of the most significant limitations hindering nutrition, health improvements and human development. In developing countries healthcare facilities are mostly centralized with inadequate infrastructure that inhibits millions of rural-dwelling people from receiving reliable laboratory diagnosis and treatment for IDA. Presently, a point-of-care, easy-to-use, low-cost diagnostic tool to quickly and accurately determine micronutrient status of at-risk
populations living in rural settings does not exist. Enzyme linked immunosorbent assays (ELISAs) are currently the most common detection platform for IDA biomarkers (Erhardt et al., 2004). Nevertheless, ELISAs require trained personnel, ideal laboratory infrastructure with electricity and waste disposal, expensive readout equipment and a high cost of analysis (Voller et al., 1978), thereby, limiting their use in resource-poor areas. Thus, low-cost, robust technologies that can identify individuals with IDA are needed.

**Photonic crystal (PC) based biosensors: an opportunity.** PCs are periodic dielectric surface structures that reflect a narrow band of wavelengths when illuminated by a broadband light source (Cunningham, 2002). Adsorption of biomolecules (e.g. proteins) near the biosensor surface (~200 nm) will increase the dielectric permittivity (Arakawa and Kita, 1999) and cause a wavelength shift (PWV); thus, making a simple mechanism for biomarker detection. PC biosensor detection systems have various diagnostic and screening applications in DNA microarrays (Mathias et al., 2010), cancer cell analysis (Chan et al., 2007) virus detection (Pineda et al., 2009), protein detection (Peterson et al., 2014) and pharmaceutical drug screening (Heeres and Hergenrother, 2011). PC biosensors are inexpensively manufactured from plastic materials using roll-to-roll fabrication processes, and easily incorporated into liquid handling formats such as standard 96- and 384-well microplates for single-use disposable applications. Figure 2 shows the PC biosensor in microplate formatting.
Recently, a portable smartphone application was designed for measuring analytes on the PC biosensor (Gallegos et al., 2013). Although more work is needed to measure analytes in liquid samples using the smartphone detection application, this technological advancement highlights the potential of PC biosensor as a point-of-care detection platform for IDA. In conjunction with this work, the proposed studies aim at optimizing and validating the PC biosensor for clinical diagnosis.

1.2. Long-term Goal, Research Objective and Hypotheses

My long-term goal is to bridge bioengineering and nutrition to target micronutrient deficiencies by designing and implementing innovative diagnostic technologies. The objective of this research, which is the next step toward accomplishing this long-term goal, is to develop an immunoassay based on a photonic crystal (PC) biosensor, which includes IDA biomarker detection optimization, separation from complex matrixes, selection, and platform error analysis. My central hypothesis is that by employing a PC biosensor as a diagnostic tool, it will be possible to differentiate between low and high levels of specified iron-deficiency biomarkers with similar analytical error, selectivity and sensitivity as a commercial ELISA.
Specific Aim 1. Establish parameters for detection, selection, separation and of IDA biomarkers using a PC sandwich assay with immunofunctionalized iron-oxide nanoparticles (fAb-IONs). The working hypothesis is that fAb-IONs can be used to amply signal-to-noise ratio and the magnetic properties of fAb-IONs can be used to select and separate a biomarker of IDA (i.e. ferritin and sTfR) from various matrices (i.e., buffer, BSA, and serum) and then to increase signal to noise response in the immunoassay based PC biosensor (Figure 3).

![Diagram of steps for selection and separation of IDA biomarkers in various matrices.]

Specific Aim 2. Evaluate the analytical error of PC biosensor against FDA-certified in-vitro diagnostic ELISA kits at clinically relevant biomarker concentrations in patient population. The working hypothesis, based on previous studies, is the PC biosensor performs similar to FDA-certified ELISAs in terms of random error and systematic error when quantifying World Health Organization standards and Liquichek™ Control Sera Standards.

1.3. Rationale and Significance

The research herein has basic and applied significance in nutrition, material sciences, immuno-diagnostics and clinical chemistry. Regarding nutrition, this research assists achieving three of the nutrition-related Millennium Development Goals. By developing a diagnostic platform capable of determining iron status, “hidden hunger,” infant health, and maternal health
can be targeted for more improved assistance-program implementation. Currently, most nutrition diagnostic devices require a laboratory for analysis; as a result of this work, new potential methodological approaches to assess micronutrient status in field settings is possible. In the field of material sciences, these studies are significant because they address some properties of magnetic nanoparticles (i.e. size, magnetism) that improve performance of the PC biosensor in terms of assay duration, sensitivity, and selectivity. In the field of immuno-diagnostics, these studies contribute to the understanding of how a given concentration of antibody-conjugated nanoparticles extract varying concentrations of biomarkers in simple (i.e. buffer) and complex (i.e. serum) matrixes. In addition, it is essential in clinical chemistry to quantify analytical error of the PC biosensor before it can clinically diagnose patients. Moreover, these experiments are significant because no studies have quantified the analytical error of PC biosensor or attempted to magnetically separate nutritional biomarkers from sera as a means to decrease matrix interferences on the PC biosensor. The research project adds to the nutritional diagnostic literature by validating a PC biosensor method for clinical diagnosis of IDA in serum. Furthermore, the innovation of this research promotes interest in magnetic nanoparticles as potential probes for separating biomarkers from complex matrices when developing point-of-care diagnostics. The rationale of this research is that once populations suffering from IDA are identified, health- and nutrition-related problems can be addressed.

1.4. Thesis Structure

This thesis is organized into five chapters. Chapter 1 is composed of the introduction and literature review. The literature review provides a background of each area of research relevant to the dissertation research included herein. This information gives the reader sufficient background knowledge to understand the theoretical basis of the conducted research. Following
Chapter 1, Chapter 2 describes the first study testing the PC biosensor as a diagnostic device for measuring ferritin, a biomarker of IDA. This proof-of-concept study demonstrates that the PC biosensor is capable of accurate and precise detection of a protein biomarker at physiological concentrations using the standard sandwich assay (SA) (Peterson et al., 2014). These data from Chapter 1 partially satisfy aim 1 and 2 by identifying detection parameters on the PC biosensor and evaluating its accuracy and precision using the SA, respectively. Chapter 3 builds on the proof-of-concept work of Chapter 2 and adds the evaluation of a magnetic separation protocol using the inverse sandwich assay (IA) format, detection of soluble transferrin receptor (sTfR), an additional biomarker of IDA. The research in Chapter 3 contributes further to completing aim 1 and 2 by defining parameters related to selection and separation of sTfR and quantifying the analytical error associated with the IA, respectively. In addition, Chapter 3 covers information related to binding ratios of the sTfR:fAb-IONs. Chapter 4 describes the final study carried out in this thesis. The final study analyzed ~40 samples of patient sera, who had been diagnosed with clinical kidney disease, to evaluate the IA measuring sTfR and ferritin. Analytical error of the IA on the PC biosensor was compared with FDA-certified ELISA kits for each biomarker. Chapter 5 summarizes the findings from each of the previous chapters and concludes with the potential future directions of this research.
1.5. References


CHAPTER 2

LITERATURE REVIEW

2.1. Biomarkers of iron deficiency Anemia

Hulka and Wilcosky (1988) defined biomarkers (i.e. biological markers) as cellular, biochemical or molecular alterations, which are measurable in biological media such as human tissues, cells or fluids (Hulk and Wilcosky, 1988). In the case of iron status assessment, there are numerous biomarkers primarily measured in blood and tissue. Although this dissertation work focuses on two protein biomarkers measured in human serum, a more comprehensive review of the common biomarkers used to assess IDA is presented.

The gold standard for determining IDA in a clinical setting is by staining bone marrow aspirates for hemosiderin (Burns et al., 1990). Nevertheless, more recent studies have suggested using a combination of biomarkers to ensure IDA diagnosis (Jonker et al., 2014; Barron, 2001). These iron stores in the bone marrow act as the iron that is incorporated into hemoglobin during erythropoiesis. An absence of stainable iron in the bone marrow aspirate indicates IDA with high specificity and sensitivity (Cook, 2009). Despite bone marrow iron being the preferred method for evaluating IDA, the procedure is invasive and requires a skilled pathologist for accurate evaluation. Therefore, bone marrow sampling is unfit for field measurement, and as a result, other biomarkers must be measured. Table 1 adapted from Cook, 2009 describes the advantages and limitations of each biomarker in the diagnosis process of IDA.

Daily hemoglobin iron turnover in red blood cells ranges from 30-40 mg (Cook, 2009). Due to this high rate of iron turnover, hemoglobin is one of the most common biomarker used to detect anemia. However, hemoglobin levels provide low sensitivity and low specificity for IDA.
Hemoglobin has low sensitivity as a result of iron stores being nearly deplete before hemoglobin levels fall below normal limits. This means that iron deficiency can occur without anemia (WHO, 2007). Moreover, because there are several types of anemia, such as hemolytic anemia, anemia due to chronic kidney disease, macrocytic anemia, hemoglobin has low specificity for IDA. For these reasons of low specificity and low sensitivity of hemoglobin, additional biomarkers need to be measured to determine whether iron deficiency is the cause of anemia.

Table 1. Advantages and Limitations of biomarkers of iron deficiency anemia (Cook, 2009).

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hemoglobin</td>
<td>Inexpensive</td>
<td>Low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Universally available</td>
<td>Low specificity</td>
</tr>
<tr>
<td>2. Ferritin</td>
<td>Quantitative (iron stores)</td>
<td>Affected by inflammation</td>
</tr>
<tr>
<td></td>
<td>Well standardized</td>
<td>Affected by liver disease</td>
</tr>
<tr>
<td>3. Soluble transferrin receptor</td>
<td>Quantitative (tissue deficiency)</td>
<td>Affected by rHuEPO therapy</td>
</tr>
<tr>
<td></td>
<td>Unaffected by inflammation</td>
<td></td>
</tr>
<tr>
<td>4. Bone-marrow iron</td>
<td>Well established</td>
<td>Invasive</td>
</tr>
<tr>
<td></td>
<td>High specificity</td>
<td>Prone to error</td>
</tr>
</tbody>
</table>

In addition to hemoglobin, two other biomarkers that are well established and improve the specificity of IDA diagnosis are transferrin saturation and zinc protoporphyrin (ZPP). Transferrin is the protein transporter that delivers iron in circulation throughout the body. Under normal iron status, transferrin saturation with iron is near 33%. This is calculated as the ratio of serum iron to the total iron-binding capacity of transferrin (Cook, 2009). However, when iron deficiency is present, the transferrin saturation is < 15% (WHO, 2007). ZPP, on the other hand, is a reliable indicator of abnormal heme synthesis (Labbe et al., 1999). In the last step of
hemoglobin’s biosynthetic pathway, trace amounts of zinc are incorporated into the porphyrin ring instead of iron at a normal zinc:iron ratio of 1:30,000. When this ratio is > 80 µmol/mol, iron status is inadequate to maintain sufficient heme synthesis; thereby, indicating iron-deficient erythropoiesis (WHO, 2007).

Two protein biomarkers commonly used to diagnose IDA, without bone marrow aspirates, are ferritin and soluble transferrin receptor (sTfR) (Jonker et al., 2014). Ferritin is a 480-kDa symmetrical protein that has the capacity to store 4,500 iron atoms (Finazzi and Arosio, 2014). The intracellular form of ferritin is composed of a tissue-specific distribution of H and L subunits that make up 24 subunits in total. It has a cage-light structure that holds iron and prevents it from perpetuating oxidation through the Fenton reaction. Ferritin can be used as a clinical indicator of absolute iron deficiency because it is the protein that store corporal iron. sTfR, on the other hand, is an indicator of functional iron deficiency because of its role in the transport of transferrin-bound iron into tissues throughout the body. The primary method by which iron is brought into cells is through receptor-mediated endocytosis (Feelders et al., 1999). Diferric transferrin binds to the two identical transferrin receptors (190 kDa) on the cell surface before being brought into the cell. sTfR is the truncated form of the intact transferrin receptor, which is the 85 kDa extracellular domain that resulted from proteolytic cleavage between the Arg-100 and Leu-101 (Shih et al., 1990). Table 2 presents the cut-off for each biomarker to classify subjects with or without iron deficiency. For ferritin, the storage protein for corporal iron as established by the WHO, the cut-off value in serum is <30 µg/L or <15 µg/L depending on the presence of inflammation or not, respectively (WHO, 2007). The normal range for ferritin is 25-300 ng/ml. Therefore patients below these cut-off levels, and at a given inflammatory state, would be considered iron deficient. In addition to ferritin, sTfR, which is expressed along cell
membranes to import circulating iron, has various cut-off values depending on the population. For children 1-5 years old in developing countries, the cut-off value has been cited to be >8.3 µg/ml (Phiri et al., 2009), whereas the cut-off values for children and non-pregnant women in the USA are >6.0 µg/ml and >5.3 µg/ml, respectively (Mei et al., 2012). The normal range for sTfR is 1.8-4.6 µg/ml. Thus, children and adults above these cutoffs are considered iron deficient. The author’s focus on sTfR stems from the fact that it does not fluctuate as much as ferritin during acute or chronic inflammation.

### Table 2. Serum ferritin and sTfR cut-off values.

<table>
<thead>
<tr>
<th></th>
<th>Ferritin w/ Inflammation</th>
<th>Ferritin w/o Inflammation</th>
<th>sTfR Developing Countries</th>
<th>sTfR Healthy US Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>30 µg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 µg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
<td>5.3 µg/ml&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Children 1-5 y old</td>
<td>30 µg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15 µg/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 µg/ml&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0 µg/ml&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> WHO, 2007  
<sup>b</sup> Phiri et al., 2009  
<sup>c</sup> Mei et al., 2012

### 2.2. Magnetic Nanoparticles

Interferences and non-specific binding to biosensor surfaces impede widespread clinical diagnostic applications for biosensing technologies. Complex matrixes (i.e. serum and whole blood) with high amounts of interfering proteins are the root cause for non-specific binding and poor signal-to-noise responses (Kyprianou et al., 2013). As a result of these signals, patients are at risk of false-negative or false-positive results (Marks et al. 2002). These misdiagnoses, in terms of IDA can have harmful side effects or even death on patients, especially when providing iron supplementation to false-positively diagnosed children, who are also infected with malaria parasites (Sazawal et al., 2006). Although expensive, clinical diagnostic devices can analyze
serum samples in laboratory settings, no clinical diagnostic tool exists for accurate field measurement. As a solution to this problem, a simple, field-friendly approach to purify analytes of interest from complex matrixes such as serum is possible with the use of magnetic nanoparticles.

Magnetic nanoparticles provide a facile method for analyte extraction from complex matrixes due to the high magnetic moments of metal nanoparticles made of iron and/or cobalt. As a result, magnetic nanoparticles have the potential to decrease interferences; a problem associated with immunoassays (Marks et al. 2008; Kyprianou et al., 2013). Grass et al. coated cobalt nanoparticles with carbon to protect the metallic core from oxidation, maintain magnetism and provide a stable substrate for functionalization (Grass et al., 2007). They described successful functionalization of the carbon layer with chemical linkers (e.g. carboxylates) that form covalent bonds with antibodies along the nanoparticle surface. Many studies employing carbon-coated magnetic nanoparticles based on the synthetic procedure of Grass et al., have demonstrated these magnetic capabilities for interleukin-6 extraction in whole blood (Herrmann et al., 2010) digoxin detoxification in blood *ex-vivo* (Herrmann et al., 2011) (Figure 4), and antibody-antigen complex purification in serum (Kumar et al., 2013). However, although the cobalt nanoparticles synthesized by Grass et al. had a superior magnetic moment, the carbon coating does not allow for monodispersion as it has been achieved in commercial iron-oxide nanoparticle (IONs).
Iron oxide is a metal oxide that has shown promise in biomedical applications. Iron-oxide nanoparticles (IONS) can be synthesized using various methods such as co-precipitation, thermal decomposition, metal reduction, microemulsion or nanoemulsion synthesis, among others (He et al., 2014). However, the synthetic method that allows for tunable size and monodispersion of IONs is a process using thermal decomposition (Yu et al., 2004). In this synthesis process, Yu and colleagues made carboxylate salts through a neutralization reaction of FeCl$_3$ and carboxylic acids in ethanol. They found that at a given set of temperatures and times, they were able to tune the size of the IONs to a narrow size distribution (~5-10%) (Yu et al., 2004). The stability of these IONs was further improved to resist hydrolysis and enzymatic degradation by coating the IONs with a monolayer of oleic acid, an amphiphilic polymer coating and a polyethylene glycol (PEG), which is soluble in water (Gao et al., 2004). As a result, these IONs have good colloidal stability and can be used in biomedical *in-vitro* diagnostics. Xu et al. published work employing IONs functionalized with antibodies to target surface receptors of cancer cells in whole blood.

Figure 4. Magnetic separation using TurboBeads® composed of a cobalt core and carbon coating functionalized with immuno-functionalized probes (Herrmann et al., 2011)
Then, they were able to separate cancer cells based on the presence of the target antigen of cancer cells (Xu et al., 2011). Figure 5 illustrates the size distribution of 30-nm IONs and fAb-IONs synthesized by Ocean NanoTech. Xu et al. also measured the zeta potential at -51 mV and -36 mV for IONs and fAb-IONs, which indicates colloidal stability (Xu et al., 2011).

More pertinent to our studies, Wang et al. developed an assay on a surface resonance plasmon (SPR) optical biosensor in which they functionalized anti-\textit{E. coli} 0157:H7 antibodies to IONs. They then showed how they were able to amplify the signal-to-noise ratio detecting \textit{E. coli} with the fAb-IONs (Wang et al., 2012).

These studies corroborate that magnetic nanoparticles conjugated with antibodies will function to separate biomarkers of interest and amplify signal-to-noise ratio on an analogous optical biosensor to that of the PC biosensor. Applying magnetic nanoparticles to amplify signal and separate biomarkers from sera as shown in this thesis work is the first application of such...
technology for nutrition-related \textit{in-vitro} diagnostics. Successful application of magnetic nanoparticles drives the PC biosensor closer towards a viable platform for measuring serum samples in field studies.

2.3. \textbf{Diagnostic Platforms for Iron Deficiency Anemia}

The following review of diagnostic platforms for IDA is by no means exhaustive. Instead, this section aims to provide a review of the most important diagnostic platforms used in the laboratory for research and clinical applications in the context of global health.

\textit{Hemoglobin Diagnostic Platform}

Hemoglobin has been the longest known biomarker associated with iron status. Despite the lack of specificity with hemoglobin due to other potential pathologies that affect erythropoiesis, it remains an important biomarker to determine if anemia is present. Since the late 1800’s and into the early 1900’s scientists and clinicians like Drabkin and Sahli developed techniques to quantify hemoglobin in red blood cells. Drabkin’s method was published in 1935 and uses capillary or venous blood to measure hemoglobin. The reagents lyse the red blood cells and oxidizes the hemoglobin to methemoglobin with potassium ferricyanide. Then, methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which can be measured with a spectrophotometer at 540 nm (Drabkin and Austin, 1935). Although this method was developed in the early 1900’s, validity testing (Van Lerberghe et al., 1983; Han et al., 2009) and modified versions (Yang et al., 2013) continue to show promise using the Drabkin’s method.
Dating back to the 1966, Vanzetti developed an azidemethemoglobin method to quantify hemoglobin in red blood cells (Vanzetti, 1966). As a result of this method, the commercial HemoCue® has since developed into the most dependable point-of-care diagnostic tool for measuring hemoglobin and diagnosing anemia (Figure 6). Numerous validation studies have verified its accuracy and precision (von Schenck et al., 1986; Neufeld et al., 2002; Bhaskaram et al., 2003), and it is now used as a reference method for validating new experimental methods (Kim et al., 2013). In this method, the blood sample is drawn into the reaction cuvette, where a modified azide methemoglobin reaction occurs (Vanzetti, 1966). The erythrocyte membrane is lysed by sodium deoxycholate, which releases the hemoglobin. Then, sodium nitrite converts the ferrous iron (i.e. from its Fe$^{2+}$ oxidation state) found in hemoglobin to ferric iron (i.e. Fe$^{3+}$ oxidation state) to form methemoglobin, which combines with azide to form azide methemoglobin. The sample is finally read with a double-wavelength photometer at 550 and 880 to correct for turbidity (von Schenck et al., 1986).
The primary disadvantage of the HemoCue® is the relative invasiveness of the procedure, which requires capillary blood. To address this, the market for non-invasive methods is growing. Timm et al., 2009 reported the development of an LED hemoglobin sensor that uses multi-wavelength light absorption that is connected to an index finger (Timm et al., 2009). Another non-invasive method published in 2013 by Doshi and Panditrao uses a similar optical sensor where light is transmitted through the skin on a finger and the hemoglobin concentration can be determined based on the ratio of red to infra-red signal (Doshi and Panditrao, 2013). The most dependable non-invasive finger sensor is the spHb®. A review article written by Lindner and Exadaktylos in 2013 evaluated the spHb® based on previous studies (Lindner and Exadaktylos, 2013). Although the authors acknowledged the limitations of the spHb® compared to validated invasive methods, the spHb® is capable of monitoring hemoglobin in real time, which is advantageous during transfusions. Nevertheless, these noninvasive methods still require electricity and do not have the dependability as invasive hemoglobin measurements.

Ferritin and sTfR Diagnostic Platforms

Methods to quantify levels of ferritin date back to the mid-19th century. In 1956 rudimentary methods allowed for limits of detection near 1000 µg/mL (Reissmann and Dietrich, 1956). As a result of the limited specimens that could be quantified at these levels, new method development was sought after. After the discovery of radioisotopes in the early 19th century, their applications in thyroid physiology in the 1950’s, applications were being discovered each decade leveraging the ability to measure radioactivity with a gamma counter. In the 1970’s, multiple radioimmunoassays (RIAs) based methods were published, in which detection of ferritin down to single ng/mL concentrations was reported (Addison et al., 1972; Marcus and Zinberg, 1975). Essentially, the RIA is a competition-type assay where the unknown concentration of the
unlabeled antigen of interest is determined by comparing the inhibitory effect on binding of radioactively-labeled antigen to a specific antibody (Ewing and Simmons, 1974). The only requirement is that the unlabeled antigen and labeled antigen used in the RIA react in the same manner with the selected antibody. Then, the unknown samples are measured in a gamma counter to determine the concentration compared to the known standards. In contrast to ferritin, sTfR was not detected using RIA until the late 1980’s when it was recognized as being clinically relevant to patients with hematological malignancies (Kohgo et al., 1986). It was not until later when sTfR was used to assess iron status in Zairian children with malaria (Kuvibidila et al., 1995) and pregnant women in Bangladesh (Hyder et al., 2004). Although the RIA method is cost effective and highly sensitive, using radioactive labels requires special handling certifications and health risks. In addition, well-established laboratory infrastructures are essential when running RIAs. Therefore, new diagnostics approaches have moved away from RIA.

The in-vitro diagnostic platform that has replaced RIAs to measure ferritin over the past 20 years is the enzyme-linked immunosorbent assays (ELISAS). Similar to RIAs, ELISAs use the selectivity of the antibody to bind and quantify unknown concentrations of antigen. However, instead of the radioactivity being measured to quantify the antigen, an enzymatic reaction produces a chromophore or fluorophore (colorimetric or fluorometric assay), which is quantified with a spectrophotometer or fluorimeter (Voller et al., 1978). Moreover, ELISAs require a primary antibody and secondary antibody conjugated to an enzyme (e.g. horseradish peroxidase) (Figure 7).

Recent ferritin ELISAs can be purchased from biotechnology companies or developed in house (Erhardt, 2004). Sensitivity and selectivity of ferritin ELISAs are sufficient to determine ferritin within physiological ranges in sera diluted from 1:2-1:10. Numerous papers measuring
ferritin with ELISAs in individuals with iron deficiency (Erhardt, 2004; Hallberg et al., 1993; Flowers et al., 1986) have been published. Measurement of sTfR has also been evaluated and validated using ELISAs since the 1990’s to assess for iron status (Kuiper-Kramer et al., 1996; Suominen et al., 1997; Erhardt, 2004). Nevertheless, non-specific binding at high protein concentrations has been cited in commercial kits to increase the optical density in ELISAs.

![Image of ELISA format](http://www.abnova.com/copyright.asp)

**Figure 7.** Sandwich ELISA format. Determination of antigen concentration is done by measuring optical density with a spectrophotometer at given wavelength or set of wavelengths (http://www.abnova.com/copyright.asp).

As optical technology improved, immunoturbidimetry and nephelometry assays were developed for quantifying ferritin and sTfR. Immunoturbidimetry is a method that quantifies a given protein biomarker by measuring the amount of transmitted light by particles in suspension. The concentration of the given biomarker is determined by the amount bound to antibody-functionalized latex particles. Measurements using immunoturbidimetry are made with a spectrophotometer. The first publications validating ferritin and sTfR quantification using immunoturbidimetry were published in 1990’s and continued into 2000’s (Cotton et al., 2000; Hikawa et al., 1996; Dupuy et al., 2009; Borque et al., 1999). Similar to immunoturbidimetry, nephelometry immobilizes antibodies on the service of latex microparticles and leverages the
amount of scattered light to quantify a sample when it is placed in a cuvette. The primary difference is that the cuvette is placed in a nephelometer, instead of a spectrophotometer, at a particular angle to measure forward scattered light. Because more light is scattered than transmitted when passing through a turbid suspension, nephelometry is more sensitive than immunoturbidimetry. Both ferritin and sTfR have been quantified using nephelometry as well (Cotton et al., 2000; Borque et al., 1992). Such methods have minimized the need for radioactive labeling and allowed for automated measuring of ferritin and sTfR in the clinical setting.

Since the late 1980’s optical biosensors have offered another platform for detection of biomolecules, including ferritin. The most commonly used and commercially available optical biosensor is surface plasmon resonance (SPR). In 1983, the first publication indicating SPR as a potential immunosensor was demonstrated in Sweden (Liedberg et al., 1983). These authors showed that IgG antibodies could be detected down to levels of 0.02 µg/mL on a silver surface using SPR. The way by which this SPR biosensor detects biomolecules on the surface is through an evanescent field that permeates along the silver or gold surface. A surface plasmon occurs at the interface of a metal (e.g. silver and gold) and a dielectric material (e.g. oxides). The surface plasmon can be excited by light penetrating the materials at a resonance angle where photon energy is transferred to the charge energy wave (Liedberg et al., 1995). As a result, an evanescent field permeates above the metal surface. This is the principle by which all SPR can detect biomolecules along the metal surface. Since the initial SPR detection in the early 1980’s, commercial SPRs in the early 1990’s introduced a new platform besides the ELISA and RIA to detect ferritin. Flow cells act as the primary method to facilitate mass transport of molecules along the SPR detection region. (Figure 8). Because the chemical reaction is limiting and not the
mass transport due to the flow cell, kinetic data can be gathered and the equilibrium constants can be determined (i.e. $k_a$, $k_d$, and $K_d$).

![Sensorgram and schematic of gold surface plasmon resonance with 1) ligand immobilized (baseline), 2) analyte associating ligand (flow cell not shown) 3) equilibrium binding, 4) Acid wash (dissociation phase), 5) steady-state baseline.](http://web.bf.uni-lj.si/bi/sprcenter/Technology.html)

**Figure 8.** Sensorgram and schematic of gold surface plasmon resonance with 1) ligand immobilized (baseline), 2) analyte associating ligand (flow cell not shown) 3) equilibrium binding, 4) Acid wash (dissociation phase), 5) steady-state baseline. [http://web.bf.uni-lj.si/bi/sprcenter/Technology.html](http://web.bf.uni-lj.si/bi/sprcenter/Technology.html)

To date, four SPR immunoassays have been published measuring ferritin (Cui et al., 2003; Oddie et al., 1997; Wilson and Nie, 2006). However, these publications do not detect ferritin with the objective to diagnose a nutritional deficiency, they use ferritin as a model biomarker for SPR binding (Oddie et al., 1997), tumor biomarkers (Cui et al., 2003; Wilson and Nie, 2006). In addition, to the SPR optical biosensor, a less common microresistive pulse sensor was also published that used antibody-functionalized microparticles in a microfluidic system to bind to ferritin and detect it label free (Han et al., 2014).

The advancement in diagnostics and immunology has made ELISAs available in most laboratory settings. Despite this, ELISAs still require laboratories and trained individuals to carry out the experiments. As technology continues to progress, new sensing *in-vitro* devices have
imparted an opportunity to measure ferritin using different properties of light and potentially outside of the laboratory.

2.4. Photonic Crystal Biosensor

Similar to SPR, photonic crystal (PC) biosensors are optical sensors that function as a transducer to convert the dielectric permittivity of a detected molecule or group of molecules on the biosensor surface into a quantifiable light signal that can be measured by a detection instrument (i.e. spectrophotometer) (Cunningham et al., 2002). To create a sensor that can concentrate light into extremely small volumes, “sub-wavelength surfaces (SWS)” or “nanostructures”, it must be engineered so as to be smaller than the wavelength of light. Furthermore, the SWS materials must have particular refractive indexes in relation to one another to achieve resonance at a desired wavelength (Cunningham et al., 2002). More precisely, when the refractive index of the grating region is greater than the substrate region, a waveguide is created. As a result, when incident light pass through the waveguide region and propagates, the grating structure selectively couples light at a narrow band of wavelength into the waveguide. The light propagates for a distance of 10-100 µm before undergoing scattering and couple with forward- and backward- propagating light. Figure 9 shows a schematic representation of the materials of the PC biosensor surface and the electric field that emanates from the surface after incident light interacts with the PC biosensor grating.

This produces a resonant grating effect on the reflected radiation spectrum, resulting in a narrow band of reflected or transmitted wavelengths (Cunningham et al., 2002). In addition, to the physical properties of how the PC biosensor detects analytes, chemical functionalization have been adapted with anime groups that allow for bifunctional linkers that will bind to the receptor
molecule that will ultimately provide selective recognition of target analytes. As stated previously PC biosensor detection systems have various diagnostic and screening applications in DNA microarrays (Mathis et al., 2010), cancer cell analysis (Chen et al., 2013), virus detection (Shafiee et al., 2014) protein detection (Peterson et al., 2014) and high throughput drug screening (Heeres and Hergenrother, 2002).

![Figure 9. PC biosensor dielectric surface materials with sub-wavelength surface grating of 120 nm. The heat image shows high concentrations of electric fields resonating above the PC surface (Cunningham et al., 2002)](image)

In each of the following chapters of this thesis, the PC biosensor will be described in some detail as it pertains to the application of protein detection. However, specific details of the engineering aspects of the PC biosensor are not included because the engineering of the PC biosensor is not the primary focus of this work. Readers are referred to Cunningham et al., 2004 and Cunningham et al., 2002 for more information about the PC biosensor used in the following studies.
2.5. Validation of Diagnostic Methods

Method validation provides the foundation for clinical diagnostic tools. Analytical quality specifications of more than 300 biomarkers have been published (Ricos et al., 1999). Analytical quality specifications set baseline levels of the total allowable error (TE\textsubscript{a}) that clinical diagnostic instruments can exhibit when measuring a biomarker of interest. The purpose of these analytical specifications in clinical diagnostics is to provide accurate, precise and reliable diagnosis for patients to prevent misdiagnosis of disease or deficiency. As new technologies, namely biosensors, become available for clinical diagnostic applications, method validation studies are required to estimate the total analytical error (TE). The acceptability of diagnostic devices is dependent on TE\textsubscript{calc}, which is the estimated total analytical error of the test method (i.e. PC biosensor) quantified by replication experiments and comparison of methods experiments (Westgard et al., 1974). When the TE\textsubscript{calc} is less than TE\textsubscript{a} for a given biomarker, as defined by the quality specifications, the method is considered acceptable (Ricos et al., 1999). Each biomarker has a different quality specification for TE\textsubscript{a}. Therefore, the TE\textsubscript{a} for ferritin is different than the TE\textsubscript{a} for sTfR depending on within- and between patient variability (Rico et al., 1999). Error analysis is used to determine whether the TE of the proposed biosensor is acceptable at clinical cut-off concentrations. Table 3 lists how TE\textsubscript{calc} is determined in different sets of experiments, which are described below.

Replication Experiment

The imprecision or random error (RE) of the test method (PC biosensor) is evaluated in the replication experiments where within-day variability and between-day variability are measured. Means, standard deviations, confidence intervals, and relative standard deviations
(%RSD) are calculated to observe the variation expected in a test result under normal operating conditions in the laboratory (Westgard, 2008). Various matrixes should be tested to determine how systematic error (SyE) and RE vary depending on matrix effects. The within-day experimental precision should include at least 2-3 replicates per sample. The between-day experimental precision is measured 3 separate times on different days and over two weeks. These experiments account for the variability in reaction conditions such as timing, temperature, and mixing over a shorter period (i.e. within-day) and longer period (i.e. between-day) (Westgard et al., 1974).

**Table 3.** Analytical error described in terms of the experiments necessary to quantify the type of error. The experiments can be divided into two type: preliminary and final. The preliminary experiments are conducted with a small sample size to test performance of test method. The final experiments are done with a large sample size from the general population to assess the test method measuring a Gaussian distribution of analyte concentration. (Westgard 2008)

<table>
<thead>
<tr>
<th>Type of Analytic Error</th>
<th>Evaluation Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preliminary</td>
</tr>
<tr>
<td>Random Error</td>
<td>Replication Within run</td>
</tr>
<tr>
<td>Constant Error</td>
<td>Interference</td>
</tr>
<tr>
<td>Proportional Error</td>
<td>Recovery</td>
</tr>
</tbody>
</table>

**Interference and Recovery**

Interference experiments aim at quantifying the amount of constant systematic error (CE) that as a result of the lack of specific of a particular assay (Westgard, 2008). To test for interference signals an interference experiment is run. One test sample is prepared with the given analyte of interest as well as the substance suspected of causing non-specific signals (i.e.
interferer). Then, a second aliquot of the original sample is diluted with water (no interferer added). The difference between the determined concentrations is the result of the interferer.

Young compiled in a database a number of substances that have resulted in interferences (Young, 1993). Equation (1) one shows the CE in terms of bias.

\[
\text{Bias} = \text{Original Sample with Interferer} - \text{Original Sample with H2O} \quad (Eq1)
\]

Recovery experiments are designed to estimate the proportional systematic error (PE). Despite the utility of recovery experiments, clinical laboratories have often incorrectly carried out these experiments. Nevertheless, the recovery experiment is similar in nature to the interference experiment. The primary difference is that the analyte is added to one “spiked” sample (instead of interferer molecule) and that sample is compared to a second sample where water is added. Then, the recovery is expressed as a percentage by taking the difference of the concentration obtained in the “spiked” sample from the water sample and divided by the amount of analyte “spiked” in sample one (Westgard, 2008). The recovery (R) Equation (2) is expressed below. The magnitude of the PE is then calculated by the Equation (3) where \(\overline{\%R}\) is the mean recovery of a series of experiments (Westgard et al., 1974).

\[
\%R = \frac{\text{concentration recovered}}{\text{concentration added}} \times 100 \quad (Eq2)
\]

\[
PE = \overline{\%R} - 100 \quad (Eq3)
\]
Comparison of Method Experiment and Difference Plots

The inaccuracy or systematic error (SyE) of the test method is estimated through its comparison with comparative methods (i.e. commercial ELISAs) somewhat considered the traditional method or the “gold standard.” In these experiments patient samples are measured by the comparative method and the test method. Systematic differences at critical medical decision levels are the errors of interest. These systematic errors can be expressed in terms of constant error and proportional error. Equation (4) expresses this relationship.

\[
\text{Systematic Error (SyE)} = \text{Constant Error (CE)} + \text{Proportional Error (PE)} \quad (Eq4)
\]

Because FDA-certified ELISAs have met Clinical Laboratory Improvement Amendment Final Rule standards and performed satisfactorily in comparative studies against definitive methods, they serve as approved reference methods for comparison of analytical platforms. Therefore, any SyE observed in the comparison of methods will be assigned to the PC biosensor. When running a comparison of methods experiment, a minimum sample of 40 different patient specimens should be tested by the two methods (CLSI, 2002). Sufficient replicate measures for each specimen are recommended to be analyzed to identify problems from sample mix-ups, transposition errors and other mistakes. Discrepant results should be rerun to verify results. Furthermore, several analytical runs are suggested to be included over a minimum of 5 days (CLSI, 2002).
A difference plot is used to evaluate differences between the results from the test method minus those from the ELISA assay on the y-axis versus the results from the ELISA assay on the x-axis. Visual inspection of graphed data at the time of data collection highlights outliers. These outliers can be re-analyzed to confirm that the difference between the two methods is real and not a mistake (i.e. recording error or specimen mix-up). An example of a difference plot comparing a test method (FIELD) and comparative method (REF) for S-Creatinine is shown in Figure 10. (Hyltoft-Petersen et al., 1997).

![Difference Plot](image)

**Figure 10.** Difference Plot for S-Creatinine, with reference Method (REF) values on x-axis and the difference between field method (FIELD) and REF as y-axis (Hyltoft-Petersen et al., 1997).

As illustrated in Figure 10, the difference plot compares the results of measuring s-creatinine with a FIELD method and a REF method. This example is analogous to comparing the PC biosensor to an FDA-certified ELISA. Ideally, the experimental FIELD method should give the same results as the control REF method, which would correspond to all the data points of different concentrations aligning along the zero line. Thus, delineating zero difference between the two methods. However, many data points align away from the zero line and around 1σ or 2σ above and below zero. This means that the FIELD method overestimated and underestimated the
value given along the y-axis what the REF measured at a given concentration along the x-axis. The narrower the $2\sigma$ range above and below the zero line is, then, the narrower the variance of the differences between the FIELD and REF method is. This refers to the term “narrow limits of agreement”. Likewise, the more data points outside the $2\sigma$ lines indicate a higher constant error/bias in the test method.

A comparison plot using regression statistics shows the agreement between the two methods. These statistics allow estimation of the $SyE$ at the medical decision level. Using linear regression, the slope ($b$) and y-intercept ($a$) of the line of best fit and the standard deviation of the coordinate points for it ($s_{y/x}$) will be determined. Then, by using the regression line equation, the $SyE$ at a given medical decision concentration ($X_c$) will be calculated by using the corresponding Y-value ($Y_c$) from the regression line and taking the difference of $Y_c$ and $X_c$ (Westgard and Hunt., 1973). The previous information is expressed by the following Equations (5) and (6), respectively:

$$Y_c = a + bX_c \quad (Eq 5)$$

$$SyE = Y_c - X_c \quad (Eq 6)$$

Figure 11 gives an example of a comparison plot created by Erhardt et al., 2004, illustrating how linear regression can be used to calculate slope, y-intercept and ultimately, $SyE$. On the x-axis is the comparative method and on the y-axis is the test method. In this example, the $SyE$ of the test ELISA can be calculated. The components of $SyE$ are CE and PE. From the regression equation, the CE is the y-intercept of 2.89 µg/ml. This means that every sample using the test ELISA has a positive bias of 2.89 µg/ml, and is therefore, overestimated by 2.89 µg/ml. The PE can be
derived from the slope of 0.727. To calculate the PE in this example, the slope of 0.727 is subtracted from 1 (slope of complete agreement) and multiplied by 100 to arrive at 27.3%, which again is considered the PE. In practical terms, each sample being measured with the test method gives a 27.3% proportion less concentration than the comparative method. To figure out how this PE affects each sample in the regression analysis, 0.727 is multiplied by the concentration given for a sample using the regression equation. For example if the comparative method says the sample contains 10 µg/ml of sTfR, with a PE of 27.3%, the test method will measure 7.27 µg/ml (10 µg/ml × 0.727). Therefore, at a sTfR concentration of 10 µg/ml, the SyE would be 2.89 µg/ml + (-2.73) = 0.14 µg/ml.

Clinical acceptability of the PC biosensor depends on what amount of total analytical error is allowable (TEa) without affecting or limiting the diagnosis and interpretation of test results. (Westgard et al., 1974). Thus, as mentioned previously, the TE will be calculated by the following equation:

**Figure 11.** Comparison Plot for sTfR, with comparative method (Ramco ELISA) results on x-axis and the test method (Sandwich ELISA) on y-axis (Erhardt et al., 2004).
\[ TE_{calc} = SyE + RE \quad (Eq7) \]

Then the \( TE_{calc} \) will be compared to the established allowable total error for any given biomarker (Ricos et al., 1999). In the case of this proposal, the \( TE_a \) for sTfR \( TE_a \) is 17.4% (Bailey et al., 2013).
2.6. References


CHAPTER 3

A PHOTONIC CRYSTAL BIOSENSOR ASSAY FOR FERRITIN UTILIZING IRON-OXIDE NANOPARTICLES

3.1. Introduction

Iron-deficiency anemia is the most prevalent micronutrient deficiency afflicting 1 in 3 people worldwide; most of them women and children who live in rural areas of developing countries (Black et al., 2008). Iron-deficiency anemia causes deleterious effects on pregnancy outcomes, children’s cognitive and physical development, and productivity in working adults (Black et al., 2008). The identification of populations suffering from iron deficiency, however, remains a significant limitation that hinders nutrition and health improvements.

Despite their widespread use and availability, commercial test platforms like enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) are expensive and impractical in field settings considering the high cost of equipment (plate reader, glassware), the need for specialized resources in the field (electricity, radioactive reagents), and the need for highly trained personnel (nurses and technicians) (Nash et al., 2012). On the contrary, robust and field-friendly technologies in biosensing demonstrate potential for point-of-care nutrition diagnostic methodologies.

Photonic crystals are periodic dielectric surface structures, designed to reflect a narrow band of wavelengths when illuminated by a broadband light source (Cunningham et al., 2002). The adsorption of biomolecules on the sensor surface results in an increase in the dielectric permittivity of material in an evanescent electromagnetic field region in the media within ~200
nm of the surface (Arakawa and Yoshiko, 1999), which in turn causes the reflected peak wavelength value (PWV) to shift to a greater value; thus, providing a simple mechanism for biomolecule detection. Unlike ELISAs that use a colorimetric reaction between an enzyme and a substrate to measure analyte concentrations (Voller et al., 1978), and RIAs that determine analyte concentrations based on the change of radioactivity of analyte samples (Marcus and Zinberg, 1975), PC biosensors utilize simple optics and intrinsic physical properties of the analyte as the mechanism of detection. PC detection systems have various diagnostic and screening applications in DNA microarrays (Mathias et al., 2010), cancer cell analysis (Chan et al., 2007), virus detection (Pineda et al., 2009), and pharmaceutical drug screening (Heeres and Hergenrother, 2011) Furthermore, PC biosensors are inexpensively manufactured from plastic materials and incorporated into liquid handling formats such as microplates (Cunningham et al., 2004) and microscope slides (Gallegos et al., 2013) for single-use applications.

One goal of current biomedical and nanotechnology research is to develop biosensor applications for point-of-care diagnostics in field settings (Nash et al., 2012). To achieve field readiness, methods to improve biosensor sensitivity as well as to increase biosensor versatility are needed to detect physiological concentrations of analytes comparable to commercial ELISA and RIA tests, often on the order of 1.0-1000.0 ng/mL. Non-specific binding and inconsistencies in sensitivity due to the proteinaceous nature of complex matrices like whole blood, serum, and plasma limit analysis (Byrne and Diamond, 2006). Current studies aimed at practical applications using optical biosensors acknowledge the difficulty of selective analyte detection in serum (Chung et al., 2005; Kumbhat et al., 2010; Kyprianou et al., 2013) and whole blood (Bonanno and DeLouise, 2007) necessary for point-of-care applications. A promising amplification approach using IONPs has shown to increase the signal-to-noise ratio in surface plasmon
resonance optical biosensors in a chocolate bar matrix (Pollet et al., 2011) and serum and stool (Soelberg et al., 2009) matrices. Yet, to our knowledge, no studies with PC optical biosensors have tested IONPs as a method to enhance analyte detection in serum.

In the present work we describe proof-of-concept studies using IONPs to enhance sensitivity for detection of ferritin, a biomarker of iron deficiency anemia, in control serum and quality control samples using a PC biosensor.

3.2. Materials and Methods

Reagents

3-glycidoxypropyltrimethoxysilane (GTPMS), NaOH, Tween 20, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, and phosphate buffer saline (PBS) and StartingBlock blocking buffer from Pierce (Thermo Fisher Scientific). Double deionized water (DDW) was used in all experiments.

ELISA kits, antigen and antibodies (Ab)

Human ferritin ELISA kits were purchased from BioVendor (RCAN-F-4280R), GenWay Biotech, Inc. (GenWay; GWB-F4BE8D), and RayBiotech, Inc. (RayBiotech; ELH-Ferritin-001). Human liver ferritin (US Biological; F4015-21A) and Liquichek™ serum controls (Bio-Rad Laboratories) were dissolved in PBS or BSA to develop standard curves for the BIND and ELISA detection platforms, and act as quality controls for agreement and recovery studies. The monoclonal mouse anti-human liver ferritin Ab (mAb), used as the capture antibody in the BIND assay, were purchased from US Biological (F4015). As the detection Ab in the BIND, polyclonal goat anti-human liver ferritin Ab (pAb) were purchased from US Biological (F4015-17).

Iron-oxide nanoparticles conjugation protocol
Detection pAb were functionalized (FpAb) to iron-oxide nanoparticles (30 nm) as reported by vendor (Ocean NanoTech, LLC). Aliquots of 0.2 mL of IONPs were combined with 0.1 mL of Activation Buffer. Then, 100 µL of a solution containing 2 mg/ml EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 1 mg/ml NHS (sulfo-N-hydroxysuccinimide) was added to the IONPs, mixed, and left at room temperature for 5-10 min with continual stirring. The next step mixed 0.4 mL of Coupling Buffer to the activated IONPs and then added at least 1 mg of pAb. Reaction time for conjugation lasted 2 h with continual mixing. Next, 10 µL of Quenching Buffer was left for 10 minutes at room temperature and then the entire mixture was transferred to a plastic cuvette and 3 mL of Wash/Storage Buffer was added. The cuvette was inserted into a SuperMag Separator™ (Ocean NanoTech, LLC) magnetic separator to allow conjugated magnetic FpAb to separate for 5 h. Liquids were aspirated without taking magnetic contents before FpAb were re-suspended in 3 mL and the last two steps were repeated for higher extraction. Conjugation was verified by Western blot tests (data not shown).

**PC biosensor and readout system**

As described and illustrated in Cunningham et al., 2004, the PC biosensors used in this work are comprised of a plastic replica molded periodic linear grating surface structure that is over-coated with a high refractive index TiO$_2$ thin film to create a resonant reflection surface that functions as a high efficiency reflector for only a narrow band of wavelengths near $\lambda$=855 nm when covered with aqueous media. At the resonant wavelength, an optical standing wave is established at the PC surface. Adsorption of biomolecules or iron oxide tags, which have dielectric permittivity that is greater than water, results in displacement of water from the evanescent field region and an increase in the effective refractive index experienced by the optical standing wave. In turn, the augmented refractive index results in an increase in the
resonant reflected wavelength from the PC. The PC biosensor structure is fabricated on sheets of plastic film and attached to bottomless standard format microplates. The PWV of the resonant reflection is measured by illuminating the PC at normal incidence with a broadband light source, and measuring the resonantly reflected wavelength with the aid of a spectrometer. Changes in PWV induced by adsorption of biomolecules can be monitored in each well, where the magnitude of the PWV shift can be used to quantify the amount of adsorbed material.

PC microplates (384-well) were purchased from SRU Biosystems, Inc. The Biomolecular Interaction Detection system (BIND; SRU Biosystems, Inc.) was used to measure interactions of ferritin with antibodies. The BIND illuminates the microplate with a broadband light source (λ range 400-700 nm) via an optical fiber positioned below the biosensor microplate. The system contains 8 parallel readout heads, and is capable of measuring the PWV of all 384-well biosensor microplate in ~10 seconds. The microplate may be re-scanned at preset intervals to generate kinetic plots of biomolecular binding. A ~2 mm diameter region of the biosensor is illuminated. A second parallel optical fiber is bundled with each illuminating fiber to capture reflected light, which is directed into a spectrophotometer. Detailed description of the design and operation of the BIND instrument can be found elsewhere (Cunningham et al., 2002; Cunningham et al., 2004).

PC detection procedure

Epoxy-silanization of PC biosensor surface

A 0.1 M NaOH solution was dispensed (20-μL) into the wells of a 384-well biosensor microplate and left to incubate for 1 h at room temperature (23 °C). After incubation, plates were ultra-sonicated (Fisher Scientific Isotemp202 Heater Ultrasonic bath) for 15 min. Wells were then aspirated and dried under nitrogen stream. Next the plate was placed in an oxygen plasma
Planar Plasma System, Texas Instruments Inc.) for 5 min. Then, 2.5% 3-
glycidoxypropyltrimethoxysilane and 10 mM acetic acid solution in ethanol of was added (15 µL) to each well and left to incubate for 1 h at room temperature (23 °C). Finally, wells were aspirated and washed twice with ethanol and dried under nitrogen stream, before they were ready to be assayed.

Capture mAb immobilization

Capture mAb (62.5 µg/mL) was dispensed (15 µL) into all epoxy-silanized wells. The PC microplate was sealed with tape (Pierce, Thermo Fisher Scientific) and left at room temperature (23 °C) overnight. Then, wells were washed with PBS-Tween (0.5%) three times. The PWV corresponding to mAb immobilized on sensor surface was measured relative to baseline. Final mAb concentration in this assay was the result of several tests using different mAb dilutions (data not shown).

Blocking step

Several blocking agents (i.e., BSA, casein, non-protein blocker, ethanolamine, and StartingBlock) were evaluated to limit non-specific antigen binding to the epoxy-silane surface as well as to reduce detachment of blocking molecules. StartingBlock was selected from this group because other blockers such as casein detached from surface to give a negative signal and BSA, ethanolamine, and the non-protein blocker did not effectively prevent nonspecific-antigen binding. Undiluted StartingBlock (20 µL) was pipetted into all wells. PC microplates were incubated for 2 h at room temperature, and then, washed with PBS-Tween three times. The PWV shift was measured relative to baseline.

Antigen preparation
Dose response curves for ferritin were prepared in PBS, BSA, and control serum. For PBS standard curve, ferritin was serially diluted from stock concentrations (4.0 mg/mL) in PBS (137 mM NaCl, 2.7 mM KCl, pH 7.4). Ferritin concentrations ranged from 0.03 to 1.0 µg/mL. To test dynamic range, higher ferritin concentrations were included, 2.0 and 4.0 µg/mL. For BSA response curve, BSA was diluted with DDW to 10 mg/mL and spiked with the same ferritin stock used to make PBS standards. For serum standard curve, Liquicheck™ serum level 3 was diluted in PBS to 10 mg/mL of total protein (based on reported protein content) and spiked with ferritin to reach same concentrations as in the BSA and PBS standard curves. Testing samples (i.e., PBS, BSA and serum) were assayed (15 µL per well) onto PC biosensor. Active binding was measured for 30 minutes, in 1 min intervals. BioVendor ELISA quality control ferritin was assayed without dilution and had a concentration near 0.35 ± 0.088 µg/mL. This control was also used for accuracy and recovery studies.

Secondary pAb and functionalized pAb (FpAb)

Secondary pAb were used to increase assay sensitivity. Stocks of pAb and FpAb were 5 and 1 mg/mL, respectively. After 30 min kinetic reading of antigen-mAb binding step, either non- or functionalized pAb (250 µg/mL) were dispensed (15 µL) in all wells. Formation of sandwich complexes (mAb-ferritin-F/pAb) was measured for 1 h, in 1 min intervals. Final antibody concentrations in this assay were the result of several tests using different dilutions (data not shown).

Recovery and accuracy experiments.

Based on Liquichek™ manufacturer’s reported ferritin concentrations (from 11 different methods of analysis) in each of the three sera levels, we calculated the mean ferritin concentration of all these methods to establish an expected concentration and range for each
serum level. Calculated concentrations for each serum level were used as the basis for dilutions and to establish an expected concentration in the analyses of recovery, agreement and accuracy. Liquichek™ levels 1 and 2 were diluted to 10 mg/mL of total protein and spiked to achieve 42-48 ng/mL of ferritin for recovery analysis. Recovery was evaluated using the apparent recovery formula as explained by Burns et al. (2002). Liquichek™ serum level 3 was diluted 10-fold to determine accuracy and agreement of PC biosensor method against reported Liquichek™ ferritin values and ELISA measurements. For evaluation of inter-day and intra-day assay reproducibility in the PC biosensor, all assays were conducted in triplicate, in three separate days, within a 2-week period. Reproducibility within ±15% RSD was considered appropriate.

**PC biosensor comparison to ELISA.**

Three commercial ELISAs (BioVendor, GenWay, and RayBiotech) were used as detection platforms to compare against the PC biosensor in terms of detection accuracy, agreement, bias, recovery, dynamic range, limit of detection, inter-assay/intra-assay variability, and matrix effect (i.e. BSA, PBS and serum). Liquichek™ serum controls and BioVendor quality control were tested following the protocols provided by each vendor without modifications.

**Statistical procedures.**

Means, standard deviations (SD), replicates (n), confidence intervals (CI), and relative standard deviations (%RSD) presented in figures and tables were calculated using Excel 2008. All figures and linear fitted curves (slope, intercept and determination coefficient) for dose response effects in each diagnostic platform were performed in SigmaPlot (v 11.0, Systat Soft. Inc., San Jose, CA). The limit of detection (LOD) was determined using previously published analytical methods (Armbruster and Pry, 2008). Bland-Altman statistical analysis was employed to determine agreement and bias between each of the methods and reference controls (Bland and
Altman, 1986). Mean differences and post hoc analysis were carried out using paired t-test and Tukey honest significant difference test, respectively, in SPSS (v 21.0, SPSS Inc., Chicago, IL). For all tests statistical significance was set at $P<0.05$.

### 3.3. Results

*Development of a PC biosensor platform for analysis of human ferritin in serum.*

Real time binding of ferritin to antibodies in three PC biosensor modalities was conducted using a set of primary, secondary and functionalized antibodies and was monitored in the BIND system. Figure 12 shows a schematic of the three binding assays on the PC platform. It also shows real-time binding data for ferritin (1 µg/mL) in PBS collected in the BIND system. Figure 13 shows ferritin standard responses for all assay types on the PC biosensor. In the label-free assay (only capture mAb), the PC biosensor resulted in a limit of detection (LOD) of 2.43 µg/mL and was able to detect ferritin up to 4.0 µg/mL. The use of a secondary pAb, “sandwich assay”, lowered the LOD (0.38 µg/mL) for ferritin and extended the dynamic range (0.38 – 4.0 µg/mL). The assay using immunofunctionalized (FpAb) IONPs resulted in the lowest LOD (26 ng/mL), with a dynamic range between 26 – 2000 ng/mL. The LODs and dynamic ranges for all immunoassay platforms are reported in Table 4.
Figure 12. A schematic of the three immunoassay types on the PC biosensor and their real-time kinetic readings for ferritin (1 µg/mL) in the BIND system. (A) label-free assay; (B) with secondary antibody sandwich assay; (C) immunofunctionalized IONPs with secondary antibodies; (D) kinetic readings: label-free assay (◊), with secondary antibody sandwich assay (□), and immunofunctionalized IONPs with secondary antibodies (○). [Peterson et al., 2014]

Figure 13. Comparison of responses from three immunoassay types on the PC biosensor for different ferritin standard concentrations in PBS buffer. Label-free assay (black bars); sandwich assay with secondary antibody (grey bars); immunofunctionalized IONPs with secondary antibodies (white bars). Bars represent mean ±SD (n=3, average of separate assays). [Peterson et al., 2014]
**Table 4.** Intra-assay and Inter-assay variability of different immunoassay platforms.

<table>
<thead>
<tr>
<th>Sensing Platform</th>
<th>LOD (ng/mL)</th>
<th>Range (ng/mL)</th>
<th>Intra-Assay %RSD</th>
<th>Inter-Assay %RSD</th>
<th>Intra-assay %RSD&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Inter-assay %RSD&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC biosensor</td>
<td>26</td>
<td>26-2000</td>
<td>3.5</td>
<td>6.8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>GenWay</td>
<td>5</td>
<td>5-1000</td>
<td>7.9</td>
<td>14.5</td>
<td>6</td>
<td>5.6</td>
</tr>
<tr>
<td>RayBiotech</td>
<td>7</td>
<td>7-50</td>
<td>2.8</td>
<td>3.5</td>
<td>6</td>
<td>&lt;10</td>
</tr>
<tr>
<td>BioVendor</td>
<td>52</td>
<td>52-2000</td>
<td>2.4</td>
<td>7.5</td>
<td>6</td>
<td>7.3</td>
</tr>
</tbody>
</table>

<sup>1</sup>PC biosensor using IONPs functionalized secondary Ab.
<sup>2</sup>Provided by the vendor.

All detection platforms resulted in linear dose-responses (Δsensing units/Δferritin µg/mL) with high coefficients of determination ($R^2$) (Figure 14A). Fitted linear equations for ferritin dose response curves ($m$=slope; $b$=intercept) were $m=0.0028$, $b=0.11$, $R^2=0.98$ for PC biosensor; $m=0.0022$, $b=0.98$, $R^2=0.98$ for GenWay; $m=0.0029$, $b=0.06$, $R^2=0.99$ for BioVendor; and $m=0.021$, $b=0.49$, $R^2=0.90$ for RayBiotech. Most ELISA assays showed saturation of response below 1 µg/mL. GenWay and RayBiotech ELISAs had the highest sensitivities, but responses quickly saturated detection, especially in the latter. The intra- and inter-day assay variability for all test platforms are reported in Table 4. For the PC biosensor the intra-assay variability was 3.5%. The BioVendor ELISA gave the lowest intra-assay variability (2.4%), whereas the GenWay ELISA gave the highest intra-assay variability (7.9%). With the exemption of GenWay ELISA (14.5%), inter-assay variability was less than 10% for all other platforms.

Liquichek™ control serum level 3 was used to determine agreement between PC biosensor and ELISA detection platforms (Table 5). The PC biosensor and the GenWay ELISA provided results similar to expected range of ferritin (ng/mL) in Liquichek™ serum level 3 (M=32, 95% CI [24.9-39.1], Tukey, $P>0.1$). Similarly, analysis of quality control sample from
BioVendor on the PC biosensor and BioVendor ELISA resulted in ferritin values (ng/mL) within the expected range (M=350, 95% CI [262-437], Tukey, \(P>0.1\)).

**Table 5.** Detection of ferritin in control sera on PC biosensor and commercial ELISAs.

<table>
<thead>
<tr>
<th>Serum controls</th>
<th>Expected Mean &amp; Range (^1)</th>
<th>PC biosensor (^2)</th>
<th>GenWay</th>
<th>RayBiotech</th>
<th>BioVendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquichek (\text{™}) level 3</td>
<td>Mean</td>
<td>32 (^a)</td>
<td>39.9 (^a)</td>
<td>19.9 (^b)</td>
<td>49.3 (^c)</td>
</tr>
<tr>
<td>Quality Control</td>
<td>Mean</td>
<td>350 (^a)</td>
<td>316 (^a)</td>
<td>421 (^b)</td>
<td>635 (^c)</td>
</tr>
</tbody>
</table>

\(^1\) Concentration based on Liquichek\(\text{™}\) serum level 3 and protein buffer Quality Control provided by BioVendor ELISA kit. \(^2\) PC biosensor using immunofunctionalized IONPs. Means with different superscripts within each row represent statistical differences (Tukey; \(P<0.05\)).
Figure 14. Standard responses and measurement agreement/bias of four immunoassay platforms. PC biosensor data were obtained from assays with IONPs. (A) Ferritin dose-response curves indicating the linear range and saturation levels of the different sensing platforms. Point-to-point lines are presented to facilitate observation of saturation effects. Data points represent means ±SD (n=3, average of separate assays). (B) Bland-Altman plots representing agreement/bias of immunoassay platforms. For each immunoassay type, means between observed values for several ferritin concentrations and their expected control (Liquichek™) sera values (x-axis) are plotted against differences between observed values and expected control sera values (y-axis). Solid lines represent the mean difference. Dotted lines represent upper (+2SD) and lower (-2SD) limits. [Peterson et al., 2014]
Liquichek™ control serum levels 1 and 2 were used to assess recovery from PC biosensor and ELISA detection platforms (Table 6). Expected ferritin concentrations in serum levels 1 and 2 were 47.7 and 45.1 ng/mL, respectively. Ferritin recoveries from analysis of serum levels 1 and 2 on the PC biosensor were 96.9% (7.6% RSD) and 94.3% (13.1% RSD), respectively. Ferritin recoveries from these control sera on the BioVendor’s ELISA were similar to those from PC biosensor (Tukey, \( P > 0.1 \)). Detection of ferritin in both serum levels using the GenWay’s RayBiotech’s ELISAs resulted in the lowest and highest recoveries, respectively.

Table 6. Recovery of ferritin from control sera in several immunoassay platforms.

<table>
<thead>
<tr>
<th>Serum controls</th>
<th>Ferritin (ng/mL)</th>
<th>% Recovery (± %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unspiked(^1)</td>
<td>Spiked</td>
</tr>
<tr>
<td>Liquichek™ level 1</td>
<td>7.91</td>
<td>47.7</td>
</tr>
<tr>
<td>Liquichek™ level 2</td>
<td>25.5</td>
<td>45.1</td>
</tr>
</tbody>
</table>

\(^1\)Ferritin concentrations based on reported Liquichek™ serum levels 1 and 2.
\(^2\)Represents number of replicates from three experiments over two weeks.
Means with different superscripts within each row represent statistical differences (Tukey; \( P < 0.05 \)).

The Bland-Altman statistical analysis was employed to determine the bias and agreement of the PC and ELISA detection platforms compared to the reported Liquichek™ ferritin value (Figure 14B). The PC biosensor and BioVendor detection methods had the lowest biases, -1.26 (95% CI[-3.0 – 5.5]) and 1.34, 95% CI[-15.0 – 12.3], respectively. Compared against expected values, RayBiotech test had a positive bias of 17.1 ng/mL (95% CI[10.4 – 23.7]), while the
GenWay test had a negative bias of -10.7 ng/mL (95% CI[-21.0 – 0.34]). The limit of agreement (LOA) with the expected ferritin values was -11.6 – 9.1 ng/mL for the PC biosensor, -28.6 – 7.3 ng/mL for GenWay, 5.6 – 28.6 ng/mL for RayBiotech, and -25.2 – 22.5 ng/mL for BioVendor.

Matrix effect on PC biosensor response.

Detection of ferritin on both the PC biosensor (using FpAb) and BioVendor ELISA was evaluated by spiking concentrated antigen in three matrices: PBS, BSA and serum. Ferritin dose-response curves differed depending on the matrix. On the PC biosensor, ferritin (ng/mL) fitted linear dose response curves in BSA ($m=0.0024$, $b=0.192$, $R^2=0.88$, $P<0.01$) and serum ($m=0.0022$, $b=0.258$, $R^2=0.85$, $P<0.01$) were similar, but different than in PBS ($m=0.0035$, $b=0.062$, $R^2=0.98$, $P<0.01$). Similar trends were observed in experiments using BioVendor ELISA as detection platform; however, coefficients of determination were higher. Fitted linear curves for ferritin (ng/mL) dose response spiked in BSA ($m=0.0033$, $b=0.1817$, $R^2=0.97$, $P<0.01$) and serum ($m=0.0030$, $b=0.1936$, $R^2=0.93$, $P<0.01$) were similar, but different than in PBS ($m=0.0022$, $b=0.0624$, $R^2=0.99$, $P<0.01$).

3.4. Discussion

In this work, we describe proof-of-concept studies for the development of a PC biosensor assay for determination of ferritin as a biomarker of iron status. To the authors’ knowledge, this is the first known PC biosensor application that uses IONPs to enhance sensitivity for diagnosis of nutritional status. Ferritin, hemoglobin, and soluble transferrin receptor are widely used as diagnostic biomarkers of iron-deficiency anemia. However, alternative low-cost, detection platforms that could identify populations afflicted by micronutrient deficiencies at the point of care in resource-poor environment are still needed. Thus, the objective of this work was to demonstrate the PC biosensor’s capability of accurate and precise detection of ferritin down to
concentrations close to the cut-off used to differentiate populations with iron deficiency as well as show the ability of IONPs in enhancing the signal-to-noise ratio.

The principle of the PC method for analyte detection is fundamentally different than an ELISA, however both assays use antibodies for antigen recognition and binding. An ELISA (colorimetric assay) uses an enzyme-tagged detection antibody that catalyzes a reaction with an exogenous substrate, producing color as the method for antigen quantification with a spectrophotometer. Different versions of ELISA using fluorescent and luminescent probes are available in microtiter plates (Voller et al., 1978). In contrast, the PC platform uses an intrinsic physical property of the analyte (i.e. dielectric permittivity) that when illuminated with a broadband light source at normal incidence causes a change in the refractive index, which is used to quantify the analyte of interest bound onto the sensor surface (Cunningham et al., 2002). This physical binding is quantified in the form of wavelength shifts (nm). Thus, the PC platform allows for label-free and sandwich applications, without the need of a tagged enzyme. In these studies we found that the immunofunctionalization of IONPs with secondary Ab improved the sensitivity and performance of the PC biosensor when compared with label-free and secondary Ab sandwich assays. As a result, the wavelength shift used to detect biomolecules was enhanced. This mechanism is shown in Figures 12 and 13, where the kinetic binding and standard responses are presented for all three PC assays. Due to the lower dielectric permittivity in the label-free and antibody sandwich assay, the binding response is nearly constant relative to the enhanced sandwich assay with IONPs.

The use of pAb functionalized with IONPs on the PC biosensor yielded an improvement of two orders of magnitude in sensitivity when compared to the label-free assay. The LOD of ferritin on the PC biosensor (26 ng/mL) fell within the LOD range of the ELISA platforms tested
(5 - 50 ng/mL). Differences among immunoassay platforms were attributed to the characteristics of the assays such as dilution buffers, different protocol steps (e.g., washing and shaking), antibody affinity constants, and the biotinylation of the detection antibodies (Jordan, 2000). The World Health Organization (WHO) has established the cut-off points for ferritin to discriminate iron deficiency and non-iron deficiency at 30 or 15 ng/mL for subjects with or without inflammation, respectively (World Health Organization, 2007). Although the WHO cut-off point for patients without inflammation falls below the PC biosensor’s LOD, our proof-of-concept studies demonstrate potential for further optimization.

Further improvements in signal-to-noise ratio of the PC biosensor could be achieved with the functionalization of slightly larger IONPs (>30 nm) (Tsai et al., 2007; Piletska and Piletsky, 2010) as well as optimization of assay parameters such as adjusting pH, temperature, buffer matrix, and kinetic energy (Jordan, 2000). Although larger IONPs could increase sensitivity, it could also modulate protein interactions. Piletska and Piletsky (2010) demonstrated that binding affinity of streptavidin-coated surface for biotin was two orders or magnitude lower when biotin was bound to silica-based nanoparticles of larger particle size. The density of the nanoparticle and mass transfer of the FpAb may also modulate the amplification of the signal. Tsai et al. (2007) studied the ability of IONP functionalized antibodies for detection of C-reactive protein (CRP), an inflammatory response protein (~115 kDa) in serum. They found that 80 nm IONPs provided better sensitivity than 440 nm IONPs, suggesting steric hindrance interfered with FpAb-antigen binding. In another study using surface plasmon resonance optical biosensors for detection of Ara h1, a peanut allergen (65 kDa), Pollet et al. (2011) found that IONPs of 20 nm in diameter instead of 60 nm were most suitable to increase detection sensitivity. These findings suggest that diameter and density of nanoparticles play a significant role in protein interaction.
resulting in variable detection sensitivities over a large dynamic range potentially due to lower steric hindrance and facilitated mass transfer. PC biosensors, however, lack systematic optimization studies of IONP size that could lead to improved binding and sensitivity in complex matrices like serum.

Based on the analytical tests used, the PC biosensor performed well in comparison with three commercial ELISAs (Table 5). The PC biosensor demonstrated accuracy and precision in detecting ferritin concentrations in Liquichek™ level 3 and BioVendor’s quality control, where the means were similar to those reported by suppliers. Nevertheless, the Liquichek™ level 3 responses on the PC biosensor were on the higher end of the expected range. This result was attributed to the non-specific binding of interfering proteins present in the serum matrix (Pineda et al., 2009). However, the PC biosensor performed within the lower to middle range of the expected value for BioVendor’s quality control which had ferritin diluted in an unknown protein buffer solution. Based on the intra-assay and inter-assay variability, the PC biosensor had similar precision (<10%) compared to the ELISAs evaluated. RayBiotech’s ELISA had the lowest intra- and inter-assay variability, which was potentially due to the strong interaction of biotin and streptavidin, one of the strongest non-covalent biological interactions used in immunodiagnostics (Guesdon et al., 1979). Immunodiagnostic tests that use biotin-streptavidin interaction normally display stronger binding affinities and lower limits of detection (Gould et al., 1985; Jordan, 2000; Nara et al., 2008).

In spike-recovery experiments with serum, the PC biosensor performed similar to BioVendor’s ELISA, and was capable of recovering ferritin within a narrow range of the expected concentrations in Liquichek™ serum levels 1 and 2 (Table 6). In contrast, ferritin recovery in GenWay’s ELISA was consistently lower than the expected values. This could be
due to the lack of shaking in the GenWay protocol that, as a result, did not provide enough kinetic energy for antibody-antigen interaction in the serum matrix (Yolken, 1982). A high %RSD in GenWay’s ELISA was the result of this heterogeneous binding in replicate wells. RayBiotech’s ELISA presented consistently higher ferritin recoveries in both Liquichek™ sera, however with the smallest %RSD. This was attributed to the effective interaction between the biotinylated detection antibodies and the HRP-streptavidin (Guesdon et al., 1979; Nara et al., 2008). Also, this assay required larger sample dilutions for ferritin to fit within the standard curve, whose slope was an order of magnitude higher than the rest of the tests (Figure 14A).

The dynamic range of detection in the PC biosensor is larger than commercial ELISAs, especially RayBiotech’s, even without IONPs functionalization. In the sandwich assay with pAb and without sample dilution the dynamic range for ferritin detection was 0.3 to 3.2 µg/mL. Pollet et al. (2011) reported similar ranges in studies with surface plasmon resonance. A large dynamic range is useful because it allows more flexibility in modifying the kinetics and timing of the assay. It also reduces the need for dilution steps, which adds variability to final test determination. In our studies the sample matrix affected the dynamic range. The responses in the PC biosensor with IONPs saturated between 0.4-0.8 µg/mL ferritin when spiked into BSA or serum. As indicated by the lower R² values, these protein matrices may have interfered through steric hindrance with the functionalized IONPs and reduced interaction with capture Ab, which instead affected the linear response (Piletska and Piletsky, 2010). In contrast, ELISA tests showed strong linear responses at those concentrations. It is important to notice that ELISAs use a wash step, whereas the PC biosensor does not. Thus, the reading of ELISA wells is practically conducted on PBS. Despite signal saturation, iron deficient individuals have ferritin levels below
30 ng/mL; thus, signal saturation from PC biosensor has limited implications for iron deficiency diagnosis.

The Bland-Altman bias and agreement analysis was used to evaluate how well the PC biosensor experimental results compared to the expected results of 11 established diagnostic tests used to measure ferritin in the Liquichek™ sera, as well as how well it compared with the ELISAs. In the plot (Figure 14B) a low bias is reflected in values closer or around the zero reference line. In addition, limits of agreement lines (±2SD) represent how variable these results are from the mean difference. Thus, the closer these lines are to the mean, the higher the precision. These analyses showed that the PC biosensor assay had low bias and results were within 95% confidence intervals close to those expected values from the 11 established diagnostic tests. GenWay and BioVendor ELISAs yielded ferritin means lower than those expected for the Liquichek™ serum level 3 and thus had a negative bias and lower agreement. Nonetheless, when comparing these values to the AccuBind ELISA ferritin range, one of the 11 Liquichek™ analytical methods reported, the GenWay and BioVendor ELISA results fell within the expected concentration range. Therefore, it can be speculated that ELISAs may yield lower values than other analytical methods like the PC biosensor, among others. This could be due to interfering proteins that prevent the antibody-antigen interaction or the wash steps normally applied in ELISA protocols (Yolken, 1982; Jordan, 2000). In contrast, RayBiotech’s ELISA consistently had a positive bias when detecting ferritin values for both control sera, but a small 95% CI of bias and a tight limit of agreement. It is possible that this positive bias was due to analysis of ferritin concentrations on the high-end of the supplier’s recommended analyte detection (Nara et al., 2008) as this assay uses an optimized protocol for quantification of low ferritin concentrations in not only serum, but also cell culture supernatants and urine.
3.5. Limitations

Despite promising results supporting the use of PC biosensor and the BIND system as a viable diagnostic test for iron deficiency, certain limitations must be addressed in future experiments. Control sera were used to evaluate assay diagnostic parameters. Further studies will require the use of sera from a large, diverse population pool. Assay optimization is another area of improvement to maximize detection sensitivity and minimize total cost of analysis. Finally, concurrent determination of other biomarkers such as hemoglobin, soluble transferrin receptor and CRP to adequately differentiate types of anemia and inflammatory responses are needed. At the moment few ELISA platforms are capable to measure these biomarkers in tandem (Erhardt et al., 2004). However, the flexible nature of PC biosensors makes them amenable to conduct multiple determinations in a single sensor strip.

The high cost of trained personnel, facilities, sampling storage, laboratory equipment, and detection systems required to conduct ELISA type assays is a significant roadblock for field diagnosis and point-of-care applications in rural or remote areas in developing countries. Recently, the Cunningham group demonstrated the use of a smartphone as a detection instrument for a label-free PC optical biosensor. The smartphone is placed on a cradle that incorporates several inexpensive optical components in alignment with the camera. This allows the phone to work as a highly accurate spectrometer (PWV as low as 0.009 nm) for measuring the transmission spectrum from a PC biosensor (Gallegos et al., 2013). This innovative strategy will streamline our objective to bring point-of-care diagnostics of nutritional status to homes, clinics, or remote areas.

3.6. Conclusion
Our proof-of-concept studies showed accurate and precise detection of serum ferritin as a biomarker of iron deficiency using a PC biosensor assay along with immunofunctionalized IONPs and the BIND readout system in comparison to commercial ELISAs. Our group is optimizing the current PC assay platform using IONPs of different sizes, biomarker purification, antibodies of higher affinity and avidity, and improved assay protocols. The novel smartphone cradle-reading instrument will be evaluated using the PC biosensor application for diagnosis of serum ferritin along with other biomarkers of nutritional status.
3.7. References


CHAPTER 4.

ENHANCED SANDWICH IMMUNOASSAY USING ANTIBODY-FUNCTIONALIZED MAGNETIC IRON-OXIDE NANOPARTICLES FOR EXTRACTION AND DETECTION OF SOLUBLE TRANSFERRIN RECEPTOR ON A PHOTONIC CRYSTAL BIOSENSOR

4.1. Introduction

Iron deficiency is estimated to account for half of the two billion anemia cases worldwide (WHO, 2008). When untreated, iron deficiency anemia (IDA) has shown to negatively affect cognition in children (Sachdev et al., 2005), productivity in adults (Horton and Ross, 2003) and pregnancy in women (Black et al., 2008). In addition to hemoglobin, two protein biomarkers commonly used to diagnose IDA are ferritin and soluble transferrin receptor (sTfR). Although ferritin is used by the World Health Organization (WHO) as a biomarker of iron deficiency, its accumulation in the body’s acute-phase response during episodes of infection or inflammation (Worwood, 1979) limits its diagnostic value (WHO, 2008). sTfR, however, is unaffected by infection and increases when red blood cell production is upregulated (Ward, 1987). Although sTfR has diagnostic utility when combined with ferritin, it was not until 2010 (Thorpe et al., 2010) when the measurement of sTfR for diagnosis was standardized. As a result of an international collaborative effort, the WHO developed a reference standard reagent (07/202) to help standardize sTfR immunoassays.

Aside from hemoglobin determination methods, current methodologies to measure IDA biomarkers require specialized equipment, trained technicians, and medical laboratories for analysis (Erhardt et al., 2004; Pfeiffer et al., 2007). As a consequence, large-scale surveying studies are not feasible to identify people suffering from or at risk for IDA in rural areas of low-income countries. This hinders the ability of nutrition and health interventions to assist those
most in need. Thus, diagnostic platforms amenable for use in field settings and that provide quick and actionable results are critically needed to facilitate widespread diagnosis of nutrient deficiencies and support development programs.

Photonic crystal (PC) biosensors have shown potential as a prospective diagnostic platform to measure biomarkers of IDA at the point of care (Gallegos et al., 2013). PCs are periodic subwavelength grating structures that reflect a narrow band of wavelengths when illuminated by a broadband light source (Cunningham et al., 2002) at the resonance conditions. Attachment of biomolecules (e.g. proteins) upon the near field (~200 nm) of the biosensor surface increase the refractive index (Arakawa and Kita, 1999) of the resonance mode and cause a shift of peak wavelength value (PWV) to a higher value; thus, representing a simple mechanism for biomarker detection.

![Diagram of detection steps for standard sandwich assay (SA); and B) inverse sandwich assay (IA).](image)

**Figure 15.** A) Detection steps for standard sandwich assay (SA); and B) inverse sandwich assay (IA). [Peterson et al., 2015]
PC biosensor detection systems have various diagnostic and screening applications in DNA microarrays (Mathis et al., 2010), cancer cell analysis (Chen et al., 2013), virus detection (Shafiee et al., 2014) protein detection (Peterson et al., 2014a) and high throughput drug screening (Heeres and Hergenrother, 2002). Although current PC biosensor methodologies require a laboratory setting, a portable optical interface capable of converting a smartphone into a detection instrument was recently designed for the PC biosensor (Gallegos et al., 2013). Nevertheless, the most significant challenge continues to be the accurate and sensitive detection of biomarkers in complex matrixes (Marks, 2002; Kyprianou et al., 2013).

Complex matrixes (i.e. serum and whole blood) containing variable amounts of interfering proteins are the primary cause of non-specific binding and poor signal-to-noise responses (Kyprianou et al., 2013), which results in unreliable data. These can lead to false-negative or false-positive clinical results that instead increase healthcare costs and burden the health of patients. IDA misdiagnoses can have harmful side effects on patients, especially when providing iron supplementation unnecessarily to children with malaria (Sazawal et al., 2006). Thus, in the development of point-of-care tools to assess patients’ nutritional status in low-income field settings, samples must be analyzed using field-friendly protocols that do not jeopardize diagnostic reliability.

Previously, the authors established the use of PC biosensors as a standard sandwich assay (SA) to detect and quantify ferritin as a potential platform for iron deficiency assessment (Figure 15A), where the signal from bound ferritin was further amplified by the use of antibodies conjugated to colloidal iron-oxide nanoparticles (IONs) (Peterson et al., 2014a). In addition to ferritin, detection of sTfR in a dose-response manner was verified. However, in an attempt to measure the WHO reference standard of sTfR in a solution of 100 mg/mL of bovine serum
albumin, the PC biosensor overestimated the expected sTfR concentration 2x compared to the same sTfR concentration in buffer (Peterson et al., 2014b). Magnetic nanoparticles have recently been used to isolate biomolecules of interest from complex matrixes and to enhance sensitivity by concentrating the biomarker of interest on a surface plasmon resonance biosensor (Soelberg et al., 2009). However, the ability to reduce interference signals in complex matrixes (i.e. serum) using a PC biosensor and provide method validation information on inaccuracy and imprecision as part of a combined assay to extract and measure protein biomarkers has not been studied.

In the present work, the development of an inverse sandwich assay (IA) (Figure 15B) is described where antibody-conjugated IONs are used as magnetic immuno-probes to decrease interference signals for the accurate and reliable determination of sTfR in serum.

4.2. Materials and Methods

Reagents.

3-glycidoxy-propyl-trimethoxy-silane (GTPMS), and NaOH were purchased from Sigma-Aldrich; phosphate buffer saline (PBS), zeba spin columns 40K 0.5 mL and StartingBlock blocking buffer from Pierce (Thermo Fisher Scientific). Double deionized water (DDW) was used in all experiments. ELISA kits, antigen (sTfR) and antibodies (Ab). Human sTfR ELISA kits were purchased from BioVendor (RD194011100) and R&D Systems (DTFR1). Liquichek™ serum controls (Bio-Rad Laboratories, Inc. Lot #52480) were diluted in PBS to measure inaccuracy and imprecision in replication experiments and comparison of methods experiments. The monoclonal mouse anti-human sTfR was used as the capture antibody in the PC biosensor assay and was purchased from Abcam® (ab38168). A monoclonal mouse anti-human sTfR antibody was the detection Ab in the PC biosensor and was purchased from Abcam® (ab10249). Iron-oxide nanoparticle conjugation kit was purchased from Ocean NanoTech (ICK-30-005).
**PC biosensor and readout system.**

Readers are referred to Cunningham et al., 2002; Peterson et al., 2014a; Cunningham et al., 2004) where there is detailed information about PC biosensor and readout system. PC microplates (384-well) were purchased from SRU Biosystems, Inc. The Biomolecular Interaction Detection system (BIND; SRU Biosystems, Inc.) was used to measure biomolecule interaction on biosensor surface (i.e. antibody interactions with antigen). The detection instrument illuminates the PC with a broadband light source (λ range 400-700 nm) that provides collimated light at normal incidence via an optical fiber positioned below the biosensor microplate. The system contains 8-parallel readout heads, and is capable of measuring the Peak Wavelength Value (PWV) of all wells in a 384-well microplate in ~10 seconds. For all the experiments presented using the PC biosensor, the quantities are reported as PWV shifts in units of wavelength (nm).

**Transmission Electron Microscope**

Philips CM200 transmission electron microscope (TEM) was used to take images of IONs. The in-house procedure revised in 2006 by Jakstys and Klintsova was followed to take TEM images TEM (Jakstys and Klintsova, 2006). Images were taken of functionalized IONs with antibody (fAb-IONs), sTfR-fAb-IONs complexes before magnetic separation and sTfR-fAb-IONs complexes after magnetic separation.

**Preparation of PC biosensor.**

**Epoxy-silanization of PC biosensor surface.**

PC biosensor surface was functionalized similarly as previously described. Briefly, each well was incubated for 1 h at 23°C in a solution of 0.1 M NaOH, before sonication (Fisher Scientific Isotemp202 Heater Ultrasonic bath) for 15 min. After aspiration and blotting, wells
received 15 µL of 2.5% GTPMS and 10 mM acetic acid in ethanol solution and were left to incubate for 1 h at 23°C. Finally, wells were aspirated and washed twice with ethanol and dried under nitrogen stream before they were ready to be assayed.

*Capture monoclonal Antibody immobilization.*

An aliquot of 15 µL at 40 µg/mL of capture anti-sTfR antibodies was dispensed into all epoxy-silanized wells. The PC microplate was sealed with tape (Pierce, Thermo Fisher Scientific) and left at 23°C for 5 h. Then, wells were washed with PBS three times. The PWV corresponding to capture antibody immobilized on sensor surface was measured relative to baseline.

*Blocking step.*

Next, an aliquot of 20 µL undiluted StartingBlock was pipetted into all active and negative control wells. PC microplates were incubated for 45 min at room temperature and then washed with PBS three times. The PWV shift was measured relative to baseline. At this step, the PC sensor is ready to use in the SA to bind sTfR, or in the IA to bind complexes of fAb-IONs and sTfR.

*Preparation of functionalized iron-oxide nanoparticles (fAb-IONs).*

Detection antibodies were functionalized (fAb) to iron-oxide nanoparticles (30 nm) by the vendor (Ocean NanoTech, LLC) and described in a previous study without modifications (Peterson et al., 2014a; Xu et al., 2011). Conjugation was verified by gel electrophoresis from Ocean NanoTech (data not shown). Conjugations are expressed as mg of Fe per mL. TEM images were taken after fAb-IONs functionalization.

*sTfR detection using standard sandwich assay.*
In this assay the antigen was added and incubated with the capture antibody in the PC biosensor first, and then, the fAb-IONs were added to create the sandwich (Figure 15A). For this, five concentrations (0.01-0.2 µg/mL) of sTfR (15 µL) were directly applied to the PC biosensor and their respective PWV was measured relative to the PWV of the previous blocking step to develop a standard curve. After 20 min, 15 µL of fAb-IONs at a concentration of 0.0241 mg Fe/mL were added to each well and data on binding kinetics were collected for 20 hours, which allowed each well to reach steady-state binding conditions.

*sTfR detection using inverse sandwich assay.*

In the IA, the antigen-containing sample and fAb-IONs were mixed first in a different tube, then after magnetic separation, removal of supernatant and resuspension in buffer, the complex was added and incubated with the capture antibody on the PC biosensor (Figure 15B). The mixture of fAb-IONs and sTfR was incubated at room temperature (23°C) on a shaker (400 rpm) for 1 h before magnetic separation was applied using the SuperMag Multitube Separator™ (Ocean NanoTech) for 1 h in 1.5 mL micro-centrifuge tubes. Specific reconstitution and concentration information of fAb-IONs and sTfR for each experiment are described below. TEM images were taken before and after magnetic separation to compare aggregation.

*Optimization of fAb-ION concentration for removal of sTfR and binding ratio determination.*

fAb-IONs were serially diluted in BioVendor dilution buffer to final concentrations of 0.0625, 0.03125, and 0.015625 mg Fe/mL. These concentrations were chosen based on previous binding data from the SA in which a linear dose-response curve was established using 0.0241 mg Fe/mL. BioVendor sTfR standards were diluted to final concentrations of 0.2, 0.3, 0.4 and 0.5 µg/mL and assayed with each of the three aforementioned fAb-IONs concentrations. The highest sTfR concentration (0.5 µg/mL) was chosen because it is near the cut-off level of sTfR for iron
deficiency (Mei et al., 2012; Phiri et al., 2009) in serum when diluted 1:10. This is also the upper limit of the IA linear range. During the magnetic separation of each treatment, after the fAb-IONs pellet was attracted to the magnetic side of the tube and the solution was transparent, the supernatant was removed. Then, it was diluted 2.5 times before sTfR analysis with the BioVendor ELISA. Diluting 2.5 times was necessary to reach sufficient assaying volume for the ELISA. The sTfR amount in the supernatant was used to calculate extraction recovery using the fAb-IONs. Binding ratios were calculated in terms of sTfR:fAb-IONs molecules. The molar concentration of fAb-IONs was provided by Ocean NanoTech and the molecules of sTfR were calculated by using the molecular weight (85 kDa) of sTfR (Speeckaert et al., 2010).

Determination of inaccuracy and bias in the analysis of sTfR from Liquichek™ control sera.

To quantify sTfR in three Liquichek™ control sera, the fAb-IONs were diluted to a final concentration of 0.25 mg Fe/mL before incubation with all samples on the IA. The SA used a final concentration of fAb-IONs at 0.0241 mg Fe/mL to measure all samples. All other parameters described below remained constant for both the IA and SA on the PC biosensor. BioVendor standard concentrations (0.01-0.5 µg/mL) were used to develop the standard curve on the PC biosensor and determine actual concentrations of sTfR in Liquichek™ control sera. Liquichek™ control sera were diluted 25 times to total protein concentrations of approximately 2.0, 3.0, 4.0 mg/mL for Liquichek™ 1, 2 and 3, respectively. These protein concentrations are based on data provided by Bio-Rad Laboratories, Inc. Dilutions at 1:25 were necessary to bring sTfR concentration in Liquichek™ sera to within the linear range of both assay platforms. Two commercial ELISAs were used to determine the reference concentrations of sTfR in the Liquichek™ sera. During magnetic extraction in the IA the supernatant was removed and discarded, and the pellet containing sTfR complexed to fAb-ION was resuspended in PBS to
original volume before analysis on the PC biosensor. Inaccuracy and bias from measuring the Liquichek™ sera was quantified using difference plots (Petersen et al., 1997) for both PC biosensor assay types. These parameters were determined by comparing the IA and SA of the PC biosensor to the average sTfR concentration measured from the two reference ELISA methods. The inherent imprecision of the test methods was calculated following a previously published equation (Petersen et al., 1997):

\[
\sigma^2(\delta) = \sigma_T^2 + \sigma_R^2 \tag{Eq8}
\]

where \([\sigma_T^2]\) is the variance of either test method (i.e. IA and SA), \([\sigma_R^2]\) is the variance of the reference methods (i.e. ELISAs) and \([\sigma^2(\delta)]\) is the total inherent imprecision of the test and reference methods. Briefly, constant analytical standard deviations are presumed, and thus it is equal to \(\sigma_T + \sigma_R\). When the two methods are identical, it is expected that 68% of differences will be distributed around 0 between \(0 \pm 1\sigma(\delta)\) and 95% of differences will be between \(0 \pm 2\sigma(\delta)\).

**Determination of precision and limit of detection of PC platform and commercial ELISAs.**

Imprecision was determined on each platform by measuring Liquichek™ control serum 2 in replication experiments. Intra-assay imprecision was measured using triplicates for each experiment. Inter-assay imprecision was calculated using data from two experiments with either duplicates or triplicates. Limit of detection (LoD) was also determined as described previously (Armbruster and Pry, 2008).

**4.3. Results and Discussion**

*Determination of fAb-IONs concentration to achieve maximum sTfR removal and binding ratios on inverse sandwich assay.*
A 4x3 factorial design experiment was used to evaluate the ability of fAb-IONs in binding sTfR in buffer, where four concentrations of sTfR were paired with three concentrations of fAb-IONs. After evaluation of supernatants using ELISA, the highest concentration of fAb-IONs (0.0625 mg Fe/mL) recovered >89% of all the sTfR levels (Figure 16). The lowest concentration of fAb-IONs (0.0156 mg Fe/mL), however, only recovered 50% of the highest concentration of sTfR (0.5 µg/mL). All these data points had a relative standard deviation (RSD) of less than 4%. It was expected that a higher concentration of fAb-IONs would potentially remove nearly 100% of sTfR from solution. The high sTfR recoveries at 0.0625 and 0.03125 mg Fe/mL supported the experimental data observed from the SA on the PC biosensor, where a linear dose-response to sTfR was created using 0.03125 (not shown) and 0.0625 mg Fe/mL fAb-IONs (Figure 17A, -●-). Thus, fAb-IONs at 0.0625 mg Fe/mL were used to magnetically extract sTfR standards in the IA. Building on the previous data shown in Figure 16, the binding ratios of sTfR:fAb-IONs for each concentration of fAb-IONs and sTfR in the 4x3 factorial design were calculated. The binding ratios ranged from 1:1-2:1, 2:1-4:1, and 3:1-5:1 at 0.0625 mg Fe/mL, 0.03125 mg Fe/mL and 0.01562 mg Fe/mL, respectively, for each of the four concentrations of sTfR. The significance of these binding ratios will be discussed in the next section.
Optimum concentration of fAb-IONs to develop a dose-response curve measuring sTfR using the PC biosensor.

Based on the experimental data described in Figure 16, it was hypothesized that by using the highest concentration of fAb-IONs applied in the recovery experiments (i.e. 0.0625 mg Fe/mL) to magnetically extract a range of increasing concentrations of sTfR standards in the IA would lead to a similar linear dose-response as obtained in the SA. However, when three concentrations of sTfR (i.e. 0.05, 0.1 and 0.2 µg/mL) were assayed using 0.0625 mg Fe/mL of fAb-IONs in the IA, no linear dose-response curve was obtained (Figure 17A,■□).
Thus, to optimize the concentration of fAb-IONs necessary to produce a linear dose-response curve in the IA, one concentration of sTfR (i.e. 0.1 µg/mL) was assayed with six serially diluted concentrations of fAb-IONs (0.024 - 0.5 mg Fe/mL). Figure 17B shows the binding characteristics of sTfR in the IA, where increased concentrations of fAb-IONS elicited higher
PWV responses. Responses did not follow a linear pattern. At the lower end, from 0.024 to 0.1 mg Fe/mL, PWV increments were steady but low. At higher fAb-IONS concentrations, increments in PWV were larger. The evaluation of 0.25 mg Fe/mL fAb-IONS in the IA resulted in a linear dose-response curve (Figure 17A, -□-). Therefore, although 0.0625 mg Fe/mL fAb-IONS removed >89% of several sTfR concentrations (0.1-0.5 µg/mL), this was not optimal for development of a linear dose-response curve, and thus, a higher concentration of fAb-IONS was needed.

These results, although perplexing, can be explained based on the nature of the fAb-IONS and the differential binding of sTfR in either assay type. In the SA, the antigens are added 20 min before the fAb-IONS, which allows time to interact with the capture antibodies immobilized on the sensor surface. As a result, the antigen reaches steady-state binding equilibrium with the capture antibody before the fAb-IONS are added for detection amplification. This configuration enables maximum binding efficiency between the capture antibody and antigens as well as the fAb-IONS and antigens. As a result of this configuration using the SA, despite the difference in fAb-IONS concentrations, a maximum PWV response was reached when measuring the same sTfR concentration albeit with different binding kinetics (Figure 17C). In the IA, however, the antigen are first bound to fAb-IONS forming complexes, and then, magnetically extracted as a whole unit. These sTfR:fAb-ION networks formed due to the aggregation interactions continue even after re-suspension and detection on the PC biosensor. Early complexation between sTfR-fAb-IONS prevented sTfR from binding with the capture antibodies and eliciting a maximum response similar to that observed in the SA prior to adding fAb-IONS (Figure 17C). Nonetheless, as the concentration of fAb-IONS increased, and the ratio of sTfR:fAb-IONS decreased, the response approached the maximum response observed in the SA. At the fAb-IONS concentration
where a linear dose-response curve was elicited (i.e. 0.25 mg Fe/mL), the theoretical binding ratio was calculated to be <1 (less sTfR molecules per fAb-ION), which was in contrast to the lower fAb-ION concentrations shown in the previous section (i.e. 0.0625, 0.03125 and 0.01562 mg Fe/mL) where the binding ratios were >1 (more sTfR molecules per fAb-ION).

Although this binding mechanism is not completely understood, these data suggest a binding ratio <1 leads to a lower amount of sTfR:fAb-ION network formation due to the lesser amount of sTfR per fAb-ION, and thereby, promotes interactions between sTfR and the capture antibody more similar to the SA. TEM images taken after magnetic separation of the sTfR-fAb-IONs complexes and prior to assaying on the PC biosensor did not show more aggregation compared to TEM images of the fAb-IONs alone and sTfR-fAb-IONs complexes before magnetic separation (Figure 18). This lower amount of fAb-ION aggregation is considering that the 30-nm IONs are nearly superparamagnetic and carry a single magnetic domain (Lu et al., 2007). Conversely, at a ratio >1, there is more sTfR per fAb-ION, which is thought to lead to more sTfR:fAb-ION network formation between the sTfR and fAb-IONs, and thus, inhibit the sTfR from interacting with the capture antibodies on the biosensor surface. Therefore, although a lower concentration of fAb-IONs extracted >89% of sTfR through magnetic separation, a high proportion of sTfR remained “hidden” and unable to interact with the capture antibodies. Similar aggregation or network formations were observed when biotinylated IONs interacted with streptavidin in situ under a magnetic field within an SPR biosensor detection system (Lee et al., 2012). Despite these explanations, more studies are needed to examine the exact mechanism causing differential responses observed at 0.25 mg Fe/mL and those concentrations at and below 0.06125 mg Fe/mL.
A linear dose-response curve was obtained with 0.25 mg Fe/mL of fAb-IONs in the IA (Figure 19A). The linear curve represents five concentrations of sTfR, 0.02 - 0.5 µg/mL. Within this range, the sTfR dose-responses were linear, with an R² value of 0.998. The increased PWV kinetic responses as a result of binding with sTfR-fAb-IONs complexes can be explained by the nature of the PC detection system. The PC biosensor is a periodic subwavelength grating structure that can detect the existence of the attached complexes under optical resonance (Chen et al., 2014). The evanescent electric field generated by the resonance has an exponential decaying intensity versus the vertical distance above PC surface, as shown by the following equation:

\[ E(z) = E(0)^{-z/d} \]  

(Eq9)
where \( E(z) \) represents the electric field intensity at a certain distance \( z \) above the PC surface, \( E(0) \) is the intensity at the surface interface, and \( d \) is called the penetration depth, which is also the effective detection range of the PC biosensor (Chen et al., 2014). Eq. 2 indicates that the interaction between the sTfR-fAb-IONs complexes and the PC biosensor is highly dependent on their distance from the surface. In the IA, at 0.25 mg Fe/mL of fAb-IONs, the addition of the sTfR-fAb-IONs complexes in one step allows the complexes to interact with the immobilized capture antibodies and arrange themselves close to the PC biosensor surface, where the electric field is maximized, and thus, producing a strong detection signal. Based on the experimental data, the higher the fAb-IONs concentration in the IA, the greater the response will be as it approaches the maximum steady-state equilibrium of the SA (Figure 17C). As described in the
previous section, however, unless there is a <1 ratio of sTfR to fAb-IONs to amplify the signal, which was determined to be above 0.1 mg Fe/mL, no dose-response could be achieved (Figure 17A and B).

The ability to track kinetic binding on the PC biosensor allows for the development of real-time dynamic binding curves. Figure 19B shows the dynamic binding curves for three sTfR concentrations at one concentration of fAb-IONs (i.e. 0.25 mg Fe/mL) in the IA. Responses in this assay reached equilibrium by 200 min; however, differences among concentrations are significant even within 30 min. As described in Eq. 2, the mechanism for the kinetic binding of the antigen-fAb-IONs complexes involves the mass of these complexes displacing volume in the detectable area of the PC biosensor. Due to the multivalency of the fAb-IONs during magnetic separation, each fAb-ION can bind numerous sTfR (Yang et al., 2008; Saha et al., 2014). The antigen-fAb-IONs complexes with large cross-sections migrated towards the PC biosensor surface at a high velocity, and therefore reaching equilibrium within a short time.

*Inaccuracy of inverse and standard assays on the PC biosensor in measuring sTfR from reference materials.*

The interference/non-specific binding in the measurement of sTfR in a complex matrix (i.e. diluted sera) was evaluated with both assays on the PC biosensor. These experiments showed that the IA was able to extract, detect and quantify sTfR in Liquichek™ sera 1, 2, and 3 with minimal inaccuracy (i.e. bias) compared to the two reference ELISA methods. The average bias for Liquichek™ 1, 2 and 3 was 0.18, 0.19 and -0.04 µg/mL, respectively. This means the IA slightly overestimated the sTfR in two of three Liquichek™ samples compared to the mean of the reference ELISAs. The inherent imprecision of the IA and reference ELISAs was calculated as $\sigma(\delta)=0.45 \mu g/mL$. These data indicated that 68% of the differences between methods should
be between 0 and 0.45 μg/mL and 95% of differences between 0 and 0.90 μg/mL of sTfR.

Although the sample size was small, 83% of points fell within 0 ± 0.45 μg/mL and 100% of points fell between 0 ± 0.90 μg/mL (Figure 20A). Therefore, due to a small bias and inherent imprecision that fits the normal distribution, the IA method does not perform statistically different from the reference ELISA tests.

The SA, however, had a greater bias measuring sTfR in Liquichek™ sera when compared to the reference ELISA methods. As shown in Figure 20B, the biases for Liquichek™ 1, 2 and 3 were 0.66, 0.14 and -0.67 μg/mL, respectively. Consequently, the SA overestimated (positive

**Figure 20.** Difference plots comparing concentrations of sTfR in the IA (A) and in the SA (B) on the PC biosensor to those from commercial ELISAs. For both difference plots, three Liquichek™ control sera (LQC) were measured in either duplicate or triplicate on each platform in two experiments on different days. [Peterson et al., 2015]
bias) the sTfR concentration in Liquichek™ 1 and 2 and underestimated (negative bias) Liquichek™ 3. The inherent imprecision of the SA and reference ELISAs was calculated as $\sigma(\delta)=0.52 \mu g/mL$. As described above, the inherent bias indicates that 68% and 95% of the differences should fall between $0 \pm 0.52$ and $0 \pm 1.04 \mu g/mL$. Although data from Liquichek™ 2 followed this distribution, data from Liquichek™ 1 and 3 had 80% of the replicates with a difference greater than 0.52 µg/mL. Thus, the SA performed statistically different from the reference ELISA tests in measuring sTfR. The bias observed indicated that these samples contained some non-specific component, more likely serum proteins.

**Table 7.** Precision of various methods measuring sTfR in Liquichek™ sera.

<table>
<thead>
<tr>
<th>Sensing Platform</th>
<th>LoD (ng/mL)</th>
<th>Intra-assay %RSD</th>
<th>$n^1$</th>
<th>Inter-assay %RSD</th>
<th>$n^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Sandwich</td>
<td>14</td>
<td>22.2</td>
<td>3</td>
<td>3.9</td>
<td>5</td>
</tr>
<tr>
<td>Inverse Sandwich</td>
<td>21</td>
<td>8.1</td>
<td>3</td>
<td>18.2</td>
<td>6</td>
</tr>
<tr>
<td>BioVendor ELISA</td>
<td>100</td>
<td>1.2</td>
<td>3</td>
<td>12.9</td>
<td>6</td>
</tr>
<tr>
<td>R&amp;D ELISA</td>
<td>553</td>
<td>2.4</td>
<td>3</td>
<td>13.2</td>
<td>5</td>
</tr>
</tbody>
</table>

$^1$Number of replicates in intra-assay experiments.

$^2$Number of total replicates in two inter-assay experiments (i.e. 2-3 replicates per experiments).

Imprecision and limit of detection (LoD) of inverse assay, standard assay, and commercial ELISAs.

The imprecision of the detection platforms were compared to each other through intra- and inter-assay replication studies, measuring sTfR in Liquichek™ 2 serum (Table 7). These data indicated the intra-assay imprecision of the SA and IA on PC biosensor was higher than the commercial ELISA assays measuring sTfR from the reference sample. Despite this, based on the inaccuracy and inherent imprecision shown in the difference plots (Figure 20), the IA performs
similar to both ELISA tests in the quantification sTfR. To put the imprecision of the IA and SA in clinical perspective, based on biological variation data (Bailey et al., 2014), the maximum allowable random error (i.e. standard deviation) for sTfR is 0.23 µg/mL for intra-assay and 0.30 µg/mL for inter-assay at a sTfR cut-off concentration of 5.3 µg/mL. Experimentally the SDs of the SA for the intra- and inter-assay replication studies were 0.19 and 0.13 µg/mL, respectively. Likewise, in the IA, these were 0.12 and 0.23 µg/mL, respectively. Therefore, for both assays the SDs were within the total allowable error for sTfR, indicating that the random error observed has no clinical significance when measuring sTfR on the PC biosensor. The inter-assay imprecision of the SA was small due to interference that falsely homogenized the responses. The LoD of the IA and SA were lower than those from the reference ELISAs (Table 7). Due to the IONs amplification in the PC biosensor assays, the LoD on this platform is 5-fold to 40-fold lower than the commercial ELISAs. The low LoD of the PC biosensor is particularly useful when measuring small quantities of biomolecules in blood. In the case of sTfR, the low LoD is not as beneficial because in an iron-deficient individual, normal circulating sTfR concentrations increase above the cut off of 5.3 µg/mL. Therefore, the upper detection limit of the linear range (i.e. 0.5 µg/mL) is more important to evaluate the utility of this assay when assessing sTfR status. Regarding the relevance of this upper detection limit in the IA, a simple 1:10 sample dilution would be needed to determine whether the individual is above or below the cutoff; in which a value greater than 0.5 µg/mL after dilution would indicate deficiency, whereas a value less than 0.5 µg/mL would indicate sufficiency. Accurate diagnosis of IDA requires the use of at least two biomarkers, such as ferritin and sTfR. Unlike sTfR, ferritin levels can fall below 30 ng/mL in IDA individuals. Thus, a low LoD achieved through the IA method could help isolate, detect and measure this biomarker as well as we have shown previously (Peterson et al., 2014a).
4.4. Limitations.

Despite these encouraging data, there are areas of further research necessary to continue the technological process development. The optimization of functional concentrations of several assay components is required to maximize its performance. It is known that EDC-NHS functionalization used in this study to immobilize antibodies to IONs results in random orientation. Future studies will test Protein A functionalization or streptavidin functionalization to improve antibody orientation on the IONs that could potentially improve antigen detection (Soelberg et al., 2009). Furthermore, these studies require confirmation using sera from a larger sample of subjects representing populations with normal and deficient levels of sTfR. In terms of point-of-care amenability, more studies are needed to test the smartphone platform developed for the PC biosensor (Gallegos et al., 2013), and test microfluidic concepts that may improve the extraction of biomarkers from biological samples. Regarding assay time, future optimization studies are needed to determine the optimal time need for antigen-fAb-IONs binding before magnetic separation as well the optimal time needed for magnetic separation.

4.5. Conclusion

A detection protocol (inverse sandwich assay) that combines magnetic separation of a protein biomarker of iron deficiency with immuno-probes and their detection using a PC biosensor was established. The IA was able to extract sTfR from buffer and serum, elicit a linear standard curve, and perform similarly to two certified ELISAs in terms of precision and bias in the quantification of sTfR from three control sera. This agreement in quantifying sTfR in sera suggests the IA assay removes the interfering compounds that cause non-specific binding in the SA. Although, the SA was capable of reaching higher sTfR responses on the PC biosensor from
lower concentrations of fAb-IONS, it tended to overestimate (positive bias) sTfR at low concentrations and underestimate (negative bias) at high concentrations, reducing its diagnostic potential. While further optimization is needed, the experimental protocol presented herein provides a simple process whereby biomarkers are magnetically separated from a complex matrix to remove non-specific binding signals; a problem often associated with biosensors’ performance at the point of care. Future studies will examine how pre-concentration of samples after magnetic separation can improve sensitivity, and ultimately, the usefulness of this assay when measuring biomarkers at low physiological concentrations.
4.6. References


5.1. Introduction

Anemia is suspected in all chronic kidney disease (CKD) patients who are undergoing hemodialysis (National Kidney Foundation, 2006). A functional or absolute iron deficiency is estimated in 25-38% of CKD patients with anemia (National Kidney Foundation, 2001). Despite administration of recombinant erythropoietin (EPO), a hormone produced in the kidneys to stimulate red blood cell production, less than one third of hemodialysis patients with CKD achieve target hemoglobin levels of 11 to 12 g/dL (Foret, 2002). As a result, CKD patients with iron deficiency anemia (IDA) suffer secondary consequences leading to an increased risk of cardiovascular disease (Foley et al., 1996), cognitive impairments (Wolcott et al., 1989), and fatigue and mortality rates (Locatelli et al., 1998). To improve the efficacy of iron treatment, intravenous (IV) iron treatments began in the early 1990’s because they decreased the cost of EPO treatments to increase red blood cell levels (Fishbane, 2013) and improved other quality of life measures (Evans et al., 1990).

Bone marrow aspirates are the gold standard for determining iron status; however, the invasiveness of this procedure limits widespread application (Phiri et al., 2009). Therefore, measuring protein biomarkers (i.e. ferritin and soluble transferrin receptor) offers the most practical route to diagnose IDA in hemodialysis patients. IDA in CKD patients is diagnosed based on a protein biomarker and a relationship of biomarkers: serum ferritin, and transferrin saturation (TSAT), respectively (Chung et al., 2012). Ferritin is large spherical protein that stores iron atoms within a cage-like structure (Finazzi and Arosio, 2014), while TSAT is a non-protein
biomarker that reflects the iron available for erythropoiesis. However, these biomarkers do not always deliver dependable results when working with CKD patients undergoing hemodialysis due to its inflammatory nature (Chung et al., 2012).

Another IDA protein biomarker of interest is soluble transferrin receptor (sTfR). sTfR is a truncated protein found in the serum that originates from the transferrin receptor protein found within membranes of erythroid tissues (Feelders et al., 1999). The advantage associated with measuring sTfR is its stability during episodes of acute and chronic inflammation (Speeckaert et al., 2010). Despite the potential usefulness of measuring sTfR in hemodialysis patients to assess for IDA, previous studies have not provided sufficient evidence showing whether or not sTfR offers any advantage when attempting to diagnose IDA (Bovy et al., 2007; Tessitore et al., 2001).

The primary diagnostic platform employed to measure protein biomarkers of IDA (i.e. ferritin and soluble transferrin receptor) is the enzyme-linked immunoassay (ELISA) (Erhardt et al., 2004). ELISAs have high sensitivity due to the horseradish peroxidase reaction with a substrate that develops color and amplifies the signal detectable by a spectrophotometer. Although ELISAs are mainstream diagnostic tools, they are expensive, require laboratory equipment and trained personnel to run experiments. As an alternative, optical biosensor technologies provide a facile mechanism of detection by harnessing the properties of light to achieve accurate and precise IDA biomarker detection (Peterson et al., 2014a; Peterson et al., 2015).

A type of optical biosensor that has been developed to detect biomolecules is a photonic crystal (PC) biosensor. PCs are periodic subwavelength grating structures that reflect a narrow band of wavelengths when illuminated by a broadband light source (Cunningham et al., 2002) at
the resonance conditions. Biomolecule binding (e.g. proteins) within ~200 nm of the biosensor surface increases the refractive index of the resonance mode and causes a shift of peak wavelength value (PWV) to a higher value. In addition, there exists extensive literature demonstrating the PC biosensors utility in biomolecule detection ranging from cells to viruses and pharmaceuticals (Chen et al., 2013; (Heeres and Hergenrother, 2002; Mathis et al., 2010; Shafiee et al., 2014). Recent studies measuring biomarkers of IDA have aimed at validating the PC biosensor as a diagnostic tool for IDA with the hope of applying a point-of-care diagnostic device employing a smartphone detection instrument (Gallegos et al., 2013).

Preliminary findings have shown that the PC biosensor is capable of producing dependable standard curves using a sandwich assay format to detect ferritin (SA) (Peterson et al., 2014a) and an inverse sandwich assay format (IA) to detect sTfR (Peterson et al., 2015). The advantage of the IA over the SA is that the IA decreases interference signals caused by non-specific binding of proteins in a serum matrix. This might be useful to evaluate the serum from CKD patients, as it is known to contain high levels of interference molecules due to the inability of the kidneys to effectively excrete endogenous (Choi et al., 2011; Zhao, 2013) and exogenous compounds (Pichette and Leblond, 2003). Therefore, it was hypothesized the IA would be able to effectively remove ferritin and sTfR from interference molecules in sera from CKD patients and allow for detection of these biomarkers on the PC biosensor with similar inaccuracy and imprecision as FDA-certified ELISAs. The IA uses a magnetic separation protocol where iron-oxide nanoparticle functionalized with antibodies (fAb-IONs) act as immuno-probes to selectively bind antigens before being removed from the remaining serum protein matrix by magnetic separation. Preliminary validation experiments quantifying inherent analytical imprecision of the IA demonstrated that when measuring sTfR in Liquichek™ control serum,
there is no statistical difference to the results of an FDA-certified in-vitro diagnostic ELISA (Peterson et al., 2015).

Therefore, the present study was carried out to evaluate the utility of the IA on the PC biosensor for the determination of ferritin and sTfR in maintenance hemodialysis patients and its ability to distinguish individuals with IDA. The evaluation process was done by systematically quantifying the total analytical error of the IA on the PC biosensor and using FDA-certified ELISAs as the reference methods. The use of sera from hemodialysis patients offered an extreme scenario for validation of the PC platform as the stability of fAb-IONs might be compromised by highly variable amounts of proteins and other biological metabolites that may interfere with biomarker extraction and detection.

5.2. Materials and Methods

Reagents.

3-glycidoxy-propyl-trimethoxy-silane (GTPMS), and NaOH were purchased from Sigma-Aldrich; phosphate buffer saline (PBS), and StartingBlock blocking buffer from Pierce (Thermo Fisher Scientific). Double deionized water (DDW) was used in all experiments.

2.2. ELISA kits, antigens and antibodies (Ab).

Human sTfR ELISA kits were purchased from R&D Systems (DTFR1) and human ferritin ELISA kits were purchased from Alpco (25-FERHU-E01). Human serum samples were collected from hemodialysis patients all of whom had end-stage renal disease (CKD stage 5). The monoclonal mouse anti-human sTfR was used as the capture antibody in the IA and was purchased from Abcam® (ab38168). A monoclonal mouse anti-human sTfR antibody was the detection Ab in the IA and was purchased from Abcam® (ab10249). The monoclonal mouse anti-human liver ferritin was used as the capture antibody in the IA and was purchased from US
Biological (F4015). The detection antibody was polyclonal goat anti-human liver ferritin that was purchased from US Biological (F4015-17). Iron-oxide nanoparticle conjugation kit was carried out by the IONs manufacture: Ocean NanoTech. World Health Organization (WHO) reference reagent of ferritin (94/572) was used to develop the standard curves to measure ferritin in the hemodialysis sera. Standards of sTfR were purchased from BioVendor™ and measured to make the standard curves to quantify sTfR in hemodialysis sera. These standards were calibrated with WHO recombinant soluble transferrin receptor rsTfR reference reagent (Peterson et al., 2014b).

**PC biosensor and readout system.**

Readers are referred to Cunningham et al., 2002; Peterson et al., 2014a; Cunningham et al., 2004) where there is detailed information about PC biosensor and readout system. PC microplates (96-well) were purchased from SRU Biosystems, Inc. The Biomolecular Interaction Detection system (BIND; SRU Biosystems, Inc.) was used to detect biomolecule interaction on biosensor surface (i.e. antibody interactions with antigens). The detection instrument illuminates the PC with a broadband light source (λ range 400-700 nm) that provides collimated light at normal incidence via an optical fiber positioned below the biosensor microplate. The system contains 8-parallel readout heads, and is capable of measuring the Peak Wavelength Value (PWV) of all wells in a 96-well microplate in ~10 seconds. For all the experiments presented using the PC biosensor, the quantities are reported as PWV shifts in units of wavelength (nm).

**Preparation of PC biosensor.**

**Epoxy-silanization of PC biosensor surface.**

PC biosensor surface was functionalized similarly as previously described (Peterson et al., 2014a). Briefly, each well was incubated for 1 h at 23°C in a solution of 0.1 M NaOH, before
sonication (Fisher Scientific Isotemp202 Heater Ultrasonic bath) for 15 min. After aspiration and blotting, wells received 30 µL of 2.5% GTPMS and 10 mM acetic acid in ethanol solution and were left to incubate for 1 h at 23°C. Lastly, wells were aspirated and washed twice with ethanol and dried under nitrogen stream before being rinsed with PBS for assay preparation.

Capture monoclonal antibody immobilization.

An aliquot of 30 µL at 40 µg/mL of capture anti-sTfR antibodies was dispensed into all epoxy-silanized wells. Likewise, for ferritin a 100 µg/mL aliquot of capture anti-ferritin antibodies was pipetted into the epoxy-silanized wells. The PC microplate was sealed with tape (Pierce, Thermo Fisher Scientific) and left at 23°C for 5 h. Then, wells were washed with PBS three times. The PWV corresponding to capture antibody immobilized on sensor surface was measured relative to baseline.

Blocking step.

Next, an aliquot of 30 µL undiluted StartingBlock was dispensed into all active and negative control wells. PC microplates were incubated for 45 min at room temperature and then washed with PBS three times. The PWV shift was measured relative to baseline. At this step, the PC sensor was ready detect the complexes of fAb-IONs and its respective antigen.

Preparation of functionalized iron-oxide nanoparticles (fAb-IONs).

Detection antibodies were functionalized (fAb) to iron-oxide nanoparticles (30 nm) by the vendor (Ocean NanoTech, LLC) and described in a previous study without modifications (Peterson et al., 2014a; Xu et al., 2011). Conjugation was verified by gel electrophoresis from Ocean NanoTech (data not shown). Conjugations are expressed as mg of Fe per mL.

sTfR and ferritin detection using inverse sandwich assay.
In the IA, the hemodialysis patients’ serum samples and fAb-IONs were mixed first in a 1.5-mL micro centrifuge tube before magnetic separation. After being mixed thoroughly, the samples were incubated at room temperature (23°C) on a shaker (400 rpm) for 1 h. Then, the tubes were placed in a SuperMag Multitube Separator™ (Ocean NanoTech) for 1 h until complexed fAb-IONs-antigen formed pellets along the side of the microcentrifuge tube. The serum supernatant was then aspirated and the pellet was constituted with PBS buffer. Finally, the reconstituted samples were assayed onto the PC biosensor in a 96-well microplate format. The samples were read on the BIND instrument once they reached steady-state equilibrium between three and four hours. Due to the components of some patients’ serum samples, the fAb-IONs formed aggregates in solution that could not be reconstituted with PBS in a stable colloidal solution. These samples were disregarded in the analysis.

**Difference plots and comparison studies of PC biosensor with reference ELISAs**

Difference plots were constructed to determine if the IA method on the PC biosensor measured each biomarker statistically different from the ELISA method. The approach of making difference plots stems from earlier work done on calculating inherent imprecision (Petersen et al., 1997). Total inherent imprecision is described in the following equation:

\[
\sigma^2(\delta) = \sigma^2_T + \sigma^2_R \tag{Eq9}
\]

where \([\sigma^2_T]\) is the variance of the test method (i.e. IA), \([\sigma^2_R]\) is the variance of the reference method (i.e. ELISA) for each biomarker, and \([\sigma^2(\delta)]\) is the total inherent imprecision of the test and reference methods together. The null hypothesis is that the measured differences for all samples are zero. Furthermore, the acceptable limits are defined by the inherent analytical imprecisions (Petersen et al., 1997). Because constant analytical standard deviations are presumed, they equal \(\sigma_T + \sigma_R\). When the two methods are identical, it is expected that 68% of
differences will be distributed around $0 \pm 1\sigma(\delta)$, and 95% of differences will be distributed between $0 \pm 2\sigma(\delta)$.

Next, a paired $t$-test and an $F$-test were performed to quantify the systematic error (i.e. bias) and random error (i.e. standard deviation of the PC biosensor compared to the ELISA. Mean differences (i.e. bias) were evaluated by a paired $t$-test and the distribution of differences (i.e. standard deviation of differences) by an $F$-test. Although least-squares regression analysis is the preferred analysis to compare methods and determine bias over a wide analytical range, paired $t$-test statistics were used because the correlation coefficient ($r$) obtained from the results of the IA and ELISA was less than 0.99 and the concentrations of ferritin and sTfR did not have a wide analytical range (Westgard, 2008). The correlation coefficient expresses how well the results of both methods change with each other. A correlation coefficient of less than 1.0 indicates the data points were scattered around the line of best agreement, where the lower the $r$, the higher the degree of scattering. Although the correlation coefficient has been used incorrectly to describe whether a method is acceptable or not, its main function is to determine how reliable regression calculations are. In the case that $r$ is less than 0.99, the estimates of the $y$-intercept (i.e. bias) and slope (proportional error) are not reliable, and thus, it is better to use paired $t$-tests for calculations.

The paired $t$-test was carried out to determine if bias existed on the PC biosensor compared to the reference ELISA. In the paired $t$-test, three statistics are calculated, bias, standard deviation of differences, and the $t$-value. The bias was calculated using the following equation:

\[ \text{bias} = \bar{y} - \bar{x} \]  

(Eq10)

where $\bar{y}$ is the mean of ELISA method and $\bar{x}$ is the mean of PC biosensor. The standard deviation of the differences is calculated with the equation below:
Finally, the \( t \)-value is calculated by the following equation:

\[
t = \frac{\text{bias}}{SD_{diff} / \sqrt{N}}
\]  

(Eq12)

This equation shows the expression of the \( t \)-value as a ratio of the systematic error (bias) and the random error (standard deviation) (Westgard, 2008). In other words, the \( t \)-value expresses the systematic error in multiples of random error (i.e. \( SD_{diff} \)). For example if the \( t \)-value is 3, this means the systematic error term is three times larger than the random error term. The null hypothesis (\( H_0 \)) is that for each biomarker determination there is no statistical difference between systematic error of the PC biosensor and the ELISA. Conversely the alternative hypothesis (\( H_a \)) is that there is not sufficient evidence to accept the \( H_0 \) and state there is no statistical difference between the systematic error of the PC biosensor and the ELISA.

Following the \( t \)-test, an \( F \)-test was conducted. \( F \)-tests in method validation studies are used to compare the variance of the test method (PC biosensor) against the variance of the reference method (ELISA). Variance is the square of the standard deviation. The \( F \)-test will show if there is a difference between the variances of both methods. The following equation was used to perform an \( F \)-test.

\[
F = \frac{(s_1)^2}{(s_2)^2}
\]  

(Eq13)

Where \( s_1 \) is the variance of the more imprecise method and \( s_2 \) is the variance of the less imprecise method. The null hypothesis is that there is no difference (\( H_0 \): \( F \)-test = 1) between the variances of the PC biosensor and the ELISA. If the calculated \( F \)-value is greater than critical \( F \)-value (obtained from the \( F \) distribution), then the null hypothesis is rejected and a difference between variances is present at a given degrees of freedom and probability. This means, the
larger variance from the method in the numerator (i.e. PC) of the $F$-test equation has a statistically significant larger error than the variance of the method in the denominator (i.e. ELISA). If the calculated $F$-value is less than the critical $F$-value, then the null hypothesis cannot be rejected and there is no difference between the variance of both methods. In summary, the paired $t$-test is used to evaluate differences in systematic error or inaccuracy of methods, and the $F$-test is used to evaluated differences in random error or imprecision.

The total analytical error of the PC biosensor was calculated based on the bias (i.e. systematic error) and the standard deviations (random error). The total analytical error was calculated using the following equation:

\[
TE_{\text{calc}} = SE + RE \tag{Eq14}
\]

\[
TE_{\text{calc}} = \text{bias}_{\text{meas}} + 2s_{\text{meas}} \tag{Eq15}
\]

where $TE_{\text{calc}}$ is the total calculated analytical error of PC biosensor; $SE$ is the systematic error (i.e. measured bias), and $RE$ is the random error (i.e. standard deviation). The $TE_{\text{calc}}$ is then compared to an established and agreed upon total allowable error ($TE_a$). The $TE_{\text{calc}}$ included a factor of two to inflate random error in Equation (7). This is because a method that does not fall within the $TE_a$ when multiplying the standard deviation by two is unacceptable (Westgard, 1995). Comparison against a $TE_a$ is useful to determine whether the performance of the PC biosensor is within the acceptable limits of error (Westgard, 2008). $TE_a$ is estimated based on within-individual and between-individual biological variation and clinical significance (Fraser, 2015; Perich et al., 2015). It is worth noting that the $TE_a$ can either be expressed as an absolute concentration, as a percentage of a clinical relevant cutoff, as an average of data, or as a range determined by a survey group (U.S. DHSS, 1992). For both IDA biomarkers, the $TE_a$ has been
estimated as a percentage of a clinical relevant concentration (i.e. sTfR) or average of data (i.e. ferritin).

5.3. Results and Discussion

Measured means, standard deviations, coefficient of variation and range of sample concentrations.

Table 8 shows the means, standard deviations, coefficient of variations (CV) and range of measurements when measuring sTfR and ferritin in the sera from CKD patients on each analytical platform. The means did not differ significantly for measuring either sTfR or ferritin when comparing the IA on the PC biosensor (sTfR: 1.21 µg/mL; ferritin 279.5 ng/mL) and the ELISAs (sTfR: 1.30 µg/mL; ferritin: 276.8 ng/mL). The concentration ranges of sTfR and ferritin found in this study are similar to those found in others (Beerenhout et al., 2002; Buttarelo et al., 2010; Gupta and Pawar, 2009; Tessitore et al., 2001). Furthermore, the standard deviations and CV were not substantially different albeit, the ELISAs (sTfR CV: 11.2%; ferritin: 4.1%) had less imprecision than the IA (sTfR CV: 12.8%; ferritin CV: 8.5%). These statistics, however, do not provide sufficient information to determine if the IA on PC biosensor is a reliable diagnostic platform. It is more important to look at the individual differences measured between the PC biosensor and ELISA for each sample in method validation studies instead of the overall means, standard deviations and CV. The ranges indicated the ELISAs had wider analytical ranges compared to the IA on PC biosensor, which might be indicative of other differences in measurement not apparent in the aforementioned statistics. To determine the differences of an individual sample measured on each platform, more statistical analyses were needed to determine if the IA on the PC biosensor measured without any statistical differences compared to the reference ELISAs.
### Table 8. Means, Standard Deviations, Coefficient of Variations and Range of Measurements

<table>
<thead>
<tr>
<th>Sensing Platform</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
<th>Range of Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTfR PC Biosensor&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21</td>
<td>0.16</td>
<td>12.8</td>
<td>0.62-1.93</td>
</tr>
<tr>
<td>sTfR R&amp;D ELISA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.30</td>
<td>0.15</td>
<td>11.2</td>
<td>0.82-2.47</td>
</tr>
<tr>
<td>Ferritin PC Biosensor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>279.5</td>
<td>23.7</td>
<td>8.5</td>
<td>56-414</td>
</tr>
<tr>
<td>Ferritin Alpco ELISA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>276.8</td>
<td>11.3</td>
<td>4.1</td>
<td>0-504</td>
</tr>
</tbody>
</table>

<sup>a</sup>sTfR measurements are in µg/mL for both platforms  
<sup>b</sup>ferritin measurements are in ng/mL for both platforms

**Difference plots comparing mean differences when measuring ferritin and sTfR on the PC biosensor and reference ELISAs**

Using Equation (15), the mean bias of measuring serum ferritin with the IA on the PC biosensor was 7 ng/mL when compared to the ferritin ELISA. These results for ferritin can be better visualized in the ferritin difference plot (Figure 21B). Representative calibration curves are shown for both the PC biosensor IA and ELISA formats for each both ferritin and sTfR in Figure 22. When measuring ferritin, the inherent imprecision ($\sigma(\delta)$) of both diagnostic methods was 25 ng/mL using Equation (9). As described above, the inherent imprecision indicates the range in which the mean differences must fall within order to fail to reject the null hypothesis that there are no differences between methods. That is, 68% and 95% of the differences must fall between 0±25 and 0±50 ng/mL, respectively. The actual distribution of mean differences was 43% at 0±1$\sigma(\delta)$ and 68% at 0±2$\sigma(\delta)$. In this case, based on the inherent analytical imprecision of measuring ferritin, the null hypothesis was rejected because the distribution significantly differed from what was expected of 68% and 95% at 0±1$\sigma(\delta)$ and 0±2$\sigma(\delta)$, respectively.
A similar analysis for sTfR using Equation (15) resulted in an average bias of 0.09 µg/mL when comparing the IA on the PC biosensor against the ELISA. The inherent imprecision of the IA and sTfR ELISA was $\sigma(\delta)= 0.23$ µg/mL (Figure 21A). Therefore, these data denote that 68% of the differences between the IA and sTfR ELISA should fall between 0 and 0.23 µg/mL and 95% of differences between 0 and 0.46 µg/mL of sTfR to fail to reject the null hypothesis. The actual distribution was 62% at 0±1$\sigma(\delta)$ and 96% at 0±2$\sigma(\delta)$. Therefore, based on the inherent imprecision of both methods, the null hypothesis cannot be rejected because the distribution does not significantly differ from what was expected at 0±1$\sigma(\delta)$ and 0±2$\sigma(\delta)$. 
Figure 21. Difference plots comparing measured concentrations of sTfR (A) and ferritin (B) using the IA on the PC biosensor to those from commercial ELISAs. The measured samples were from hemodialysis patients and were run in triplicates.
Figure 22. Standard curves using the IA on the PC biosensor for A) ferritin and B) sTfR. Standard curves using an Alpco ELISA to measure C) ferritin and an R&D Systems ELISA to measure D) sTfR
Determination of systematic error and random error

Next the statistical differences in bias and random error were calculated. Least squares linear regression statistics were not used to evaluate differences because the comparison plots used to associate measured values for each of the IDA biomarkers from each detection platform (Figure 23) showed the correlation coefficient (Pearson moment correlation, r) value was less than 0.99. This is probably due to the limited analytical range of samples used in this study. The comparison plots (Figure 23) showed the spread of data points and the range of concentrations measured for each biomarker using both analytical methods. These plots were essential to determine whether regression statistics would be the best approach to quantifying the analytical error or if paired t-test and F-tests would be the best. Because, r was less than 0.99, paired t-test and F-tests were the best. Furthermore, these plots assisted in the identification of those hemodialysis patients with CKD that had sufficient iron status based on each biomarker.

A paired t-test was calculated for both biomarkers using Equation (12). Critical values of t were selected at p=0.05 and N-1 degrees of freedom for each biomarker using a t-table. The calculated t-value for sTfR was 2.23 compared to the critical t-value of 2.05 (p<0.05). Thus, the null hypothesis was rejected showing that there was a difference between the two means and significant systematic error was observed. For ferritin, the calculated t-value was 0.87 compared to the critical t-value of 2.06 (p>0.05). Thus, there is not sufficient evidence to reject the null hypothesis that there is no difference between the two means. Despite the empirical usefulness of running t-tests to determine whether a difference exists between the two means of the IA and the ELISA, these results can be misleading if not interpreted correctly. Referring back to Equation (12) when rearranged (see below) it is observed that a large $SD_{diff}$ can lead to a small t-value (e.g. in the case of ferritin). Likewise, a large bias term or $\sqrt{N}$ term can lead to large t-values.
Therefore, it is important to consider the clinical significance of these errors when diagnosing patients, not solely whether systematic error exists. In the case of ferritin, a large $SD_{diff}$ made the $t$-value small.

**Figure 23.** Comparison plots showing the analytical range and correlation coefficient of sTfR (A) and ferritin (B) measured in sera from hemodialysis patients.
\[ t = \text{bias} \frac{\sqrt{N}}{S_{\text{diff}}} \]  

(Eq12)

An F-test was conducted for both biomarkers using Equation (13) to determine random error. Critical values of \( F \) were selected at \( p=0.05 \) and \( N-1 \) degrees of freedom for the denominator and numerator for each biomarker using a \( F \)-table. The variance (i.e., standard deviation squared) of the IA when measuring ferritin was 563.6 ng/mL (i.e., \( s_f \)) and the variance of the ELISA was 128.7 (i.e., \( s_2 \)). The calculated \( F \)-value was 4.38 and was compared to the critical \( F \)-value of 2.06 (\( p<0.05 \)). Therefore, the null hypothesis was rejected because a difference between the variances was observed and random error was observed. For sTfR, the variance of the IA when measuring sTfR was 0.034 µg/mL (i.e., \( s_f \)), and the variance of the ELISA was 0.021 µg/mL (i.e., \( s_2 \)). The calculated \( F \)-value was 1.62 and was compared to the critical \( F \)-value of 1.93 (\( p<0.05 \)). Thus, there was not enough evidence to reject the null hypothesis that there was a difference between the variances of both methods measuring sTfR.

*Total calculated analytical error compared to total allowable analytical error*

Based on the bias and standard deviation, the \( TE_{\text{calc}} \) was calculated using Equation (15). Once the \( TE_{\text{calc}} \) was determined for the IA on the PC biosensor, it was compared to the allowable total error (\( TE_a \)) for each IDA biomarker. Table 9 includes calculated data on \( TE_{\text{calc}} \) and the established \( TE_a \). The \( TE_{\text{calc}} \) of the IA on the PC biosensor measuring sTfR was 0.53 µg/mL. Based on National Health and Nutrition Examination Survey (NHANES) 2003-2010, the cutoff for sTfR in healthy populations is 5.3 µg/mL (Mei et al., 2012). Although hemodialysis patients with CKD are not considered “healthy”, no quality specification for CKD patients exists. Therefore, the clinical cutoff used by NHANES was applied. Because the \( TE_a \) of sTfR is a percentage, the absolute concentration observed in this study (0.52 µg/mL) must be converted to a percentage of the clinically relevant cutoff (5.3 µg/mL). Thus, the \( TE_{\text{calc}} \) of the IA on the PC...
biosensor measuring sTfR is 9.8%, which is lower than the \( TE_a \) of 17.4%. The latter \( TE_a \) was estimated by biological variation studies conducted previously (Bailey et al., 2014). Therefore, the \( TE_{calc} \) was less than the \( TE_a \) at the clinical cutoff of 5.3 \( \mu g/mL \). This demonstrated the performance of the IA compared to ELISA resulted in less error than would be observed in biological variation within and between individuals. However, because the range of sTfR concentrations in the experiments was between 0.6-2.5 \( \mu g/mL \) in the CKD patients, and did not include 5.3 \( \mu g/mL \), it is inappropriate to extrapolate the error outside the range that was measured in the experiment. In that case, it is recommended to use the mean of the measured samples instead of the clinical cutoff.

**Table 9.** Total Analytical Error (\( TE_{calc} \)) for IDA biomarkers using the Inverse Sandwich Assay on the PC biosensor.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>n(^1)</th>
<th>( TE_{calc} )(^2)</th>
<th>( TE_{calc} ) (%)(^3)</th>
<th>( TE_a ) (%)</th>
<th>( TE_{calc} ) (%)(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTfR</td>
<td>27</td>
<td>0.52 ( \mu g/mL )</td>
<td>43.0</td>
<td>17.6(^5)</td>
<td>9.8%</td>
</tr>
<tr>
<td>Ferritin</td>
<td>23</td>
<td>94 ( ng/mL )</td>
<td>33.6</td>
<td>16.9(^6)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\(^1\)Number of samples included in analysis  
\(^2\)\( TE_{calc} \) as absolute concentration  
\(^3\)\( TE_{calc} \) as a percentage  
\(^4\)\( TE_{calc} \) for clinical cutoff at 5.3 \( \mu g/mL \)  
\(^5\)\( TE_a \) cited by Bailey et al., 2013  
\(^6\)Cited on Westgard online available: http://www.westgard.com/biodatabase1.htm

The mean of the measured samples was 1.21 \( \mu g/mL \) and thus, the \( TE_{calc} \) using Equation (15) was 43% when comparing it to the mean. Contrary to the \( TE_{calc} \) of 9.8% when using the clinical cutoff of 5.3 \( \mu g/mL \), the \( TE_{calc} \) of 43% is not less than the established \( TE_a \) of 17.6% (Bailey et al., 2014). Although the \( TE_{calc} \) was greater than what is allowed, the clinical decision of diagnosing IDA is not affected because all sTfR values are below the cutoff at which deficiency would be suspected (i.e. 5.3 \( \mu g/mL \)).
For ferritin, the $TE_{calc}$ of the IA on the PC biosensor was 94 ng/mL. As a percentage of the mean found in the experimental data, the $TE_{calc}$ was 33.6%. Compared to the total $TE_{a}$, previous biological variation studies demonstrate that a $TE_{a}$ of 16.9% is an acceptable amount of error. Therefore, the observed $TE_{calc}$ was greater than the $TE_{a}$. As in the case of sTfR, it is important to take into account the clinical relevance of this error in the context of how the diagnosis and treatment may change.

This study showed that ferritin was a better indicator than sTfR to identify patients who needed IV iron treatment. Although these patients with low ferritin (i.e. <100 ng/mL) had higher values of sTfR, no sTfR values exceeded the clinical cutoff (i.e. >5.3 µg/mL) for iron deficient erythropoiesis. In this particular data set, the majority of individuals had ferritin levels between 200-500 ng/mL. The National Kidney Foundation has stated that ferritin levels >200 ng/mL are ideal to lower the risk of an absolute iron deficiency. To ensure EPO therapy is not limited by iron, the serum ferritin cutoff at which IV iron treatment is recommended for CKD patients is <100 ng/mL. After applying this cutoff to our data, from both diagnostic platforms, it was found that two hemodialysis patients with CKD should receive IV iron therapy to prevent the consequences of an iron-deficient erythropoiesis that ultimately would lead to IDA. This was not the case for sTfR.

sTfR values from all samples measured on the ELISA and PC biosensor were not above or near the cutoff indicative of deficiency (i.e. 5.3 µg/mL), which is similar to other studies that have measured sTfR in CKD patients (Beerenhout et al., 2002; Buttarello et al., 2010; Gupta and Pawar, 2009; Tessitore et al., 2001). Low sTfR levels may be the result of patients who had recently received EPO, which causes ferritin levels to drop quickly, as it is now used in hemoglobin synthesis, before sTfR is upregulated (Tessitore et al., 2001). Nevertheless, more
measures of iron status such as TSAT would be needed to reach a reliable diagnosis of iron
deficiency (Chung et al., 2012). As a result of the evidence presented from these experiments and
those of others it is suggested that ferritin remains a key biomarker to determine whether
hemodialysis patients need IV iron therapy and sTfR does not provide any additional evidence of
IDA.

The process of method validation undertaken in this research was derived from the
Stockholm Consensus Conference in 1999 when global analytical quality specifications were set
(Fraser et al., 1999) and have since been updated yearly based on ongoing studies collecting
biological variation data of new biomarkers (Ricos, 1999). Although the PC biosensor had an
unacceptable amount of total analytical when measuring sera from CKD patients, quantifying the
total analytical error and comparing it to established quality specification is essential when
evaluating a new assay platform. The method validation process is long and labor intensive, yet
appropriate when considering clinical diagnoses have treatment consequences for patients. Even
high-end validated clinical diagnostic instruments (e.g. Centaur XP and Abbott Architect i2000,)
have been rejected for measuring certain analytes due unacceptable analytical errors (Holmes et
al., 2013). In this study, over 160 specimens containing 25-hydroxy-vitamin D were measured by
both the Centaur and Architect as the test methods and liquid chromatography mass spectroscopy
and a radioimmunoassay as reference methods. Due to unacceptable levels of random variability
and a high-degree of positive bias (systematic error), as a result of a suspected interfering
antibody, both test methods (i.e. Centaur and Architect) did not meet the exhibited unacceptable
error when measuring 25-hydroxy-vitamin D (Holmes et al., 2013). New point-of-care
technologies as well need to undergo similar scrutiny before being launched as dependable
clinical diagnostics (Murata et al., 2015). Although a rapid turn-around time is important in
point-of-care diagnostics, accuracy and reliability supersede turn-around time due to the potential consequences of misdiagnosis in susceptible populations. As new innovative biosensors continue to be recognized and published, it is critical a standard method validation process is followed to ensure proper evaluation of analytical performance.

5.4. Limitations.

Based on the total analytical error observed when measuring ferritin and sTfR on the PC biosensor, optimization of the IA is required to meet the quality specifications established when quantifying ferritin and sTfR in serum. It is worth noting minimal optimization of the IA has been conducted to date. Areas of optimization for performance of the IA that may decrease the $TE_{calc}$ include adding wash steps during magnetic separation, adjusting capture and detection antibody concentrations, and changing dilution buffers, which may enhance fAb-ION stability. Assay time may be decreased by following fAb-ION-antigen binding using dynamic light scattering instruments and determining the minimum time required for fAb-ION-antigen association. In addition, functionalizing protein A or streptavidin to the IONs and conjugating the detection antibodies with a predictable orientation may improve selective binding and decrease non-specific binding near the fAb-IONs surface. Furthermore, depending on the target population for using the PC biosensor for clinical diagnosis, hemodialysis patients may not have been the best population to perform method validation experiments using fAb-IONs because they are known to have various metabolic byproducts and wastes in their sera. Previous studies conducted with IONs have shown colloidal stability can be decreased in the presence of serum proteins and other biomolecules (Hirsch et al., 2013; Petri-Fink, 2008). Another potential route to improve the performance of the PC biosensor for this specific population is using the standard sandwich assay (Peterson et al., 2014a) and establishing a wash protocol to remove interfering
agents. The loss of colloidal stability was not observed until after magnetic separation using the IA, and the standard sandwich assay does not involve this step. Therefore, employing the standard sandwich assay on sera from hemodialysis patients may decrease the \( TE_{calc} \) because the colloidal stability of the fAb-IONs would be maintained.

5.5. Conclusion

This study was the first thorough method validation study quantifying total analytical error using a PC biosensor and comparing it to established quality specifications. Despite previous studies on the PC biosensor showing harmonization of quantifying sTfR using World Health Organization standard reference materials (Peterson et al., 2014b), the interference molecules in CKD caused significant analytical error. Nevertheless, applying the inverse sandwich assay on the PC biosensor to measure ferritin and sTfR in hemodialysis patients with end-stage renal failure indicated that more than 90% of the patients in the sample had sufficient iron stored in ferritin and did not differ from the reference ELISAs. Future studies will aim at optimizing the protocol of the inverse sandwich assay and test the sandwich assay measuring biomarkers of IDA in sera from healthy individuals to quantify the total analytical error of PC biosensor when measuring sera from non-hemodialysis patients.
5.6. References


CHAPTER 6.

SUMMARY, DISCUSSION, AND CONCLUSION

6.1. Summary

In this dissertation study, considerable progress was made towards validating a PC biosensor as a clinical diagnostic tool for IDA. In the first study, it was discovered that amplifying a label-free detection format was essential when measuring protein biomarkers of IDA on the PC biosensor. Due to the minimal change in dielectric permittivity without amplification, the change in the peak wavelength shift did not elicit a dose-response curve when measuring multiple concentrations. Employing antibody-functionalized iron-oxide nanoparticles as the amplification label led to a reproducible linear dose-response curve that allowed for accurate and precise quantification of ferritin in Liquichek™ control sera compared to the results of three ferritin ELISAs from different companies (Peterson et al., 2014).

The second study of this dissertation aimed at applying a magnetic separation protocol to selectively remove another biomarker of IDA (i.e. sTfR) from a complex sample matrix. A magnetic separation protocol was necessary as a means to remove sTfR from other interfering proteins that cause a high degree of non-specific binding on the PC biosensor. After demonstrating the magnetic separation protocol was successful in extracting nearly 100% of sTfR from buffer, the magnetic separation protocol was tested to determine if it could be used to decrease non-specific signals from serum samples on the PC biosensor. The results of the inverse sandwich assay (IA) indicated that the magnetic separation protocol led to accurate and precision detection of sTfR in Liquichek™ control sera compared to an FDA-certified ELISA. Further characterization included the determination of the binding ratios between the fAb-IONs and
sTfR, and size of functionalized and non-functionalized IONs by transmission electron microscopy (TEM). The former was useful to identify the fAb-ION concentration at which nearly 100% of sTfR was removed from buffer solution. The latter was instrumental to verify aggregation of fAb-IONs did not occur after magnetic separation in a serum matrix (Peterson, 2015). TEM images verified that nanoparticle spacing and colloidal stability were not influenced before or after magnetic separation.

The final study contained in this dissertation quantified sTfR and ferritin in chronic kidney disease (CKD) patients on hemodialysis using the IA. The objective of this study was to quantify the total analytical error of the PC biosensor compared to an FDA-certified ELISA for each respective biomarker. The results of this study indicated the IA on the PC biosensor had more total analytical error than the defined total allowable error from the quality specifications of each biomarker. However, due to the fAb-IONs losing colloidal stability and precipitation, one third of the CKD samples could not be measured for either biomarker. The loss of colloidal stability in the fAb-IONS was suspected to be the result of excess metabolic wastes or other biomolecules in the sera interacting with the fAb-IONs and potentially causing cross-linkages between fAb-IONs and, ultimately, aggregation. As a result of this study, it is suggested that before the PC biosensor is used to diagnose IDA, further optimization is needed.

6.2. Discussion

The strides made towards improving the capacity of the PC biosensor to measure protein biomarkers in an immunoassay format are highlighted in the previous three chapters of this dissertation. Employing IONs to amplify the signal from antigens adhered to the PC biosensor surface was the first time they had been applied to a PC biosensor (Peterson et al., 2014). Similarly, the development of a magnetic separation protocol to extract protein biomarkers and
then its combination with detection of antigens on a PC biosensor had never been described (Peterson et al., 2015). Nevertheless, as was observed in the third study, the PC biosensor did not meet the quality specifications of measuring ferritin and sTfR for clinical diagnosis. It is worth noting, however, the sera in the third study came from hemodialysis patients who are known to have abnormal blood profiles. Consequently, the colloidal stability of the fAb-IONs was disrupted and one third of the serum samples could not be measured. This problem could have potentially been avoided by using sera from a healthy population, which would have eliminated the metabolic waste products in the sera from hemodialysis patients. On the other hand, the intent was to test the magnetic separation protocol against a variety of sample matrixes to determine its applicability in various clinical pathologies. The final study of the research indicated the fAb-IONs are not stable in all types of serum matrixes. It is possible that other factor affect reliability of results beyond wastes in CKD patients. For example, it is known that proteins are prone to degrade during sample preparation or during several freeze-thaw cycles.

A current challenge in the field of nutrition and global health is to develop technologies that are capable of surveying large samples of the population for micronutrient deficiencies. The best data available come from large epidemiological studies extrapolating prevalence of micronutrient deficiencies to larger populations, which in some cases, is inappropriate and does not illustrate specific areas that are in dire need or not of nutritional interventions. Nonetheless, they are the best data available for government officials to make public health decisions (i.e. fortification, biofortification, etc.) regarding micronutrient deficiency. In the case of IDA, all segments of the population are negatively affected. Children have lower cognitive functioning, pregnant women have more complications during pregnancy and at birth, and working adults have lower work productivity. Horton and Ross in 2003 attempted to quantify the economic
impact of IDA and they estimated the cost-benefit was $1 cost of intervention for a repayment of $36 that the children and adults would return to the economy of a country (Horton and Ross, 2002). This study among others, clearly demonstrate the economic feasibility of interventions against IDA, yet adequate identification of those individuals and communities most in need of such interventions remains a hurdle to ameliorating widespread IDA.

Although clinical laboratories in hospitals of major cities often have the technology to give reliable diagnoses to patients, access to such facilities is often limited to individuals of higher socio-economic status that carry a lower risk for IDA. Individuals who are in need and at much greater risk live in rural communities and often have physical and financial constraints to reaching well-equipped healthcare facilities. For these reasons, the field of biosensors and bioelectronics provides an opportunity to bring needed diagnostic capabilities to clinicians and healthcare professionals, and most importantly, promote health among the patients they serve.

Since the introduction of smartphones in 1997, the central processing unit, cameras and Internet connectivity have improved remarkably. Moreover, the user base continues to grow rapidly in developing countries. As a result, clinician-friendly healthcare applications have the potential to transform medical care and bring point-of-care diagnostics to patients throughout the world. Optical biosensors that harness properties of light to transduce photons into a meaningful signal after it interacts with the sensor surface have already employed smartphones as detection instruments (Gallegos et al., 2013). Thereby, providing proof-of-concept data that optimization of such assays may lead to future clinical point-of-care diagnostics.

Despite the prospect around innovative approaches to detect biomarkers of IDA using smartphone devices, simple detection of biomarkers is not enough. Evaluation of detection platforms should go beyond reporting limit of detection, accuracy and linear reportable range.
More emphasis should be placed on the quantification of error and whether the new platform meets the defined quality specification for each respective analyte. Unfortunately, these types of studies can be expensive and time intensive. Furthermore, in engineering programs funding is as dependent on the innovation of the platform as it is on the broader impacts of the platform. Therefore, engineers of these detection platforms need to keep the broader impact pillar as well as the innovation pillar to maintain funding. Consequently, engineers cannot conduct thorough method validation experiments to determine the performance of a biosensor or bioelectronic. Then, more broadly, researchers are vetted to develop innovative detection platforms capable of measuring a wide array of biological analytes, albeit with little clinical utility in terms of accuracy and precision.

The result of this funding paradigm, in the fields of biosensors and bioelectronics, has led to hundreds of articles on new biosensing methods for different analytes and further claiming to offer complete validation studies, without even comparing it to any established quality specification for a given analyte. This is similar to trying to allocate an amount of spending without defining a budget. Furthermore, substantial human and financial resources are being funneled into research that has potential to be groundbreaking. Nonetheless, due to either time constraints or lack of clarity about the proper method validation experiments to conduct, it never goes beyond the bench and proof of concept. The question arises then, whether private industries should be the funders of late stage bioassay development? The current R&D system is designed in this way. Not to say this system has not led to accurate and precise method development, (e.g. Hemocue and Piccolo), but whether such a system is sustainable to efficiently use limited resources to streamline new detection platforms into the market, , is debatable.
In the field of nutrition, no substantial advancement has occurred to allow clinicians and researchers to survey populations outside of standard laboratories methods (i.e. ELISA and RIA). Therefore, it is suggested engineers follow the method validation process outlined in this dissertation and more directly in the book by Westgard (Westgard, 2008) to guide evaluation of new method performance. This way, biosensor platforms that meet quality specifications can be evaluated for measuring relevant nutrition biomarkers, agricultural markers, inflammatory biomarkers, and beyond.

6.3 Future Studies and Conclusion

To determine the true capability of PC biosensor as a clinical diagnostic device more optimization studies are essential. It should be noted that minimal optimization studies were carried out using the PC platform in the aforementioned studies because of the many key factors that would require optimization and the high cost associated with each of such experiments. In the next lines, the author describes some key experiments that could improve valid quantification of IDA biomarkers in serum.

*Particle size.* An interesting observation is that the aggregation and precipitation did not occur until after applying the magnetic field for 30 minutes from a permanent magnet. Therefore, smaller IONs with diameter ~20 nm may offer an opportunity to avoid aggregation in similar complex matrixes. The reason is because at an average size of 30 nm, the nanoparticles range in size from 20-40 nm, and above 30 nm, the nanoparticles lose their superparamagnetic property, which promotes aggregation. Thus, shifting the sizes of nanoparticles below 30 nm may improve the fAb-ION stability in future studies. The downside to this approach is that the magnetic separation of smaller nanoparticles takes longer. Also, if the aggregation was not due to magnetic
moment itself, but to nonspecific agents in the sera that changed the surface charges of the IONs, using smaller nanoparticles may not be effective.

**Washing.** In addition to altering the magnetic nanoparticle size, one simple approach that may improve the performance of the PC biosensor is to wash the pellet of fAb-ION-antigen complexes while the magnetic field is still being applied. By washing the pellet, any interfering proteins bound non-specifically to the fAb-ION-antigen complexes would be removed. A downside of washing is that it could disturb the pellet of fAb-ION-antigen complexes, which would increase the time required for separation.

**Antibodies.** Another opportunity for optimization is in the concentration of capture antibodies and fAb-IONs. On the PC platform, biomolecules are detected within ~200 nm from the surface. Therefore, steric hindrance that prevents the analytes of interest from reaching the near-surface field will not be detected and elicit a peak wavelength shift. Steric hindrance can become problematic when fAb-IONs-antigen complexes with a large dynamic volume collide with other fAb-IONs-antigen complexes and form aggregates. It can be further compounded if a high concentration of capture antibodies is immobilized on the PC surface. To ensure all available analytes are contributing to the response and interacting specifically with capture antibodies, studies should be carried out to test numerous concentrations of both fAb-IONS and capture antibodies that elicit the optimized peak wavelength shift for a range of analyte concentrations.

An alternative approach to reducing the risk for aggregation in complex matrixes involves using streptavidin as the functionalization molecule along the surface of IONs. Then, the detection antibodies can be biotinylated and bound stereo-specifically onto the surface of the IONs. Using the streptavidin-biotin functionalization system would further reduce other proteins
from binding nonspecifically to the surface of the IONs because the coverage of IONs would be specific to biotinylated antibody. Biotinylation would also maximize the binding sites of the detection antibodies that bind to a specific epitope and protein. As a result this would dramatically decrease the percentage of antibodies unable to bind specifically due to their orientation.

**Sample dilution.** A final optimization alternative would be to attempt the regular sandwich assay as shown in Chapter 3, after dilution of serum samples. Because most non-specific binding happens at concentrations above 20 mg/mL of total protein, sera can be diluted when measuring biomarkers of IDA. As discussed previously, standardizing the dilution to reach a clinically relevant level makes for a simple decision to either classify a patient as iron deficient or not. This is a significant improvement from blanket supplementation with iron pills, which has been shown to result in adverse side effects or even death in children where malaria parasites are present (Sazawal et al., 2006).

In conclusion, this dissertation carried out proof-of-concept studies where each study provided evidence to support the use of a PC biosensor platform to quantify protein biomarkers of IDA within a clinically relevant range. Using both a standard sandwich format and an inverse sandwich format, which employs a magnetic separation protocol, low imprecision and low inaccuracy were obtained when measuring IDA biomarkers within buffer and in Liquichek™ control sera. However, when validating the clinical capabilities of this platform after comparison of methods experiment (i.e. ELISA vs. PC) with sera from hemodialysis patients, the PC biosensor did not meet the quality specifications for measuring ferritin and sTfR. The work from this dissertation invites new perspectives and approaches both to optimize the PC biosensor to meet the quality specifications required to make reliable clinical diagnoses for IDA and to
continue development of biosensing platforms to diagnose critical biomarkers of nutrition status at the point of care.
6.4. References


