HIGHLY SENSITIVE BIOSENSORS USING PHOTONIC CRYSTAL ENHANCED FLUORESCENCE

BY

YAFANG TAN

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Electrical and Computer Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Professor Brian T. Cunningham, Chair
Assistant Professor Songbin Gong
Associate Professor Gang Logan Liu
Professor Lila O. Vodkin
ABSTRACT

Due to the importance of fluorescence assays in clinics, life science and genomic research, many nanostructures have been proposed to enhance the weak fluorescence signals from low abundance analytes. Among these structures, photonic crystal (PC) has attracted particular attentions because of its capability to produce high electric fields without quenching fluorescence emission. Properly designed PC surface can efficiently couple laser illumination to fluorescent dye molecules (enhanced excitation) and meanwhile effectively collects fluorescence emission (enhanced extraction).

In this dissertation, photonic crystal enhanced fluorescence (PCEF) was used for disease biomarker dissertation. We applied PCEF to multiplexed microspot immunoassays for detection of allergen-specific antibodies. The PCEF system was able to detect low concentrations of Fel d1 specific IgE ~0.02 kU/L, which is 5-fold to 17-fold more sensitive than the commercially available FDA-approved analyzers.

We also aimed to improve the current platform by incorporating a PC surface with a microfluidic chip in order to enable a rapid and automatic biomarker analysis at microliter sample consumption. We fabricated a plastic microfluidic device for holding the PC using a low-cost and flexible process and developed a leak-free automation system to introduce the correct sequence of fluids through the microfluidic device. The PCEF platform was successfully automated to enable more rapid, high sensitivity detection and a simplified output that will be readily usable by a clinician.

Besides microarray immunoassays, the PCEF platform was also adapted to a molecular beacon assay for miRNA detection, where the PC surface was incorporated
with a submicron-height fluid channel. The use of the PCEF platform results in a reduction of the detection limits and an increase in the signal-to-noise ratio (SNR) with a small, inexpensive detection instrument.
ACKNOWLEDGMENTS

First and foremost I would like to thank my adviser, Professor Brian T. Cunningham, for giving me the opportunity to work on such an exciting and interdisciplinary project. His enthusiasm, patience, and encouragement are truly inspirational. And his knowledge and guidance are instrumental in this dissertation. I would also like to thank my committee members Prof. Lila Vodkin, Prof. Logan Liu and Prof. Songbin Gong for their help, encouragement and valuable comments. I would like to acknowledge Prof. J. Gary Eden for his advice and support.

I am also thankful for all the current and past members in the Nano Sensors Group for their valuable discussions on this project, including Meng Lu, Tiantian Tang, Haisheng Xu, Chenqi Zhu, Weili Chen, Hojeong Yu, Jui-Nung Liu, and Meng Zhang. Also, I would like to thank the staff at the Micro and Nanotechnology Laboratory, especially Yaguang Lian, Edmond Chew, and Michael Hansen for their kind help and helpful discussions.

I also want to thank my friends for their support and good times we shared: Cheng Ouyang, Aiguo Han, Rui Guo, Tianying Jiang and Sheng Ye. Although there were some downfalls in graduate school, your companionship gave me the courage and confidence to be successful.

Finally I want to deeply thank my parents, who brought me into this world and educated me. They love, believe and encourage me no matter who I am. Also, I would like to thank my husband Rongkuo Zhao for his love and belief throughout my graduate study.

This dissertation was partially supported by the National Science Foundation and National Institutes of Health.
3.3 Methods................................................................................................................................. 44
  3.3.1 PC Fabrication and Characterization ............................................................................... 44
  3.3.2 Source of Materials ......................................................................................................... 44
  3.3.3 Preparation and Allergen Printing on the PC Surface ..................................................... 45
  3.3.4 Testing Procedures .......................................................................................................... 47
  3.3.5 Image and Data Acquisition ............................................................................................. 48
3.4 Results.................................................................................................................................... 49
  3.4.1 Selectivity of the Assay ..................................................................................................... 49
  3.4.2 Sensitivity of the PCEF Array System ............................................................................. 50
  3.4.3 Calibration of Assay: Translating PCEF Readings into IgE Concentrations .................. 52
  3.4.4 Application of Calibrated Results to Additional Sera ..................................................... 53
3.5 Conclusion............................................................................................................................... 54
3.6 Figures..................................................................................................................................... 57
CHAPTER 4 PHOTONIC CRYSTAL ENHANCED FLUORESCENCE MICROFLUIDIC CHIP FOR HIGH SENSITIVITY AUTOMATED MULTIPLEXED IMMUNOASSAYS ......63
  4.1 Chapter Introduction ............................................................................................................. 63
  4.2 Motivation ............................................................................................................................ 64
  4.3. Materials and Methods ...................................................................................................... 66
    4.3.1 Design and Fabrication of a PC-Embedded Microfluidic Device ................................. 66
    4.3.2 Computer-Controlled Microfluidic System ................................................................. 68
    4.3.3 Immunoassay Procedure Using Microfluidic Sample Handling ................................. 69
    4.3.4 PCEF Scanner and Image Acquisition ......................................................................... 71
  4.4. Results and Discussions .................................................................................................... 72
    4.4.1 Raw Fluorescent Intensity .......................................................................................... 72
    4.4.2 Fluorescence Enhancement of a PC-Embedded Microfluidic Device ......................... 74
    4.4.3 Standard Curves and Limit of Detection .................................................................... 76
  4.5 Conclusion............................................................................................................................ 77
  4.6 Figures.................................................................................................................................... 79
CHAPTER 5 PCEF FOR HOMOGENEOUS ASSAY USING SUBMICRON FLUID CHANNELS FABRICATED BY E-JET PATTERNING ...................................................84
  5.1 Chapter Introduction ........................................................................................................... 84
5.2 Motivation .................................................................................................................. 84
5.3 Methods .................................................................................................................... 87
  5.3.1 Device Structure and Detection Principle ......................................................... 87
  5.3.2 E-Jet Printing of the Submicron-Channel ......................................................... 89
5.4 Results ...................................................................................................................... 92
  5.4.1 Fluorescence Enhancement Characterization ...................................................... 92
  5.4.2 Bio-Detection Demonstration by Molecular Beacon Assay ............................. 93
5.5 Discussion and Conclusion ..................................................................................... 97
5.6 Figures .................................................................................................................... 99

CHAPTER 6 CONCLUDING REMARKS ........................................................................ 103
  6.1 Summary of Current Work ....................................................................................... 103
  6.2 Future Work ............................................................................................................ 105
REFERENCES ............................................................................................................... 109
CHAPTER 1: INTRODUCTION

1.1 Chapter Introduction

There is an urgent need for blood-based molecular tests to assist in the detection and diagnosis of diseases in a cost-effective manner at an early stage. Additionally, detection of molecular biomarkers in blood can classify diseases into distinct molecular subtypes and monitor their relapse and response to treatment. Increasingly, biomarker strategies are becoming critical to identify a specific patient subpopulation that is likely to respond to a new therapeutic agent. The improved understanding of the underlying molecular features of many challenging diseases and the availability of a multitude of recently developed technologies to interrogate the biomarkers in biological fluid have made it possible to develop clinically applicable and cost-effective tests for many diseases, even cancers. Overall, the paradigm shift toward personalized and individualized medicine relies heavily on the increased use of diagnostic biomarkers and classifiers to improve diagnosis, management and treatment.

Fluorescence sensing is the most widely used detection technique in molecular diagnostics [1-3] and genomic/proteomic research [4-6], due to the wide availability of dyes that can be easily conjugated to broad classes of biomolecules and detected with high sensitivity. By labeling biomolecules with fluorescent molecules, researchers can obtain useful information such as spatial distribution of biomolecules in cells, abundance of biomolecules in a test sample or hybridization reaction between biomolecules, among a multitude of other
potential applications. The novel tool ensures that the practice of molecular biology becomes increasingly quantitative.

There is a strong need to improve the performance of the dyes because of their importance in molecular biology. Researchers have made great efforts in the past decades to augment the performance of these molecules both in biological and non-biological contexts. Many novel nanostructures have been proposed to enhance the weak fluorescent signals from the low-concentration analyte [7-10], including metal-coated slides [11], plasmonic gratings [12, 13], two-dimensional (2D) photonic crystals (PCs) [14] and nanoantennas [15, 16]. By performing the assay protocol upon the surface of the nanostructures, sensitivity gains of over two orders of magnitude have been demonstrated. Among these structures, photonic crystals have attracted particular attention because of their capability to produce highly surface-bounded electric fields without quenching the fluorescence emission.

Recently, one-dimensional (1D) PCs have been engineered by Cunningham’s group to enhance the signal from the common microarray dye Cyanine [17, 18]. The 1D PC utilizes high-quality factor resonances from a periodic dielectric surface structure to enhance the fluorescence output. Photonic crystal enhanced fluorescence (PCEF) allows the fluorophore to be excited by a small, compact, and inexpensive semiconductor laser instead of a high-power laser. Likewise, the greater fluorescence emission provided by PCEF can be readily detected with inexpensive imaging cameras that do not require costly liquid nitrogen cooling or electron multiplication. Using these advantages, we
have demonstrated 0.1 – 1.0 pg/ml detection of protein biomarkers in serum using an instrument we assembled at a retail cost of ~$10K [19]. Our current work involves applications of the PCEF system in the detection of plant expression, soluble protein, human autoantibody, and miRNA, with the goal to improve sensitivity and minimize the sample consumption.

This chapter provides background on molecular diagnostics, fluorescence assays and the progress to date in the field of enhanced fluorescence including photonic crystal enhanced fluorescence (PCEF). The significance of molecular diagnostics, especially the detection of biomarkers in blood, is clearly stated. Then, two types of fluorescence assays that play important roles in biomarker detection are discussed. Special attention is given to enzyme-linked immunosorbent assay (ELISA) microarray and fluorescence resonant energy transfer (FRET) assay, as they are closely related to our current work. This is followed by a brief discussion on the basic mechanisms of fluorescence enhancement and the recent work in this field attributed to the progress of micro and nanotechnologies.

1.2 Molecular Diagnostics

Diagnostics tests inform a wide range of medical decision making, therefore they are an integral and critical part of our health care system. There are two primary areas of health care diagnostics: “in vivo” imaging or “in vitro” diagnostics (IVD). Imaging technologies include X-rays, ultrasonic waves, magnetic resonance, or radio-nuclear method that produce images of the body and its organs and other structures. IVDs are tests performed on a sample
taken from the body (blood, tissue, sputum, urine, etc.). One growing subset of IVDs is molecular diagnostics, which has captured particular attention in recent years because these types of tests bring deep insights to diagnosis and treatment. Molecular diagnostics is one of the most dynamic and transformative areas of diagnostics, leading to advances in research and treatment that are revolutionizing healthcare across a wide range of diseases and health conditions.

“Molecular diagnostics” refers to a class of diagnostic tests that assess a person’s health literally at a molecular level, as it detects and measures specific genetic sequences in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or the proteins they express. Molecular diagnostics identifies gene, RNA, and protein variations that shed light on whether a specific person is predisposed to have a disease, whether they actually have a disease, or whether a certain treatment option is likely to be effective for a specific disease. Before molecular diagnostics, doctors made medication decisions based on the patients’ history and their symptoms. This traditional “evidence-based” diagnostics is heavily dependent on the doctors’ experiences and could delay the medical treatment since decisions can only be made after diseases are fully developed. Molecular diagnostics, however, is capable of detecting diseases at their early stage and even predicting their severity and patients’ survival rates, thus saving valuable time and resources for the patients. In addition, molecular diagnostics plays important roles in drug discover and finding the effective treatment for patients. With the help of molecular diagnostics, it is possible to realize “personalized
medicine” which allows clinicians to avoid treating every patient based on what he or she broadly has in common with other patients to treating them as individuals with optimized treatment for every individual patient. For example, by understanding underlying molecular mechanisms, a patient with a specific gene mutation in their cancer can be divided into a subgroup with a specific type of lung cancer and receive a specialized treatment.

Of course, the ultimate power of personalized medicine is the ability to treat each individual patient with therapies tailored to the molecular profile of his/her individual disease (e.g., cancer). When specific proteins or genetic sequences have a known association with a specific health condition or disease, they are often referred to as “biomarkers” because they are markers of that condition or disease. Molecular diagnostics is a tool that is driving the continuing discovery of biomarkers at the research level, which in turn leads to treatments designed around these biomarkers. Then molecular diagnostics plays an additional critical role by ensuring that these new therapies are delivered to the right patient through more accurate diagnosis of the exact nature of their individual disease. This has led to the emerging field of companion diagnostics, in which a molecular diagnostic test is used to identify whether a specific therapy (a companion to the diagnostic) is likely to be effective for an individual patient.

Molecular diagnostics examines and assesses functions of DNA, RNA, and proteins that were produced because diseases disrupt the function of cells. These biomarkers exist in patients' tissues, fluids, tumors, or in the infectious
agents themselves that cause the disease. For example, when specific mutations, or sets of mutations, are known to be biomarkers associated with a disease or condition, molecular diagnostic tests can examine a patient’s genes to determine whether those mutations are present. These tests may look only for those certain gene variants, or map the entire sequence of a targeted portion of DNA to detect all mutations in the sequence. The test results, therefore, can assess a person’s risk of developing a disease, determine whether a person is a carrier of a hereditary condition, screen for diseases that are present but not yet symptomatic, or provide a diagnosis of existing symptoms.

1.3 Biomarker Detection in Blood

Blood has been regarded as a source of information on illness and health since ancient times. Biomarkers in blood have a great deal of promise for facilitating personalized medicine, including detection, diagnosis, prognosis, and monitoring of therapy. Although the broadest definition of a biomarker also includes the measurement of physiological and anatomical criteria, for molecular diagnostics the term refers to measuring the levels of biological molecules associated with the disease, such as specific proteins or genes. If these biomarkers are detected in the blood in abnormally high concentrations, it may suggest a diagnosis.

A key unmet need for biomarker detection to achieve clinical relevance is improved sensitivity. Early diagnostic applications for cancer, for example, require detection of biomarkers that are produced by, or in response to a small
number of cells. The biomarker is diluted throughout the volume of blood within a person, so that when a sample of peripheral blood is drawn the biomarker concentration can be <1-10 pg/ml. (For reference, 1 pg/ml of a typical cytokine such as IL-6 is equivalent to ~45 fM. A 10 ml sample at this concentration contains 0.45 attomoles of IL-6.) In addition to high sensitivity, clinical diagnostic laboratories require high throughput automated platforms that can perform multiplexed biomarker analysis that can quantify small changes in concentration that can be performed rapidly at low cost/assay. Moreover, it is widely recognized that these circulating biomarkers are highly variable across individuals, and that it will likely be necessary to utilize a panel of blood biomarkers to accurately detect or characterize a disease. Consensus is emerging that multiple, mutually exclusive biomarkers in an assay will lead to better clinical management of disease, compared to assessment of a single biomarker, and allow subtle differences in patient populations (gender, race, age) to be understood [20-22].

Current methodologies for multiplexed, sandwich ELISA analysis are the bead-based approaches (Luminex, $100K instrument capable of 500x multiplexing, and ~$3/assay for each target) and electrochemiluminescence technology (MesoScale Discovery – $190K for the Sector Imager 6000 instrument and $1500 for a 24-well plate that images 100 spots/well). However, these approaches typically use >50 ml of serum, require an expensive non-automated detection instrument, provide limited multiplexing capability, and have lengthier assay protocols than microarrays. While Luminex systems
deliver similar detection limits as conventional ELISA, (1-10 pg/ml for common cytokines), they require ~7 hour assay protocols to achieve them [23-26]. Another promising technology for high sensitivity biomarker detection is the Simoa system offered by Quanterix [27], which uses a fluorescent reporter molecule attached to antibody-functionalized magnetic beads isolated in 50-fl reaction chambers to achieve non-multiplexed fM-scale detection limits with a more complex protocol than microspot ELISA. Likewise, the Erenna immunoassay technology offered by Singulex claims 1 fM detection limits using functionalized magnetic microparticles, fluorescent dye tags, and a custom format flow cytometer, but is not capable of multiplexed assays. All these systems utilize expensive purified antibodies and secondary antibodies to capture and label each target analyte.

The goal of our research work is to develop a biomarker detection platform to enable rapid, high-sensitivity, multiplexed and automatic detection and a simplified output that can be readily usable by a clinician. Fluorescent assay, especially molecular beacon assay and sandwich antibody microarray, is chosen for biomarker detection as they have been reported to optimally use a small volume of sample to detect analytes at low concentrations that lack assay cross-reactivity and demonstrate the fastest binding kinetics. By enhancing fluorescence from the assay using a nanostructured surface PC, higher sensitivity can be achieved. The goal of this dissertation is to develop a more compact, inexpensive and practical instrument than current, alternative approaches. While clinically relevant concentrations for many biomarkers are in
the range of 10-100 pg/ml [28], the ability to reduce limits of detection through
the development of a more sensitive technology potentially results in even
earlier disease diagnosis, while increasing the signal-to-noise ratio (SNR) for
analyte concentrations in samples that are currently near the limits of existing
approaches. Significantly, amplification of fluorescence signals allows for
detection of fluorescent emission that could not otherwise be performed without
expensive high-power lasers and cooled electron-multiplied CCD cameras, thus
yielding a low-cost detection instrument that uses an inexpensive
semiconductor laser and an inexpensive sensor. The approach developed here
may be broadly applied to a wide variety of multiplexed assays, including
fluorescent microspot sandwich ELISA for soluble protein antigens or nucleic
acid microarrays for detection of miRNA-based biomarkers [29-31].

1.4 Fluorescence Assays

Fluorescence is associated with the electron transition. An electron at the
ground state can be excited to a higher energy state with absorbing a photon,
and subsequently relaxed to a lower energy state with emitting a photon.
Typically the emitted photon has less energy than the absorbed photon, which is
observed as the Stokes shift. The energy difference between absorbed and
emitted photons is accounted for by vibrational relaxation. Fluorescence assay
typically involves fluorophore labeled biomolecules. It is a powerful scientific tool
to resolve the spatial distribution of biomolecules in a cell or determine the
abundance of biomolecules in a sample. Fluorescence assays can be either
surface-based or liquid-based as illustrated in Figure 1.1. Surface-based
fluorescence assays are generally measured with a single endpoint scan, after all assay steps are completed and the assay surface is dry. They are typically utilized for multiplexed assays in an array format. Liquid-based fluorescence assays, in contrast, are performed in a “homogeneous” format, with all assay components in a liquid environment, not coupled to a surface. They are typically measured in the cuvette.

1.4.1 ELISA Microarray

The most widely used surface-based assays are the microspot-based sandwich immunoassays, or ELISA microarrays. ELISA microarrays permit the simultaneous measurement of many substances in a small sample volume, therefore providing an attractive alternative approach for quantifying multiple components in serum for clinical applications and detecting cancer biomarkers [22, 32-34]. The sandwich ELISA using secondary antibodies with fluorescent tags has demonstrated the ability to classify metastatic breast cancer based on a blood test with sensitivities in the sub-pg/ml range.

As illustrated in Figure 1.2, the microarray ELISA chip is manufactured by printing an array of individual capture antibodies in micro-spots, where each antibody is printed multiple times to enable evaluation of experimental variability. After a “blocking” step inhibits subsequent nonspecific protein binding, the chip is exposed to a test sample, providing an opportunity for analyte molecules to bind with their corresponding capture antibodies. A mixture of all the biotin-linked detection antibodies is prepared and incubated with the chip, where they bind an unoccupied epitope of the targeted captured antigen. All ELISA reagents must be
rigorously tested to prove there is no assay cross reactivity. The final assay step is the introduction of fluorophore-labeled streptavidin that attaches only to the biotinylated detection antibodies.

1.4.2 Molecular Beacon Assay

One representative example of liquid-based fluorescence assays is fluorescence resonant energy transfer (FRET) assays. They are mainstays for disease diagnostics, pathogen detection, life science research, and toxin detection. They involve the energy transfer from an excited molecular chromophore (the “donor”) to another chromophore (the “acceptor”), in which the magnitude of the fluorescent output is determined by the concentration of a target analyte [35-37]. FRET requires the donor and acceptor to be separated from each other by 0.1 – 10 nm, and that the fluorescence emission spectrum of the donor overlaps with the fluorescence absorption spectrum of the acceptor. When two biomolecules are tagged with separate donor and acceptor fluorophores, and subsequently mixed together in solution, their binding interaction can be sensed by illuminating the sample with a laser within the absorption band of the donor, and by observing a decrease in donor emission, combined with an increase in acceptor emission. Likewise, when a single biomolecule is tagged with both donor and acceptor fluorophores, the dissociation of the biomolecule can be inferred through an increase in donor emission, combined with a decrease in acceptor emission. FRET interactions scale with the sixth power of the separation between donor and acceptor, and
thus represent an extremely sensitive “molecular ruler” for measuring biomolecular binding, protein folding, and DNA folding.

While FRET is an important molecular biology research tool, it has also evolved into a powerful diagnostic technology through the use of DNA “molecular beacons” [38, 39]. As demonstrated in Figure 1.3, molecular beacons (MB) contain a stem-loop hairpin probe that is flanked by two short self-complementary sequences. A fluorescent dye and a suitable quencher for that dye are attached at each terminal of the short sequences. The dye and the quencher are close to each other, resulting in quenching of the fluorophore in the stem-loop structure. If we add the target molecules that are complementary to the hairpin probe, hybridization between the molecular beacon and the target will occur, which opens the hairpin probe and allows the fluorophore to emit. Fluorescence from the dye would be observed. MB probes have been designed and validated for a wide range of diagnostic assays, including detection of PCR products, detection of sexually transmitted disease, viral load assays, and single nucleotide polymorphisms [40].

1.5 Enhanced Fluorescence

Enhanced fluorescence is typically associated with optical resonances. The most popular optical resonance employed for fluorescence enhancement is the surface plasmon resonance observed in metal structures, but ring resonances and guided-mode resonances observed in dielectric structures have been used as well. Optical resonances used for enhanced fluorescence are associated with strong evanescent electric fields upon illumination at the
resonance wavelength. The amplitudes of evanescent electric fields are many times higher than that of the incident light illuminating the sample and decay exponentially as they move away from the surface of the nanostructure. The high amplitudes of the evanescent fields result in enhanced fluorescence for two reasons. Firstly, the increased local excitation photon density results in more excited electrons. As fluorescence is essentially a spontaneous emission process, the proportion of excited molecules in a fixed population of fluorophores is directly proportional to photon density. We call it “enhanced excitation” throughout the text. Secondly, the resonator not only can interact with the excitation but also can directly affect fluorophore emission. For example, the high photon density of the evanescent electric field will increase the probability of photon emission. The presence of an optical resonator provides a pathway for a fluorophore to transfer its photons more efficiently than would be possible in free space. This effect has been showed by Purcell as early as 1946. Fluorophore’s emission is also influenced by directional emission of the resonance. The emitted photons are concentrated at a certain direction which is determined by the structure dispersion. The concentrated photons further increase the emission intensity. We call it ‘enhanced extraction’ throughout the text.

1.5.1 Metal Enhanced Fluorescence

Metal nanostructures have long been studied due to their extraordinary ability to manipulate incident lights. The very first metal structure that was studied for enhanced fluorescence was a thin metal film atop of a dielectric substrate [41-43]. The surface of metal can support surface plasmons, the collective oscillation
of electrons that propagates along the surface. The surface plasmons in thin metal films can be resonantly excited by photons whose wave vector component parallel to the interface matches that of the surface wave, using a prism or grating coupler. At a resonant condition, the evanescent fields of the surface plasmons can interact with the fluorophores. Fluorescence enhancement of ~3-7 folds has be reported using gold or silver thin films [41].

While the first demonstration of the enhanced fluorescence relies on the flat metal surface, recent research efforts, however, are mostly focused on metal nanoparticles with localized surface plasmon resonance (LSPR), possibly because of the higher magnitude of enhancement and ease of using the instrument. Localized surface plasmons (LSP) are electron density oscillations confined to metallic nanoparticles and nanostructures. LSPR occurs when the LSP is excited at the resonant incident wavelength. LSPR is typically associated with strong light scattering, with the appearance of intense surface plasmon absorption bands and an enhancement of the local electromagnetic fields. Various metal nanostructures have been demonstrated to enhance fluorescence output. For example, enhancements of silver nanoprisms were reported when the wavelengths of LSPR of the particles overlapped excitation and emission wavelengths of various dyes [44]. Significant enhancement (greater than 300x) of cyanine dyes in the presence of silver nanoparticles on silicon substrates has been demonstrated [45].

The field of surface-based metal enhanced fluorescence is hindered by several key limitations. First, enhancement factors are strongly influenced by the
distance between the radiating dipole and the metallic surface or particle. On the one hand, the fluorophore must be placed close to the surface because of the exponential decay of the electric field. On the other hand, the fluorophores cannot be too close to the surface since the quenching effect dominates when they are in proximity. Therefore, distance of tens of nanometers from the metallic surface must be achieved to obtain a maximum enhancement. Furthermore, metals are lossy and cannot support very high-quality factor resonant modes. The inability to store a large amount of energy places an upper limit on the enhancement that can be achieved through plasmon assisted fluorescence excitation. In order to overcome these limits, dielectric nanostructures have been proposed for enhancing the fluorescence.

1.5.2 Dielectric Enhanced Fluorescence

Dielectric-based devices have also been used in an attempt to improve the sensitivity of fluorescence detection. The first demonstration of the dielectric-based enhanced fluorescence is based on total internal reflection fluorescence (TIRF) [46]. It is a well-established technique in which light is coupled into a waveguide where it evanescently excites fluorophores on the device surface. The light is coupled into the waveguide with either a grating, a prism, or a high numerical aperture objective. By exciting only the fluorophores on the surface using evanescent coupling, background fluorescence can be minimized. The inherent spatial discrimination can yield dramatic improvements in signal-to-noise of the collected fluorescence.
Photonic crystal (PC), a corrugated waveguide structure with a periodic change of the refractive index, is an alternative substrate for enhancing fluorescence. The PC slab also demonstrates “evanescent resonance” as TIRF but could provide even higher enhancement of fluorescence compared to that of TIRF, because of the stronger evanescent electric field. The PC slab has a period that is smaller than the wavelength, resulting in the evanescence nature of the non-zero-order mode. A resonance in this structure is excited when these evanescent modes are re-radiated in-phase with the reflected zeroth-order wave and out-of-phase with the transmitted zeroth-order wave. Excitation of the resonances must be a certain combination of the incident wavelength and angle, which are usually obtained from the PC dispersive curves. These resonances are capable of enhancing fluorescence in a similar fashion to surface plasmon resonance, taking advantage enhanced excitation and enhanced extraction. The study of PC slabs by Budach and colleagues in 2003 demonstrated over 100x fluorescence enhancement for certain signals on a cDNA microarray on the PC slabs [47].

Recently, PCs have been engineered by our group to enhance the signal from the common microarray dye Cyanine-5 (Cy5) by more than three orders of magnitude [19] when scanned by a system that is optimized for coupling light to/from the PC. We have successfully demonstrated the detection for a panel of breast cancer biomarkers using these PC slabs, achieving a lower detection limit compared to that on glass slides [8], [18], [48], [49]. The resulting capability will address the needs of clinical diagnostics laboratories that currently lack the
capacity to easily measure the concentrations of large panels of biomarkers in blood for purposes of early diagnosis/screening, therapy selection, and routine treatment follow-up. As a greater number of autoantibody biomarkers are identified and validated for viral infection, cancer, asthma, and autoimmune diseases, such a platform will have broad utility.

1.6 Conclusion

This chapter introduces the concept of Photonic Crystal Enhanced Fluorescence (PCEF) and how it can be applied to solve the current problems in biomarker detection. Throughout this thesis, the field enhancement property of the 1-D PC is applied to enhance the signal intensity emitted from fluorophores that are tagged to different biomarkers. Chapter 2 will detail how the PCEF technology works and various approaches to advance this technology.
1.7 Figures

**Figure 1.1.** Applications and classifications of the fluorescence assays. There are mainly two types of fluorescence assays: the surface-based one and the liquid-based one. Surface-based fluorescence assays are typically utilized for multiplexed assays in an array format where the dye molecules are bounded to the surface. Liquid-based fluorescence assays, in contrast, are performed in a “homogeneous” format, with all assay components in a liquid environment, not coupled to a surface.
Figure 1.2. Schematic diagram of the ELISA micro-immunoassay. First, ELISA microarray is produced by printing an array of individual capture antibodies in micro-spots. After a “blocking” step inhibits subsequent nonspecific protein binding, the chip is exposed to a test sample, providing opportunity for analyte molecules to bind with their corresponding capture antibodies. A mixture of all the biotin-linked detection antibodies is prepared and incubated with the chip, where they bind an unoccupied epitope of the targeted captured antigen. The final assay step is the introduction of fluorophore-labeled streptavidin that attaches only to the biotinylated detection antibodies. Reprinted from http://www.hoelzel-biotech.com.
Figure 1.3. Schematic diagram of the molecular beacon assay. Molecular beacons (MB) contain a stem-loop hairpin probe that is flanked by two short self-complementary sequences. A fluorescent dye and a suitable quencher for that dye are attached at each terminal of the short sequences. The dye and the quencher are close to each other, resulting in quenching of the fluorophore in the stem-loop structure (1). If we add the target molecules that are complementary to the hairpin probe, hybridization between the molecular beacon and the target will occur, which opens the hairpin probe and allows the fluorophore to emit (2). Fluorescence from the dye would be observed. Reprinted from http://www.sigmaaldrich.com.
CHAPTER 2 PHOTONIC CRYSTAL ENHANCED FLUORESCENCE

2.1 Chapter Introduction

The Photonic Crystal Enhanced Fluorescence (PCEF) provides a low-cost, high sensitivity and multiplexed detection platform for biomarker research and disease diagnostics. A wide variety of PC structures have been studied and fabricated for an enormous range of applications since the periodic nanostructure was first proposed to enhance fluorescence [50]. In this research work, the PCs designed to enhance fluorescence are comprised of a periodically modulated low refractive index dielectric surface structure coated with a high refractive index dielectric thin film. The PC surfaces are engineered to interact strongly with any optical wavelengths of interest through selection of their materials and the parameters of their geometry [51, 52]. It takes advantage of PC resonances associated with two phenomena: “enhanced excitation” and “enhanced extraction”. The simultaneous occurrences of both enhancement mechanisms has been shown to boost the emission from dye molecules by greater than three orders of magnitude [17].

A complete PCEF system is comprised of a PC surface and detection instruments. The design of the PC is critical to achieve large fluorescence enhancement. To confirm the optical properties of the fabricated devices, we build an optical system to measure the reflection spectrum of the PC. As for the fluorescence detection, our custom detection instrument can effectively couple PC and measure fluorescence. In this chapter, various aspects of PCEF platform
are discussed. The effects of enhanced excitation and enhanced extraction will be explained first. Following that, a standard manufacturing process for integrated circuit that is implemented to fabricate PCs will be described. Then, two fluorescence detection instruments will be discussed including a confocal laser scanner and a laser line scanner. Applications discussed throughout this thesis are based on the exact same PC device and detection instruments as described in this section. Finally presented is a brief review of the previous work where PCEF have been utilized to improve the sensitivity of detection of a biological assay.

2.2 Principle of PCEF

The PC is designed to enhance the fluorescent intensity though the enhanced excitation and enhanced extraction mechanisms. In order to vividly show PCEF, Figure 2.1 is provided to demonstrate the effect of enhanced electromagnetic field intensity on the emission intensity of a surface-bound fluorophore (quantum dots, QDs 405). Quantum dots were dispersed on the PC surface (right) and the un-patterned surface (left) surrounding the PC (acting as the control for comparison) as marked by the circles. The quantum dot emission intensity image on the top (Figure 2.1(a)) represents the condition when the PC is resonant (resonant wavelength $l = 405$ nm at $q = 11.2^\circ$) with respect to the incident laser beam ($l = 405$ nm), showing an enhancement factor of over 108 times with respect to the control. This enhancement is the result of both enhanced excitation and enhanced extraction. Even if PC is not resonant, some enhancement still exists due to the effect “enhanced extraction”. The image on
the bottom (Figure 2.1(b)) is the emission intensity image when the PC is not resonant with the incident laser \( (q = 0^\circ) \), i.e. without any pockets of strong localized electromagnetic fields. For this case the PC shows enhancement of 13 times with respect to the control. Both enhanced excitation and enhanced extraction (the off-resonant enhancement) will be explained in the following section.

2.2.1 Enhanced Excitation

The increase in the evanescent electric field intensity of the excitation is called “enhanced excitation”. The enhanced excitation can be achieved by illuminating the PC at a specific incident angle for a given laser illumination wavelength [53]. At the resonance coupling condition of the PC, the enhanced electric fields are confined to the surface of the device and extend into the adjacent media with exponentially decaying intensity, and thus only surface-bound fluorophores will be exposed to enhanced energy from the laser. In addition, these optical resonances can be engineered to have a very high electromagnetic field density on the surface of a PC during their finite lifetime. The magnitude of this electromagnetic density localized within the PC is directly proportional to the quality factor of the resonance or its lifetime, which in turn is controlled by adjusting the device parameters. The intensity of emission from the fluorophores can be greatly enhanced by placing them in the proximity of the PC surface which has its resonant wavelength overlapping the absorption spectrum of the fluorescent species due to an enhanced absorption rate. Since the resonant response of the structure is different depending on the polarization
of the incidence, with Transverse Magnetic (TM) resonances being narrower in linewidth, implying a higher quality factor for the same device structure, TM resonances have higher electromagnetic field density on their surfaces and are preferred to Transverse Electric (TE) resonances for the purposes of enhanced excitation.

2.2.2 Enhanced Extraction

Due to the resonant effect, the emission from a fluorophore near a PC surface can couple to one of the resonant modes of the PC and then emerge out in an angle-dependent direction. This provides us with a powerful mechanism where the emission can be concentrated within specific directions and therefore detected with much higher sensitivity. Such a scheme is particularly useful in improving the detection efficiency of instruments and can further help in reducing the detection limits of the fluorescent species. In most fluorescence detection instruments the detection optics are kept directly above the emitting species. When fluorescent species bound to the PC surface are imaged with such instruments, the PC is engineered so as to direct the coupled radiation normally toward the detection optics. This enables the majority of the emitted radiation to be detected efficiently, unlike the radiation emitted by a fluorophore on the glass substrate with its emission almost uniform in all directions. Experiments have been performed to verify this process and, understand its role, in fluorescence enhancement for PCs possessing different symmetries [54]. It has been shown that for a narrow spectral and angular detection instrument, a PC with increased symmetry leads to greater
enhancement of emitted radiation in the normal direction. This has been attributed to increase in the number of diffraction planes that can interact with the omnidirectional radiation.

The effects of “enhanced excitation” and “enhanced extraction” act independent of each other. Hence when they act together, their effects get compounded. These two effects can be independently optimized to design a PC that provides very large fluorescence enhancement factors. With the combined effect of the two, enhancement factors as large as 7500 have been demonstrated [17], making PCs a very desirable platform for a wide array of fluorescent biological sensing applications.

2.3 PC Structure Design and Fabrication

The PC is comprised of a periodic surface structure fabricated in a low refractive index (RI) silicon dioxide (SiO₂) layer on a silicon substrate. The grating structure is coated with a high RI titanium dioxide (TiO₂) thin film. The schematic diagram of the PC is shown in Figure 2.1(a). Simulation results dictated the use of a structure with a period of 360 nm, a duty cycle of 36%, a grating depth of 40 nm, and a TiO₂ thickness of 120 nm.

A commercially available simulation tool for rigorous coupled wave analysis (DIFFRACTMOD, RSoft) was used to aid in the optimal design of a PCEF device. The end goal of the design is to achieve high enhancement and overlap the resonant peak of PC with the excitation peak of the fluorophore. Two factors mainly determine the resonant peak location including the period of the
grating and the thickness $t$ of the TiO$_2$ layer. Because of the phase match condition, the resonant wavelength $\lambda$ must satisfy: $\lambda = n_{\text{eff}} \Lambda$. Here $n_{\text{eff}}$ is the effective index of the resonant mode at the resonant wavelength of $\lambda$ for a PC with grating period $\Lambda$, which is affected by thickness $t$. The resonant peak location is far more sensitive to the change of the period $\Lambda$ than thickness $t$. In the simulation, the period $\Lambda$ was first determined to overlap with the emission peak of Cy5 while thickness $t$ was slightly modified to fine tune the resonant peak. The enhancement is mainly determined by grating depth $d$. The grating in a PC is a diffractive element that couples the light into and out of the PC and thus is a source of the diffraction loss in the PC. The diffraction loss is directly related to its diffraction “strength” which is essentially related to its depth $d$. As the depth of grating decreases, the linewidth of its resonance also decreases thus increasing the Q-factor of the structure and the enhancement provided by the grating. A linewidth of 0.02 degree is achieved at the grating depth of 40 nm. Given that the detection instrument has the angle tolerance of 0.02 degree, we set the grating depth to be $d = 40$ nm.

Advantages of using silicon as the substrate material of PC include its relatively low autofluorescence and its wide use in industrial manufacture. Previously, we fabricated plastic PC by nano-replica molding and demonstrated enhancements of SNR $\sim$6.6 folds for detection of a panel of cancer biomarkers in a protein microarray format [8]. However, the plastic substrate with high autofluorescence increases background and limit the fluorescence detection. Then we switched to a quartz substrate [18, 55] fabricated by a nano-imprint
lithography process. Although quartz substrate has extremely low autofluorescence thus showed a 330X SNR improvement for the detection of spotted Alexa-647 labeled polypeptide on PC, the nanoimprint approach is complicated, slow and unstable. That is why we finally fabricated these PC devices over the area of 8” diameter silicon wafers using lithography. A thermal oxide (thickness of 800 nm) was grown on each Si wafer and a deep UV photolithography process (193 nm, ArF-line) was used to create the grating pattern in the oxide layer. SEM image of the PC surface is presented in Figure 2.2(b)(c). The device was then coated with TiO$_2$ and diced into 1.0 x 0.5 inch$^2$ pieces. Photo of the diced piece is presented in Figure 2.2(d).

The effectiveness of the light coupling into PC will determine the fluorescence enhancement. The process of determining the coupling condition of a PC involves the measurement of the wavelength spectra associated with the PC. It was experimentally determined by illuminating the PC by a collimated white light source, while the reflected light was detected by a spectrometer (Ocean Optics Inc.). The device was mounted on a rotational holder, and the reflection spectra at different incident angles were recorded utilizing a commercial software package (Spectra suite, Ocean Optics Inc.). The spectrum with the resonant peak at $\lambda = 637$ nm indicates the condition where the laser can be efficiently coupled into the PC structure. The incident angle corresponding to this spectrum is recorded as the excitation angle to obtain maximum fluorescence enhancement.
2.4 Detection Instruments

2.4.1 Confocal Laser Scanner

A confocal microarray laser scanner (LS Reloaded, Tecan Inc.) was the first detection instrument used to study PC enhanced fluorescence. The schematic of the scanner is shown in Figure 2.3. This system is equipped with a He-Ne laser and a solid-state Nd-YAG laser as the excitation sources and a photomultiplier tube (PMT) to detect the emitted fluorescence signal. The angle of incidence of the lasers can be tuned from 0° to 25°. In order to form an image, the substrate is scanned and the fluorescence signal intensity for each pixel is acquired. The instrument uses a lens to focus the laser beam onto the sample and collects the fluorescence signal resulting from this excitation. Due to the focusing effect, the laser beam has a beam divergence ~ 2.5°. Since the beam is not collimated, a substantial portion of the excitation energy does not get coupled into the resonant mode of the PC, thus compromising the enhancement performance of the PC.

2.4.2 Laser Line Scanner

A custom detection instrument was built to optimize coupling of laser illumination to the PC surface. As described in previously published reports [56], the excitation laser is collimated in the plane perpendicular to the grating lines, but focused in the plane parallel to the grating (Figure 2.4). According to the simulation model, we need collimated light in order to most efficiently couple with the PC resonant mode, but the light need only be collimated with respect to a single axis. Along the orthogonal axis, light can be focused without
compromising coupling efficiency to the PC, and thus focus along one axis is used to achieve high illumination intensity. The dispersive curves of PC in Figure 2.5 prove that the coupling efficiency is only related to incident angle along one axis.

The system is designed for optimal interaction with the PC for both enhanced excitation and enhanced extraction, using design principles discussed, modeled, and demonstrated in [19]. The focal point of the cylindrical lens is located at the back focal plane of the objective, resulting in a line of illumination in the PC that is collimated along the angle perpendicular to the PC grating lines, but focused in the orthogonal direction. Linear translation of the cylindrical lens results in adjustment of the incident angle to achieve “on resonance” illumination. A semiconductor laser diode (AlGaAs, 70 mW, $\lambda = 637 \text{ nm}$) is expanded to a diameter of 1 mm, and focused to an 8 µm wide line onto the PC surface by a cylindrical lens. A mirror coupled to a computer-controlled linear translation stage enables adjustment of the incident angle from 0-20 degrees with 0.01 degree minimum increments. The grating lines of the PC are oriented perpendicular to the scan line, allowing the laser (polarized output perpendicular to the grating) designed to excite the TM mode of the PC.

2.5 PCEF for Multiplexed Protein Biomarker Detection

With the use of PCEF and the customized detection platform, we have successfully detected a panel of >20 breast cancer biomarkers in a protein microarray format [57, 58]. Our results show that the resonant excitation effect increases the signal-to-noise ratio by 3.8-fold to 6.6-fold, resulting in a decrease
in detection limits of 6-89%, with the exact enhancement dependent upon the antibody-antigen interaction. Due to the enhancement provided by photonic crystal antibody microarrays, we detected common cancer biomarkers in the <2 pg/mL concentration range within a mixed sample.

Capture antibodies were first printed on the slide; replicate arrays are printed to assess the experimental variability in each slide. Following this, the slide was blocked to limit the non-specific binding of analytes in subsequent steps. The slide was then incubated with a test sample consisting of a mixture of biomarkers. Next, the slide was washed to remove all unbound biomarkers and then incubated with a mixture of biotinylated secondary detection antibodies. Finally, the secondary detection antibodies were labeled by incubation with streptavidin-Cy5 (Figure 2.6). We generated a standard curve for all the biomarkers by assaying a concentration series that covers a 10000-fold range of protein concentrations.

The PC enhancement of fluorescence was determined by comparing the fluorescence output when the PC is illuminated with the excitation laser at an incident angle matched to the PC resonant angle (“on resonance”) to that when the illumination angle is not at the PC resonance (“off resonance”). The array exposed to the second-highest concentration in the dilution series were used to determine the enhancement factor. Their fluorescence images at on-resonance condition and off-resonant condition were collected and their fluorescence intensities were quantified by ImageJ. The fluorescence signal intensity was
enhanced by a factor of 11-fold to 20-fold by illuminating the PC at its resonant condition (Figure 2.7).

Any fluorescence within the evanescent field region can be enhanced by PC, whether the source of the fluorescence is a Cy5 molecule bounded to the analytes, a Cy5 molecule captured by surfaces outside spots region, or autofluorescence from either the cover material or chemical functionalization layer. In fact, the background intensity is 4 to 5 times higher when the PC is on-resonance compared to that at the off-resonance condition. However, we still observed an increase in SNR by 3.8-folds to 6.6-folds for the assays due to the fact that the magnitude of the PC enhancement within the capture spots region is greater than that in the regions between the spots. It is important to confirm enhancements in SNR for detecting antigens at low concentrations as this indicates an improvement in detection limit. For example, two antigens EGFR and uPAR were detected at concentrations as low as 3.6 and 7.1 ng/mL with PC on-resonance (SNR > 8), while the spot signals for EGFR and uPAR at these same concentrations were noise-limited (SNR < 3) and could not be differentiated from the local background fluorescence at off-resonance condition. Improved detection sensitivity of such antigens is beneficial to the early diagnosis of cancers, because cancer biomarkers in general are present at very low concentrations in serum. Standard curves were generated with the use of the signal intensities from each dilution in the concentration series. A representative standard curves for TNFa when the PC is on-resonance and off-resonance is presented in Figure 2.8. We found that when on-resonance, the PC
demonstrated better precision as indicated by the steeper slope in the linear region of the standard curves, and ~10-fold reduced limit of detection.

### 2.6 Conclusion

Our work is focused on applications of PCEF, for both surface-based fluorescent assays and homogeneous fluorescent assays. Following the work of the detection of soluble protein in human by applying PCEF to protein microarray, our research efforts are devoted to the detection of autoantibodies in human serum. Autoantibodies detection attracts increasing attentions due to their importance in infected diseases, allergy and cardiovascular diseases. Chapter 3 shows that it is feasible to detect allergy-specific antibodies in human at low concentrations using the PCEF array system. In order to transfer the PCEF technology from a laboratory setting to a clinical tool or even a point-of-care (POC) device, our next improvement of the system is focused on assay automation that requires integration of the PC surface and microfluidic devices. In Chapter 4, we successfully demonstrate a platform that integrates photonic crystal enhanced fluorescence (PCEF) detection of a surface-based microspot fluorescent assay with a microfluidic cartridge to achieve simultaneous goals of high analytic sensitivity (single digit pg/mL), high selectivity, low sample volume, and assay automation. On the other side, PCEF can also be used for homogeneous assay. Combined with the sub-micron-height channel, the PC surface successfully enhanced the fluorescence output from a molecular beacon assay. The sensitive detection of miRNA was realized on the PCEF platform and the results are summarized in Chapter 5.
2.7 Figures

Figure 2.1 Fluorescence (pseudocolor) scan images of the PC with quantum-dots dispensed on the surface. The circular regions represent the area where the quantum dots are located. (a) Fluorescence from the PC surface was enhanced by 108 times compared to that on glass, when the PC is resonant with respect to the incident beam ($q = 11.2^\circ$). This enhancement effect is the combination of enhanced excitation and enhanced extraction. (b) Scan taken when the PC is not resonant with the incident beam ($q = 0^\circ$), showing an enhancement factor of over 13 times. This enhancement effect only includes enhanced extraction effect. (Reprinted from [53].)
Figure 2.2. (a) Schematic of the silicon PC device design. (b) Cross-sectional view of grating pattern in SiO\(_2\) layer before TiO\(_2\) coating. The grating line width and grating depth were measured to be 131 nm and 37.7 nm, respectively. (c) Top view of PC after TiO\(_2\) coating. The grating period was measured to be 366 nm. (d) Picture of finished device diced into a 1 x 0.5 in\(^2\) piece. Images are reprinted from [19].
Figure 2.3. Schematic diagram of the confocal laser scanner. Reprinted from [59].
Figure 2.4. Schematic of the objective-coupled, line scanning instrument used to acquire fluorescence data at the precise PC resonant angle. Reprinted from [19].
Figure 2.5 (a) schematics of PC structure. Incident angles that are perpendicular to grating (Φ) and parallel to grating (θ) are specified. Dispersive curve of PC along (b) θ and (c) Φ are provided. Reprinted from [56].
Figure 2.6. (a) Schematic diagram of the protein microarray assay format. Each microscope slide is divided into 16 wells and each well consists of 20 capture antibodies. (b) A representative fluorescence image of one block for 20+ biomarkers; spot color is representative of biomarker concentration.
Figure 2.7 (a) Fluorescence images of protein microarray spots after exposing to a mixture of 20 biomarkers. The left image was obtained when PC was illuminated at resonant angle while the right image was detected at off-resonance condition. (b) Comparison of the replicate-averaged fluorescence intensity at PC resonance and off PC resonance for all functional assays in the array. Error bars indicate +/- one standard deviation of 8-replicate spots. (Reprinted from [57].)
Figure 2.8 Dose-response curves for TNF-a at PC resonance condition (solid curve) and off resonance (dashed curve) condition. The detection sensitivity, which is represented by the slop in the linear region, is higher at its resonance condition compared to that at off-resonance condition. Sensitivity here is defined as the change in fluorescence signal per unit change in its concentration. (Reprinted from [57]).
CHAPTER 3 APPLICATION OF PHOTONIC CRYSTAL
ENHANCED FLUORESCENCE TO ALLERGY TEST

3.1 Chapter Introduction

We demonstrate detection of low-concentration allergen-specific Immunoglobulin E (IgE) in human sera using a Photonic Crystal Enhanced Fluorescence (PCEF) microarray platform. The PC surface, designed to provide optical resonances for the excitation wavelength and emission wavelength of Cy5, was used to amplify the fluorescence signal intensity measured from a multiplexed allergen microarray. Surface-based sandwich assays were used to detect specific IgE antibodies against a highly purified cat allergen (Fel d1). A comparison of the lowest detectable concentration of IgE measured by the PC microarray system and a commercially available clinical analyzer demonstrated that the PCEF microarray system provides higher sensitivity. The PCEF system was able to detect low concentrations of specific IgE ~0.02 kU/L, which is 5-fold to 17-fold more sensitive than the commercially available FDA-approved analyzers. In preliminary experiments using multi-allergen arrays, we demonstrate selective simultaneous detection of IgE antibodies to multiple allergens. The PCEF microarray system holds great promise in future point-of-care diagnostic tests using very small blood samples from patients with clinical allergy symptoms.

3.2 Motivation

In industrial countries, more than 20% of the population suffers from type I allergies (i.e. Immunoglobulin E), representing a major health problem in the
Western world [60-62]. The clinical evaluation of allergic disorder typically involves use of the clinical history, physical examination and a test to confirm sensitization to the allergen [63]. Sensitization can be measured by skin testing with allergen extracts [64-66] or blood tests [67-71]. A skin test is done by monitoring a patient’s reactions after a small amount of a suspected allergen is placed on or below the skin, while a blood test is an immunoassay that measures the concentration of Immunoglobulin E (IgE) against specific allergens in the patient’s blood. Blood tests have several advantages over skin tests. First, they are more convenient as they involve only a standard blood draw, and might be safer since they are performed *in vitro*, and thus do not expose the patient to allergens [72, 73]. Second, blood tests are performed instead of skin tests for patients who have severe symptoms and cannot stop taking medication [74]. Moreover, studies have shown that the total amount of IgE against some allergens can predict the severity of symptoms [75, 76].

The currently available analysis systems for blood tests primarily rely upon crude allergen extracts prepared from various allergen-containing biological materials (e.g. pollens, foods, etc.). These extracts contain a variety of allergenic and non-allergenic components and are often difficult to standardize with respect to their allergen content or potency. Therefore, extracts-based diagnostics may not adequately discriminate between patients who are sensitized to different allergen sources [77, 78]. For the same reason, it is difficult to provide accurate allergy therapy to individual patients if poorly defined allergen extracts are used during diagnostics. However, by applying current protein and DNA technology to
the field of allergen identification, it is now possible to produce the major allergens for the most prevalent allergies in a purified form [79, 80]. Using these purified allergens, each individual patient’s treatment can be tailored according to his/her sensitization profile. Since a variety of recombinant allergens is available, a comprehensive monitoring of the patient’s IgE reactivity profile to a great number of different allergen molecules requires a new type of test that can provide multi-allergen detection.

In addition to the fact that the present platforms fail to meet the multiplexing need for personalized therapy, there is substantial interest in reducing the limits of detection (LOD) and generally increasing the signal-to-noise ratio (SNR) in order to diagnose allergies in patients whose IgE levels are at very low concentrations. Because specific IgE levels depend on age, total serum IgE and the time of year tested, some patients are diagnosed as “negative” for an allergy due relatively low IgE levels that are below the detection threshold of existing technology [81, 82]. Moreover, for point-of-care methods with capillary blood, the specimen may need to be diluted below the test instrument’s lower limit of quantitation [82, 83]. However, most commercial autoanalyzer systems to measure specific IgE have detection limits in the 0.10 to 0.35 kU/L range (1 U = 2.4 ng) [82, 84, 85]. To achieve the needed sensitivity, a platform with sufficient signal amplification, employing highly purified and/or recombinant allergens is needed [86].

As shown in Chapter 2, we have demonstrated a photonic crystal enhanced fluorescence (PCEF) microarray system that can achieve high
sensitivity for multiplexed cancer biomarker detection at low sample volume [8, 19, 53]. In this dissertation, we extend the application of the PCEF technology to a prototype allergy testing platform to achieve multiplexed, sensitive and specific IgE detection. This approach allows successful detection of allergen-specific IgE at low concentration, using small volumes of human serum. We found that our platform has higher sensitivity than a standard analyzer used for allergy immunoassays throughout the world [87]. In addition, the PCEF platform permits multiplexing of allergens, provides signal quantification, and detects several allergen-specific antibodies simultaneously on a single chip. It holds great promise in future point-of-care diagnostics for patients with allergy symptoms.

3.3 Methods

3.3.1 PC Fabrication and Characterization

The PC is designed and fabricated as described in Chapter 2. The PC resonant coupling can be observed by measuring the reflected intensity of laser illumination as a function of incident angle, as shown in Figure 3.1, where the peak reflected intensity corresponds to the angle at which optimal coupling occurs. Here, an incident angle of $4.12^\circ$ achieves the resonance condition. Our previous work demonstrated that the PC also provides a second resonance at $\lambda \sim 685$ nm that provides an enhanced extraction effect [49].

3.3.2 Source of Materials

We sought to test the feasibility of PCEF for selective characterization of the presence of allergen-specific IgE using three different allergen materials
(Timothy grass extract (Hycor Biomedical), cat hair & epithelium extract (Hycor Biomedical) and Fel d1, a highly purified protein from cat extract (Indoor Biotechnologies, Charlottesville). The human sera used in these studies were provided by Viracor-IBT Laboratories and Hycor Biomedical, Inc. These discard sera had previously been tested for various specific IgEs with a standard clinical analyzer used for allergy testing (ImmunoCAP, ThermoFisher, Fremont CA).

3.3.3. Preparation and Allergen Printing on the PC Surface

A single 0.5 x 1.0 in$^2$ PC die holds 10 subarrays, and each subarray contains four sets of four replicate spots per protein for a total of 16 spots. Before allergen printing, the PC surface was cleaned and activated with a vapor-phase epoxysilane process. The epoxysilane chemistry was chosen for its low background fluorescence [88] and high binding capacity to capture antibodies [89]. The devices were first cleaned by sonication in 2" petri dishes of acetone, isopropanol, and deionized (DI) water for 2 minutes each. The devices were then dried in a stream of N$_2$ and then treated in an oxygen plasma system (Diener, Pico) for 10 minutes (power of 100 W, pressure of 0.75 mTorr). The backside of each device was then adhered to the inside of a screw top lid of a 2" glass container. At the base of the container, 100 µL of (3-Glycidoxypropyl) trimethoxysilane (GPTS, Sigma Aldrich, Saint Louis, MO) was placed and the screw top lid was securely placed over the dish. After securely tightening the lids, each dish with a device adhered to its lid was placed in a vacuum oven for an overnight incubation at a temperature of 80 °C and a pressure of 30 Torr. The devices were then detached from the lids and
sonicated in 2" petri dishes of toluene, methanol, and DI water for 2 minutes each and dried under a stream of N₂.

The allergen microarrays were printed by a commercially available instrument (Arrayit NanoPrint LM60 Microarrayer) which provides a controlled environment (ambient temperature and 50% relative humidity). Four pins (946MP3 Microarray Printing Pins) were used to print the sixteen spots. Printing pins were cleaned between sample pickups with 15 s sonication, four cycles of washing (2.5 s) in DI water and drying (1 s). Measured spot diameters were 79.00 ± 2.22 µm. Row spacing was 149.25 ± 3.26 µm and column spacing was 200.75 ± 0.82 µm. The printed slides were incubated in a sealed box with a desiccant overnight at ambient temperature before use.

It is well known that proteins deposited onto solid surfaces display distinct characteristics due to differences in charge, molecular structure, acidity, specificity, affinity, hydrophobicity and stability. The diversity of protein structures poses a challenge for identifying a universal assay surface that maintains capture protein functionality equally for all the capture probes in a microarray, as discussed in the literature [90, 91]. In this dissertation, we studied the optimized spotting concentrations by incubating the serum with different concentrations of allergen spots. Both allergen extracts (cat and Timothy grass extract) and a highly purified allergen (Fel d1) were used to produce the probe spots. Fel d1 is the major allergen in cat hair and epithelium extracts, and is expected to have better specificity than the cat extracts, which are complex mixtures of proteins. The initial protein concentrations for the cat
extract were 0.22 mg/mL, 1.3 mg/mL and 3.4 mg/mL respectively. Each of the allergen solutions was spotted over four concentrations in a two-fold dilution serial and each subarray had 16 spots with four replicate spots per each concentration. A fluorescent image of the assay that was incubated with positive sera was used to identify the printing concentration with the highest fluorescent intensity. The results (Table 3.1) indicate that the highest concentration was optimal for these three allergens to obtain strong fluorescent signals. Therefore, allergen spots were printed at the highest available concentrations for the remainder of our study.

<table>
<thead>
<tr>
<th>Printing concentration</th>
<th>Average signal from cat extract spots</th>
<th>Average signal from Fel d1 spots</th>
<th>Average signal from grass extract spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original concentration</td>
<td>10365±1544</td>
<td>23482±212</td>
<td>11089±228</td>
</tr>
<tr>
<td>2-fold dilution</td>
<td>5971±310</td>
<td>17342±3900</td>
<td>6114±342</td>
</tr>
<tr>
<td>4-fold dilution</td>
<td>3169±337</td>
<td>16525±2689</td>
<td>4450±1415</td>
</tr>
<tr>
<td>8-fold dilution</td>
<td>3821±260</td>
<td>13001±2408</td>
<td>2644±188</td>
</tr>
</tbody>
</table>

### 3.3.4 Testing Procedures

The slides were placed in a 10-well custom-made slide module assembly (Figure 2(b)) where each well can hold a 10 µL test sample. The arrays were blocked with a blocking buffer that contains 10 mM sodium phosphate, 0.5 M sodium chloride, 0.02% (w/v) tween 20, 1% (w/v) human IgG, 25% (w/v) human serum albumin and 1% (v/v) ProClin 950 (Hycor Biomedical, Inc.) for 1 h. All incubations were performed at room temperature. The arrays were then
washed five times with a wash buffer that contains 4.5% (w/v) NaCl, 0.25% (v/v) Tween-20, 15% (v/v) Propylene glycol and 0.05% (v/v) ProClin 950 (Hycor Biomedical, Inc.). Next, each well was incubated with 10 μL human serum or serum dilution in blocking buffer overnight. This was followed by five rinses after which the PC surface was incubated with only 10 μL of the biotinylated detection antibody mixture (mouse monoclonal B3102E8 anti-human IgE and mouse monoclonal HP8029 anti-human IgE, at the mixing ratio of 1:1, Abcam Inc.) in each well of the slide module assembly for 4 hours. The PC substrates were then washed five times followed by incubation with a 1 μg/mL solution of Cy5 conjugated streptavidin (Invitrogen) for 30 minutes. Finally, the devices were washed five times and dried in the ambient environment.

3.3.5 Image and Data Acquisition

The substrates were scanned with a commercially available confocal laser microarray scanner (Tecan LS Reloaded, as described in Chapter 2). This scanner was fitted with a 632.8 nm 5 mW laser for Cy5 excitation and a Cy5 emission filter (bandpass, 670-715 nm). The incident light was TM polarized and made incident on the substrates at an angle of 4.12° so that maximum laser coupling efficiency could be achieved. Scans were obtained at a resolution of 10 μm and the photomultiplier tube (PMT) gain was adjusted to 80 so that the largest fluorescence intensities did not saturate the PMT. Fluorescent images were analyzed using ImageJ to compute spot and local background intensities as well as standard deviations for each spot.
3.4 Results

3.4.1 Selectivity of the Assay

We constructed a testing well on the PC chip by creating four-plexed allergen arrays for simultaneous allergen-specific IgE antibody detection with low sample volumes (10 μL). As shown in Figure 3.2(a), the first row is cat hair extract, the second row is Fel d1, the third row is Timothy grass extract and the last row is Alexa-Fluor-555 fluorescent streptavidin conjugates to identify location of protein spots (Life technologies). As discussed earlier, the cat hair extract, the Fel d1 allergen, the Timothy grass extract and the positive control were printed at concentrations of 0.22 mg/mL, 1.3 mg/mL, 3.4 mg/mL and 10 ug/mL respectively.

To validate our platform using low sample volumes, we obtained serum samples that had been tested previously with a standard clinical immunoassay analyzer. Approximately 10 μL of human serum was used to expose the PC arrays. After processing, we analyzed the chips in a microarray scanner to detect and quantify the signals. Some of the rows of spots had high variances across spots after quantifying the fluorescence images. This issue is possibly related to problems with sample application and washing in the prototype platform or with the allergen extract variabilities.

The assays exhibited excellent selectivity. If a patient’s serum contained specific IgE antibodies against one or more allergens, we detected positive signals specifically on the respective allergen spots. With this technique, we were able to distinguish the serum samples with different amounts of grass-
pollen and Fel d1 specific antibodies (Figure 3.2(c)). For example, as shown in Figure 3.2(d), we detected strong fluorescence on grass pollen spots for 4-fold dilution of serum #85228 which had high-level grass-pollen specific antibodies, while the fluorescence was invisible for 4-fold dilution of serum #924365 that had a concentration less than 0.1 kU/L. In addition, we observed fluorescence from Fel d1 spots for both sera that was previously shown to contain IgE antibodies against Fel d1. No fluorescence was detected for either grass pollen or Fel d1 spots in the cases of negative serum incubation.

3.4.2 Sensitivity of the PCEF Array System

Two human sera (serum #85228 and #924365) that contain cat hair-specific IgE were tested over a range of eight concentrations in a 2-fold dilution series. To assess the sensitivity of IgE testing by PC microarray, the same original sera were also analyzed by ImmunoCAP, an automated instrument system widely used for allergy testing [92, 93].

The fluorescent images of microspots at different antibody concentrations are presented in Figure 3.3. Note that Fel d1 spots have higher fluorescence intensities than cat extract spots for the same serum dilution, which confirms that purified allergen has stronger binding affinity and thus higher sensitivity than the crude extracts. To quantitatively characterize how easily a spot can be distinguished from the noise, we define signal to noise ratio (SNR) as the net signal intensity divided by the standard deviation of the background intensity. A spot with SNR larger than 3 is regarded as detectable. Figure 3.3 shows that all of the Fel d1 spots were detectable over the 0.005 –
4.000 kU/L range. Even the average SNR over four Fel d1 replicates at the lowest concentration (0.005 kU/L) is 5.16. However, in order to determine the lowest detectable concentration, we need to create a dose response curve and compare fluorescence intensities at different concentrations with the background intensity.

The signal intensities from each dilution in the concentration series were used to generate a standard curve (Figure 3.4) for the Fel d1 allergen using Origin. The limit of detection (LOD) is defined as the concentration corresponding to the background intensity that is the blank intensity (i.e., the intensity of the negative control spot of diluent) plus 3 standard deviations from all assay spots. Negative controls performed by exposing the capture allergens to a negative serum sample resulted in no observable fluorescence signal above the background. The black solid line in the inset represents the background intensity, while the dashed line indicates the blank intensity from the negative control. From the inset of Figure 3.4, we can tell that fluorescence intensities from Fel d1 spots assayed with diluted sera with antibody concentrations of 0.005 kU/L and 0.011 kU/L were below the background intensity although they were observable in the image. Therefore the LOD value for antibody specific to Fel d1 is ~0.02 kU/L, which is lower than the lowest detectable concentration of 0.1 kU/L measured by ImmunoCAP system. With regard to the model allergen used, PCEF technology appears to be more sensitive than the ImmunoCAP system.
Fluorescent images of grass-positive serum (Serum#85228, Figure 3.5) were also used to characterize the LOD value for grass extracts. The starting serum is diluted by 2 times. As shown in Figure 3.5, the 32-folds serum dilution is the lowest detectable one, since the three highest dilutions are all below the lower bound of the fitted curve. The strong fluorescence from the dilutions that were below the detection limit is likely due to the non-specific binding from the crude extracts. We expect the availability of the purified grass allergen will results in lower detection limit using the PCEF system.

3.4.3 Calibration of Assay: Translating PCEF Readings into IgE Concentrations

To facilitate the use of the described PCEF assay in clinical labs, we calibrated the assay by reference to specific IgE concentrations determined by the ImmunoCAP assay. This assay generates results in kU/L specific IgE and is based on a IgE curve using an international standard IgE preparation. The kU/L results are used by clinical allergists to stratify their patient sensitivities and to make decisions about treatment. By fitting the standard curve in Figure 3.4, a four-parameter logistic function was derived as

\[ F = \frac{46489.3}{(1 + 101.7^{(0.5 - \text{[con]})})} + 865.7, \quad (3.1) \]

where F is the fluorescence intensity and \([\text{con}]\) is the concentration of the antibody. Using equation (3.1), we were able to calculate the IgE concentration of a serum sample by detecting the fluorescence intensity of its specific allergen spots. The previous sera that were used to generate the standard curves were
tested again on each PC chip in order to normalize intensities across chips for the other assays. The fluorescence signal from the immunoassay that has the smallest deviation from the value in the standard curve was used for normalization.

**3.4.4 Application of Calibrated Results to Additional Sera**

We obtained human sera from 15 individuals for whom we had previously determined specific IgE values and used the PCEF microarray system to test them. Fluorescence images were analyzed and their readings were translated into values of antibody concentrations. Figure 3.6 contains representative images of the allergen spots assayed with sera at various Fel d1-specific IgE levels ranging from 0.089 kU/L to 40 kU/L. These IgE levels were measured by the ImmunoCAP system. The calculated concentrations of Fel d1-specific antibody from the PCEF array system are shown in Table 3.2. Note that Fel d1-specific IgE in serum #9 was only detectable by the PCEF array system, which confirms its high sensitivity. The deviation of calculated IgE levels between the PCEF array system and ImmunoCAP system might be attributed to differences in surface chemistry, allergen activity after binding, fluorescence reporter and detection instrument.
Table 3.2. Autoantibody concentrations from 15 serum samples measured by the ImmunoCAP system and PC microarray system

<table>
<thead>
<tr>
<th>Serum Number</th>
<th>Fel d1 ImmunoCAP Value (kU/L)</th>
<th>Fel d1 PC Microarray Value (kU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.835</td>
<td>0.497</td>
</tr>
<tr>
<td>2</td>
<td>0.645</td>
<td>1.486</td>
</tr>
<tr>
<td>3</td>
<td>1.345</td>
<td>2.422</td>
</tr>
<tr>
<td>4</td>
<td>1.680</td>
<td>1.467</td>
</tr>
<tr>
<td>5</td>
<td>1.475</td>
<td>1.189</td>
</tr>
<tr>
<td>6</td>
<td>2.385</td>
<td>2.966</td>
</tr>
<tr>
<td>7</td>
<td>BLOD*</td>
<td>BLOD</td>
</tr>
<tr>
<td>8</td>
<td>BLOD</td>
<td>BLOD</td>
</tr>
<tr>
<td>9</td>
<td>BLOD</td>
<td>0.089</td>
</tr>
<tr>
<td>10</td>
<td>5.920</td>
<td>6.816</td>
</tr>
<tr>
<td>11</td>
<td>4.000</td>
<td>8.520</td>
</tr>
<tr>
<td>12</td>
<td>6.300</td>
<td>15.594</td>
</tr>
<tr>
<td>13</td>
<td>5.240</td>
<td>13.216</td>
</tr>
<tr>
<td>14</td>
<td>4.560</td>
<td>4.134</td>
</tr>
<tr>
<td>15</td>
<td>40.000</td>
<td>28.986</td>
</tr>
</tbody>
</table>

* BLOD means the concentration of Fel d1-specific IgE is below LOD.

3.5 Conclusion

Allergy blood tests measure levels of IgE against specific allergens such as foods, inhalants, medications, latex and venoms. These tests can confirm the diagnosis of an allergy disorder, supplementing a clinical history consistent with an immediate allergic reaction. Unfortunately, recognition of some food or inhalant allergens by IgE, which constitutes the least abundant class of immunoglobulins, requires a highly sensitive detection platform that is not currently available commercially. In addition, multiplexing capability is needed to fulfill the requirements for component-resolved diagnostics and personalized therapies.
In this work, a PCEF microarray platform successfully detected low-concentration IgE in human sera. The PC surface designed to provide optical resonances for the excitation wavelength and emission wavelength of Cy5 was used to amplify the fluorescence signal intensity measured from a multiplexed protein microarray. A surface-based sandwich assay was used in which secondary antibodies were exposed to the array after analyte hybridization, while a SA-Cy5 label was used to tag the secondary antibodies. Comparison of the LOD measured by a commercially available antibody analyzer to the PC microarray system clearly demonstrates that the PCEF microarray system provides lower limits of detection. Dose-response characterization of the assays demonstrates a lowest detectable concentration of 0.02 kU/L for Fel d1-specific IgE. In addition to the high sensitivity, the PCEF microarray platform allows simultaneous detection and quantification of antibodies to various allergens. While ImmunoCAP testing requires several milliliters of blood, the microarray platform requires only 10 μL of serum, for compatibility with less invasive sample collection via a finger-prick method. Finally, our results demonstrate the efficacy of using purified allergen components as the selective capture agent, which delivered greater sensitivity and reproducibility than raw allergen extracts.

In addition to addressing the current clinical need for improved allergy testing, we believe that this technology will enable a broad range of advances in basic and clinical research that were not previously feasible. For example, serial monitoring of antibodies in patients undergoing new treatment might predict the effectiveness of the therapy, as the IgE levels might correlate with the severity
of the patients’ symptoms. Furthermore, tracking antibody levels at high resolution in high-risk patients would yield great insight into the onset of allergy symptoms. Ultimately, we believe that this technology could be deployed to facilitate screening for allergen-specific IgEs and preventative therapies.
3.6 Figures

Figure 3.1. Reflection spectrum of PC when it is illuminated by a Transverse Magnetic (TM) polarized laser at the wavelength of $\lambda = 637$ nm. The peak location of the spectrum indicates the resonance condition is achieved at the incident angle of $4.12^\circ$. 
**Figure 3.2.** (a) A PC holds 10 subarrays, and each subarray contains four sets of four replicate spots per protein for a total of 16 spots. Inset: schematic of the microarray layout. The first row is the cat hair extract, the second row is the Fel d1, the third row is the Timothy grass extract and the last row is a set of array location fluorescent spots comprised of Alexa-Fluor-555 fluorescent streptavidin conjugates. (b) A 10-well format custom-made slide module assembly in which the PC is inserted during the assay steps. (c) Fluorescence images of the arrays tested with different sera. (d) Average fluorescence intensities from Fel d1 and Timothy grass spots. The result indicates that the Fel d1 and grass pollen assays exhibited excellent selectivity. Strong fluorescence was observed on grass pollen spots for 4-fold dilution of serum #85228 which has a high concentration of grass-pollen specific antibodies, while no fluorescent signal is observed for 4-fold dilution of serum #924365 that has low concentration. No fluorescence was detected on either grass extract or Fel d1 spots in the case of negative serum incubation.
Figure 3.3. Fluorescent images of microspots at different antibody concentrations for (a) a serum sample provided by Hycor and (b) a serum sample provided by ViraCor-IBT. Fel d1 spots have higher fluorescence intensities than cat extract spots, confirming that purified allergen has stronger binding affinity and thus higher sensitivity than crude extracts. The images also indicate that all the Fel d1 spots were visible over the 0.005 – 4.00 kU/L range. For example, average SNRs over four Fel d1 replicates at the lowest concentration (0.005 kU/L) is 5.16.
Figure 3.4. Standard curve for Fel d1 allergen detection. The black solid line in the inset shows the background intensity, which is the blank intensity from the negative control (indicated by the dashed line) plus three times the standard deviation. We consider fluorescence signals above the background intensity as detectable. Therefore, the lowest detectable concentration for Fel d1-specific antibody is ~0.02 kU/L, which is lower than that measured by the ImmunoCAP system.
Figure 3.5. Does response curve for the grass extracts. Grass-positive serum was tested over a range of 8 concentrations in a 2-fold dilution series. The starting serum is diluted by 2 times. As shown in the figure, the detection limit is 32-fold dilutions serum.
Figure 3.6. Representative images of the allergen spots assayed with patient sera at various IgE levels ranging from 0.089 kU/L to 40 kU/L. Note that the PCEF array system is capable of detecting the low concentration of IgE in serum #9, which is not observable using the ImmunoCAP system.
CHAPTER 4 PHOTONIC CRYSTAL ENHANCED FLUORESCENCE MICROFLUIDIC CHIP FOR HIGH SENSITIVITY AUTOMATED MULTIPLEXED IMMUNOASSAYS

4.1 Chapter Introduction

We demonstrate a platform that integrates photonic crystal enhanced fluorescence (PCEF) detection of a surface-based microspot fluorescent assay with a microfluidic cartridge to achieve simultaneous goals of high analytic sensitivity (single-digit pg/mL), high selectivity, low sample volume, and assay automation. The PC surface, designed to provide optical resonances for the excitation wavelength and emission wavelength of Cy5, was used to amplify the fluorescence signal intensity measured from a multiplexed biomarker microarray. A plastic microfluidic device for holding the PC was created using a low-cost and flexible process. An assay automation system that utilizes a leak-free fluid interface was built to introduce the correct sequence of fluids through the microfluidic device. Through the use of the assay automation system and the PC embedded within the microfluidic device, we demonstrate pg/mL-level limits of detection by performing a representative biomarker assay (for IL3 and TNF-α). The results are consistent with limits of detection achieved without the use of the microfluidic device with the exception that coefficients of variability from spot-to-spot are substantially lower than those obtained by performing assays with manual manipulation of assay liquids. The system’s capabilities, when fully developed, are compatible with the goal of diagnostic instruments for point-of-care settings.
4.2 Motivation

Rapid detection of immune-related biomarkers in serum or plasma to pathogenic antigens, tumor antigens, or autoimmune antigens, is critical for diagnosis, monitoring, and biomarker assessment of the immune response [28, 94]. In addition, soluble protein biomarkers in blood have also been useful for understanding the form of a disease that can be most effectively treated by a particular drug [95]. Both of these technologies facilitate the combination of therapeutics with diagnostics as a means of guiding treatment for an individual patient [96]. Biomarker detection is used most heavily in oncology for early detection of several forms of cancer [97-105] and as a tool for companion diagnostics, but it also has significant application in other diseases with high unmet needs. For example, biomarkers for cardiovascular disease [106, 107], autoimmune disease [28], neurological disorders, and infectious disease have been identified and are undergoing clinical validation [108].

A key unmet need for biomarker detection to achieve clinical relevance is improved sensitivity. Early diagnostic applications for cancer, for example, require detection of biomarkers that are produced by, or in response to, a small number of cells. The biomarker is diluted throughout the volume of blood within a person, so that when a sample of peripheral blood is drawn, the biomarker concentration can be <1-10 pg/mL. (For reference, 1 pg/mL of a typical cytokine such as IL-6 is equivalent to ~45 fM. A 10 µl sample at this concentration contains 0.45 attomoles of IL-6.) In addition to high sensitivity, clinical diagnostic laboratories require automated, high-throughput platforms that can perform rapid multiplexed biomarker analysis at low cost/assay and that can quantify small changes in concentration. Consensus is emerging that
multiple, mutually exclusive biomarkers in an assay will lead to better clinical management of disease, compared to assessment of a single biomarker, and will allow subtle differences in patient populations (gender, race, age) to be understood [20-22].

Among the current methodologies of biomarker detection, sandwich antibody microarrays hold great potential because of their capability to detect analytes at <10 pg/mL concentration [28, 109] with the use of 3-10 µL of sample, no assay cross-reactivity, and fast binding kinetics. Using a photonic crystal (PC) surface to enhance the fluorescence output from a biomarker microarray, we have published a number of studies that successfully achieved high sensitivity for multiplexed cancer biomarker detection [8, 17, 19, 53]. More recently, we developed a high sensitivity, compact, and inexpensive detection platform that used a silicon-based PC surface and a custom laser line-scanner in an effort to minimize the background auto-fluorescence and maximize the coupling efficiency of light into PC structure [19]. Microspot fluorescent immunoassays for TNF-α and IL-3 performed on this platform demonstrated that the system can achieve pg/mL-level sensitivity. Although only a 10 uL sample was consumed with the use of 2 mm-diameter microwells, manual sample handling presented difficulties in injecting liquid, required dedicated time and labor, and introduced bubbles that were difficult to remove and decreased the incubation efficiency. Furthermore, the performance and implementation characteristics of performing PCEF microarray assays with manual liquid sample handling greatly limited its scalability and accessibility to clinicians, including a disjointed workflow that required manual intervention across multiple steps and used the PC surface area
inefficiently. Therefore, the development of a simpler and automatic format is desired for clinical applications such as point-of-care diagnostics.

Here we present a new platform that combines photonic crystal enhanced fluorescence (PCEF) and microfluidic sample handling to achieve high analytic sensitivity (1.57 pg/ml), high selectivity, low sample volume, and assay automation. A low-cost and flexible process that combines stereolithographic 3D printing [110-112] and laser-cutting [113-115] was developed to create a plastic microfluidic device for holding the PC in place during an assay and for providing a stable platform during the fluorescence scanning. An assay automation system that involves a custom built, leak-free fluid interface was built to introduce the correct sequence of fluids through the microfluidic device. We performed a representative biomarker assay and demonstrated 1.57 pg/mL level detection sensitivity with the use of the assay automation system and the PC embedded microfluidic chips. The coefficients of variability across spots are decreased for the low concentration assays compared to previous results. This rational engineering design strategy incorporates innovative elements that are required for a system that can rapidly provide information in a clinical setting, thereby potentially allowing for quicker, more personalized medical intervention.

4.3. Materials and Methods

4.3.1 Design and Fabrication of a PC-Embedded Microfluidic Device

The PC is designed and fabricated as described in Chapter 2. An SEM image showing the surface structure of the PC is presented in the inset of Figure 4.1(d). As shown in Figure 4.1(a), the microfluidic chip was assembled using a stack of plastic,
Double-Sided Adhesive (DSA), and a nonfluorescent glass coverslip. The top plastic layer was designed by three-dimensional (3D) design software (PTC Creo Parametric 2.0) and fabricated by stereolithography of optically clear resin (WaterClear Ultra 10122). Stereolithography is a high-speed optical fabrication process in which model layers are built by curing photo-reactive resin with a laser. A Viper SLA System was chosen for its high resolution (2.5 μm) and small laser beam diameter (75 μm), which was critical to fabricate a precise microfluidic device. The top layer was 75 mm long, 25 mm wide, 6 mm thick, and contained a recessed region (2.2 mm x 8.2 mm), into which the PC chip of the same dimension was attached by adhesive. Two through-holes extend through this top layer to serve as fluid inlets/outlets. The middle layer was comprised of DSA (3M™ Optically Clear Adhesives 8212) with a ~2 x 12 mm wide and 150 μm thick channel in the center which matched the shape of the fluid inlet/outlet and the PC. In order to ensure device cleanness, accurate non-contact laser cutting was used to cut the channel area on the DSA. The bottom layer is a thin transparent coverslip through which a camera monitored flow inside the channel and a laser excited the fluorophores during the detection stage. Shott Glass (#1098576 24 x 60 x 0.17 mm) was chosen for its tight thickness tolerance, which minimizes spherical aberration, and its low background fluorescence, therefore promoting high-quality images of the fluorescence signal during scanning. The three layers were attached by the DSA layer in a custom-built mechanical alignment fixture and pressed by a roller at room temperature to seal them together. Figure 4.1(c) shows a photo of a fabricated microfluidic chip in which red food dye was introduced through the inlet.
4.3.2 Computer-Controlled Microfluidic System

Using components shown in Figure 4.2(a), the entire assay process was automated with no intervention from the user other than introduction of a droplet of serum into the inlet reservoir (volume ~10 µL). The 3-to-1 valve (Cole-Parmer Manifold Mixing Solenoid Valve) introduced compressed air, buffer solution, or detection antibody labeled with Cy5 into the channel. The fluid selection was determined by an automated and programmable microfluidic controller (FlowTest™ Programmable Microfluidic Controller). The fluid driving force was supplied by compressed nitrogen, which was regulated by a pressure controller (Flui gent MFCS-EZ) that provided pulseless and highly stable (<0.1% CV) flows. A digital camera (Dino-Lite AM3111T) located beneath the fixture monitored the flow through a hole in the fixture. Users can control the pressure and microfluidic flow through a computer, and users can also monitor the flow on-screen from the camera.

To ensure leak-proof sealing and zero dead volume, flanged end tubes and a custom-machined metal frame were used in our system [116]. The seal was based on a forced fit between the flanged ends of chemically inert polytetrafluoroethylene (Cole-Parmer PTFE EW-06605-27 1/16”) tubing and the flat outer surface of the microfluidic chip. The flange was created by heating the end of the tubing and pressing it against a metal washer. The metal frame maintains alignment between the tubing and the inlet/outlets of the microfluidic chip, while screws between the upper and lower metal holding plates of the fixture apply sufficient pressure for a leak-proof seal. A schematic of the sealing fixture is shown in Figure 4.2(b).
4.3.3 Immunoassay Procedure Using Microfluidic Sample Handling

To demonstrate the capability of the microfluidic system, microspot immunoassays for two cytokines (interleukin 3 (IL-3) and tumor necrosis factor alpha (TNF-α)) were performed using PCs embedded in the microfluidic device. Before printing spots, the PC surface was cleaned and activated with a vapor-phase epoxysilane process as described in Chapter 3. After silanization, the protein arrays were printed onto the PC surface. A PC holds two subarrays, and each subarray contains four sets of four replicate spots for each protein, resulting in an array with 16 spots. The first row is a fluorescent-tagged protein for array orientation/location (Alexa-Fluor-555 fluorescent streptavidin conjugates, Life Technologies), the second row is the TNF-α antibody (R&D systems Inc.), the third row is a negative control (PBS, pH = 7.4), and the last row is the IL-3 antibody (R&D systems Inc.). The antibody microarrays on the PC were printed by a desktop nanofabrication system (Arrayit NanoPrint LM60 Microarrayer) while the chip was in a free format. The printing was performed under a controlled environment using an environmental chamber (ambient temperature and 50% relative humidity). Four pins (946MP3 Microarray Printing Pins) were used to print the two cytokines at a concentration 1 mg/mL, the positive control at 10 µg/mL, and the negative control on an epoxysilane modified PC. Printing pins were cleaned between sample pickups with 15 s sonication and four cycles of washing (2.5 s) and drying (1 s). Measured spot diameters were 79.00 ± 2.22 µm. Row spacing was 149.25 ± 3.26 µm and column spacing was 200.75 ± 0.82 µm. The printed substrates were incubated in a sealed box with a desiccant overnight at ambient temperature. Next, the arrays were blocked with casein blocking buffer (BioRad, Hercules, CA) for 1
h before they were washed three times with 0.02% (v/v) tween 20 in PBS (PBST). The substrates were then dried and put in a sealed box for future use.

Following array printing and surface blocking, a PC substrate with antibody microspots was glued into the recess of a plastic holder that was made by stereolithography. This PC embedded plastic layer was then bonded to a glass cover slip by a DSA layer with the channel pattern to complete the fabrication process. After that, this complete microfluidic device was used to perform the immunoassay for mixtures of different antigen concentrations in casein buffer. Ten µL of the mixture of TNF-α and IL-3 was manually introduced into the microchannel inlet by a pipette and incubated for 3 hours. The hydrophilic property of the inner channel surface facilitates liquid flow, so the droplet of test sample covers the PC array without any intervention from the user. The cytokine TNF-α (R&D systems Inc.) was assayed at the following seven concentrations: 1 ng/mL, 0.25 ng/mL, 62.50 pg/mL, 15.62 pg/mL, 7.8 pg/mL, 3.9 pg/mL, 1.9 pg/mL. The concentration of IL-3 is 10 times higher than that of TNF-α (R&D systems Inc.) in all assayed antigen mixtures. The microfluidic device filled with the test sample was then loaded in the assay automation system, so that the wash and the labeling steps can be performed. The device was washed by flowing PBST for 1 min with an applied pressure of 25 mBar to remove the unreacted antigens. Next, the 1 ug/mL fluorescent-labeled secondary antibody solution (Alexa Fluor® 647 Goat Anti-Human IgG, Life Technologies) was introduced into the microchannel with an applied pressure of 25 mBar for 30 s. After 1 h incubation, the microchannel was washed again by flowing PBST for 1 min to remove the unreacted fluorescent-labeled secondary antibody solution. Finally, an air flow was produced in the microchannel by
applying high-pressure Nitrogen gas (345 mBar) to the inlet to remove remaining liquid. The PC surface was completely cleaned and dried in 10 s due to its hydrophobicity, as observed by the imaging camera. Following the immunoassay process, the microfluidic chip was removed from the automation system and transferred to our customer-built scanner for fluorescent imaging.

4.3.4 PCEF Scanner and Image Acquisition

We have designed and constructed a PCEF microarray detection instrument that provides collimated illumination and the ability to tune the incident angle to precisely match the resonant coupling condition [59] while focusing the light from a fiber-coupled semiconductor laser to an 8 µm line with a cylindrical lens [117]. A custom detection instrument was built to optimize coupling of laser illumination to the PC surface. As described previously in Chapter 2, the excitation laser is collimated in the plane perpendicular to the grating lines, but focused in the plane parallel to the grating (Figure 4.3). Collimated light with electric field polarization perpendicular to the grating lines is able to couple most efficiently with the PC resonant mode, but the light need only be collimated with respect to a single axis. Along the orthogonal axis, light can be focused without compromising coupling efficiency to the PC, and thus focus along one axis is used to achieve high illumination intensity. The system is designed for optimal interaction with the PC for both enhanced excitation and enhanced extraction, using design principles discussed, modeled, and demonstrated in reference [19]. The focal point of the cylindrical lens is located at the back focal plane of the objective, resulting a line of illumination in the PC that is collimated along the angle perpendicular to the PC grating lines, but focused in the orthogonal direction. Linear translation of the cylindrical
lens results in adjustment of the incident angle to achieve “on resonance” illumination. A semiconductor laser diode (AlGaAs, 70 mW, \( \lambda = 637 \text{ nm} \)) is expanded to a diameter of 1 mm, and focused to an 8 \( \mu \text{m} \) wide line onto the PC surface by a cylindrical lens. A mirror coupled to a computer-controlled linear translation stage enables adjustment of the incident angle from 0-20 degrees with 0.01 degree increments. The grating lines of the PC are oriented perpendicular to the scan line, allowing the laser (polarized output perpendicular to the grating) designed to excite the TM mode of the PC.

Using the scanner described above, a fluorescent image of the PC surface was obtained by adjusting the incident angle of the laser illumination line upon a region of the PC adjacent to the microspots and then translating the PC holding stage in increments of 2 \( \mu \text{m} \) past the array region, gathering a fluorescent intensity image of the line for each motion increment. Then, the fluorescent images of each line were assembled into a two-dimensional image of fluorescence intensity by using a custom-built C# user interface. Spot segmentation and intensity calculations of the constructed fluorescence images were performed using ImageJ. Net spot intensity was calculated as the local background subtracted spot intensity where the local background is an annular region around a given spot. Spot signal-to-noise ratio (SNR) was calculated as the local background subtracted spot intensity divided by the standard deviation of the local background.

4.4. Results and Discussions

4.4.1 Raw Fluorescent Intensity

Even if fluorescence was detected through the glass window, its influence on the fluorescent images and the final assay result is hardly observed, indicating that it is not
necessary to remove the glass to perform any scans. From the viewpoint of operating procedure simplicity, this is a big advantage that other fluorescent microfluidic devices do not have. In order to quantitatively show this, we investigated the effect of scanning through the glass window on the fluorescence intensity of the microarray image. In the experiment, we performed the immunoassay of 1.9 pg/mL TNFα solution using the PC-embedded microfluidic device, and we scanned the microarrays before and after removing the glass window. The illumination angle (incident angle = 4.12 degree) and camera settings (sensitivity gain = 25, exposure time = 40 ms) were the same for both scans. Although we scanned the same area twice, the intensity decay after the first scan is less than 0.001% [118] and can be safely ignored. This is because the laser only illuminated the area for 120 ms, given that the exposure time of the camera was 40 ms and oversampling rate was 3. Fluorescence intensities of each microspot and its surrounding background were obtained by quantifying fluorescent images (Figure 4.3(a)), and subsequently averaged over the four replicates. Interestingly, the spot intensity obtained by scanning through the glass window (the first red bar in Figure 4.3(b)) is 19.93% higher than that without any obstacle (the first black bar in Figure 4.3(b)). The increased intensity was attributed to the fluorescence from the glass window. In addition to autofluorescence, fluorescence from a small amount of fluorescently labeled secondary antibody that was absorbed onto the glass during the assay procedure was also a contributing factor. We can confirm this assumption by examining the average background intensity, which decreased from 2236.64 counts to 1744.08 counts by removing the glass (Figure 4.3(b)). Net intensity was calculated as the background subtracted spot intensity, through which any fluorescence from the
glass was removed. In contrast to the increase in the average spot intensity and background intensity, the average net intensity decreased to 93.23% if spots were scanned through the glass (the third bars in Figure 4.3(b)). This small signal loss was caused by light reflection at the interface between air and glass. Given that refractive index is $n_1 = 1.00$ for air and $n_2 = 1.50$ for glass, the transmittance of the fluorescence after passing the upper surface of glass is 96.00%, calculated by the equation:

$$T = 1 - \left(\frac{n_1 - n_2}{n_1 + n_2}\right)^2.$$

Ignoring the phenomena of multiple internal reflection in the glass, transmittance of the fluorescence through the glass, adding the signal loss at the second surface, is 92.16%, which is close to the percentage of the signal we obtained in the experiment. Considering the small thickness and good flatness of the glass, any absorption or scattering can be neglected. Although there was a slight decrease in the net fluorescence signal, the fluorescent spots were still distinguishable from the background, even for the assay with the lowest concentration.

4.4.2 Fluorescence Enhancement of a PC-Embedded Microfluidic Device

The PC is designed to increase the fluorescence intensity of Cy5 dyes through the enhanced excitation and extraction mechanism described previously. The enhanced extraction effect is always present, regardless of the illumination conditions. In a previous report [8], we demonstrated that enhanced extraction resulted in an approximately 4.8-fold increase in fluorescence intensity compared to detection on unpatterned glass. The effect of enhanced excitation can be determined by comparing the fluorescence output under the following two conditions: (a) when the incident angle
of the excitation laser was adjusted to illuminate the PC at the resonant angle (“on resonance”), and (b) when the angle of incidence was selected not to coincide with the resonant coupling condition (“off resonance”). Although fluorescence enhancement by a silicon PC has been demonstrated previously [19], it is still necessary to characterize the enhancement factor and show that the same enhancement can be observed using a PC embedded in a microfluidic device that is covered by a glass window. In the experiment to characterize the enhanced excitation of the PC in the microfluidic device, 0.15 ng/mL of IL-3 was assayed, and the microspots were scanned at both on- and off-resonance conditions. First, the reflection spectrum of the PC (Figure 4.4(c)) was acquired by illuminating the surface over a range of incident angles at the fixed excitation wavelength $\lambda = 637$nm. The on-resonance angle of illumination was approximately 4.12 degrees, as indicated in the spectrum, while the off-resonance angle was chosen to be 3.00 degrees. The fluorescent images shown in Figure 4.4(a)(b) were obtained at on- and off-resonance conditions, respectively. Because of the low fluorescence at the off-resonance condition, exposure time of the camera was set to be 400 ms, 10 times higher than that of the on-resonance condition. Therefore, the on-resonance value was multiplied by a factor of 10 before direct comparison to the off-resonance value. It can be observed in Figure 4.4(d) that by scanning the PC at its resonant angle, the fluorescence intensity was enhanced by a factor of approximately 26, which is similar to what was reported previously [8, 118]. In general, we expect a PC embedded in a microfluidic chip to perform identically to a PC in the “free” chip format, as the absorption and reflection of light by the thin glass cover are minimal.
4.4.3 Standard Curves and Limit of Detection

The performance of the PC-embedded microfluidic chip was studied in the context of a microspot-based fluorescent sandwich immunoassay. Seven concentrations of antigen mixtures were assayed by the PC microfluidic system as described in the previous sections. Because of the flanged tube that connects the microchannel to the control instrument, no liquid leakage was observed throughout the process. The assay procedure was controlled by a computer with minimal human intervention, which makes it highly repeatable. Figure 4.5(a) shows representative fluorescence images of microspots on the PC microfluidic chip at four sets of assayed concentrations. Compared with previously reported results obtained by manually handling the liquids [19], one visible improvement by using the automatic system is the intensity uniformity within spots of the same cytokine. In order to quantitatively characterize how easily a spot can be distinguished from background noise, we defined SNR as the net signal divided by the standard deviation. A spot with SNR larger than 3 is regarded as detectable. Figure 4.5(a) shows that all the cytokine spots were detectable over the 1.9 pg/mL-10 ng/mL range. For example, SNRs of the TNF-α spots and the IL-3 spots at the lowest concentrations are 49.96 and 78.31, respectively.

The signal intensities from each dilution in the concentration series were used to generate standard curves for both TNF-α (Figure 4.5(b)) and IL-3 (Figure 4.5(c)) using Prism. The limit of detection (LOD) is defined as the concentration corresponding to the blank intensity (i.e. the intensity of the negative control spot of PBS buffer) plus 3 standard deviations from all assay spots. Negative controls
performed by exposing the capture antibodies to a casein sample resulted in no observable fluorescence signal above the background. Therefore LOD values for the two cytokines TNF-α and IL-3 are 1.57 pg/mL and 17.96 pg/mL, respectively. While the LOD values are similar, the intensity uniformity within the spots is greatly improved compared to that obtained without the use of the microfluidic device. Coefficients of variance (CVs) were calculated for the IL-3 spots at the lowest four concentrations in order to quantitatively compare the intensity uniformity (Table 4.1). It is obvious that CVs decreased dramatically by using the microfluidic system for sample handling. For example, the CV decreased from 89.82% to 10.54% for the microspots that were assayed at the lowest IL3 concentration.

Table 4.1. Coefficients of variance (CVs) for the IL-3 spots at the lowest four concentrations

<table>
<thead>
<tr>
<th>Assay concentration</th>
<th>~2 pg/mL</th>
<th>4 pg/mL</th>
<th>8 pg/mL</th>
<th>16 pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV for PC-embeded microfluidics chip</td>
<td>10.54%</td>
<td>4.23%</td>
<td>2.93%</td>
<td>25.96%</td>
</tr>
<tr>
<td>CV for a PC chip</td>
<td>89.82%</td>
<td>22.92%</td>
<td>34.20%</td>
<td>91.64%</td>
</tr>
</tbody>
</table>

4.5 Conclusion

In this discussion, we designed and fabricated a new platform, the PC microfluidic chip, for performing automatic and rapid immunoassays. The PC microfluidic chip contains a photonic crystal surface that enhances fluorescence signals from the labeling dyes and improves the detection sensitivity. We have demonstrated a single-digit pg/mL detection limit for cancer biomarkers (TNF-α and IL3) with small intensity variances across the replicate spots by performing a surface-
based sandwich assay on this PC microfluidic chip. The chip is held in a microfluidic system where a computer-controlled pressure adjuster and three-way valve automatically perform the immunoassay, and a leak-free fluid interface ensures smooth liquid flow. The PC microfluidic platform has ideal features for a practical immunoassay: it is automatic and easy-to-use; it can perform multiple assays simultaneously; only a small volume of samples and reagents (10 μL) are needed; and high analytic sensitivity and high selectivity are achieved without any chemical amplifications. Moreover, the use of the automation system greatly reduces spot-to-spot coefficients of variability compared to manual handling.

In addition to addressing the current clinical need for improved biomarker detection, we believe the PC microfluidic chip developed here has great potential for practical immunoassay and point of care diagnostics. Because of the high sensitivity and small sample volume, it is possible to perform the assay using a droplet of blood from a finger prick, which eliminates the need to visit clinical labs for blood drawing. If the microfluidic chip can be integrated with a plasma filter, the blood testing process can be further simplified and a patient might be able to perform a test at home. Indeed, we are in the process of developing a compact system which combines a photonic crystal surface, microfluidic handling, a plasma filter, and a detection instrument. Furthermore, we are examining the liquid handling system and incorporating an ultrasonic transducer in order to introduce external agitation and reduce the total assay time to an hour.
4.6 Figures

Figure 4.1. Schematics of the PC microfluidic chip (a) before and (b) after assembling. It was assembled using a stack of plastic, Double-Sided Adhesive (DSA) and a nonfluorescent glass coverslip. The top layer was 75 mm long, 25 mm wide and 6 mm thick, and contained a recessed region (2.2 mm × 8.2 mm) into which the PC chip of the same dimension was attached. The middle layer was made of DSA (3M™ Optically Clear Adhesives 8212) with a ~2 x 12 mm wide and 150 μm thick channel in the center. The bottom layer was a thin transparent coverslip (24 mm x 60 mm x 0.17 mm) through which a camera monitored flow inside the channel and a laser excited the fluorophores during the detection. (c) Left: a photo of a fabricated microfluidic chip in which red food dye was introduced throughout the inlet; Right: SEM of the PC surface.
Figure 4.2. (a) Schematics of the microfluidic control system. The 3-to-1 valve introduced compressed air, buffer solution or detection antibody labeled with Cy5 into the channel. The selection was determined by an automated and programmable microfluidic controller. The fluid driving force was supplied by compressed nitrogen which was regulated by a pressure controller (Fluigent MFCS-EZ) that provided pulseless and highly stable flows. A digital camera monitored the flow through a hole in the fixture. (b) Schematics of the leak-proof seal. The seal was based on force fit between flanged ends of chemically inert tubing and the flat outer surface of the microfluidic chip.
Figure 4.3. (a) Fluorescent images obtained by scanning a microarray assayed with a 1.9 pg/mL TNF-alpha sample before (the upper row) and after (the bottom row) removing the glass window. (b) Average fluorescent intensities over four replicates with (black bar) and without (red bar) glass window. Although the spot intensity obtained with a glass window is higher, the net fluorescent intensity is 6.77% lower compared to that without a glass window.
Figure 4.4. (a) Fluorescent images obtained at (a) on-resonance and (b) off-resonance conditions. (c) Reflection spectrum of PC acquired by illuminating the surface over a range of incident angles at the fixed excitation wavelength $\lambda = 637$ nm. (d) Average fluorescent intensities when the PC embedded in microfluidic channel were on resonance (black bar) and off resonance (red bar).
Figure 4.5. (a) Representative fluorescence images of microspots on the PC microfluidic chip at four sets of assayed concentrations. The first row contains TNF-α spots while the second row contains IL-3 spots. Standard curves for (b) TNF-α and (c) IL-3. LOD values for two cytokines TNF-α and IL-3 are 1.57 pg/mL and 17.96 pg/ml respectively.
CHAPTER 5 PCEF FOR HOMOGENEOUS ASSAY USING SUBMICRON FLUID CHANNELS FABRICATED BY E-JET PATTERNING

5.1 Chapter Introduction

In this chapter, we demonstrate the enhancement of a liquid-based (homogenous) fluorescence assay using the resonant electric fields from a photonic crystal (PC) surface. Because evanescent fields are confined to the liquid volume nearest to the photonic crystal, we developed a simple approach for integrating a PC fabricated on a silicon substrate within a fluid channel with submicron height, using electrohydrodynamic jet (e-jet) printing of a light-curable epoxy adhesive to define the fluid channel pattern. The PC is excited by a custom-designed compact instrument that was described previously in Chapter 2. Using a molecular beacon nucleic acid fluorescence resonant energy transfer (FRET) probe for a specific miRNA sequence, we demonstrate an 8x enhancement of the fluorescence emission signal, compared to performing the same assay without exciting resonance in the PC detecting a miRNA sequence at a concentration of 62 nM from a liquid volume of only ~20 nl. The approach may be utilized for any liquid-based fluorescence assay for applications in point-of-care diagnostics, environmental monitoring, or pathogen detection.

5.2 Motivation

We have demonstrated the use of PCEF for surface-based fluorescence assay in the previous work. However, many important classes of fluorescent assays are liquid based (homogeneous). For example, Fluorescence Resonant
Energy Transfer (FRET) assays and Fluorescence Polarization (FP) assays are mainstays for disease diagnostics, pathogen detection, life science research, and toxin detection [119-123] that involve conjugation of fluorescent tags to at least one liquid-based assay component. Likewise, chemiluminescence assays and polymerase chain reaction (PCR) require detection of photon output from a LED or laser-illuminated liquid sample [40, 124, 125]. For each of these applications, homogeneous assays require the ability to observe weak fluorescent signals above background autofluorescence for detection of biomolecular analytes at very low concentrations.

A fundamental limitation for the application of nanostructured surfaces for enhancing the fluorescence of homogeneous assays is that the enhanced electric field is tightly confined to the surface, with an evanescent field volume that extends only 100-600 nm into the liquid media in contact with the surface [48, 55], [19], [53, 126]. In order to achieve a substantial enhancement of the fluorescent output from a homogeneous assay using a nanostructured surface, it is necessary to confine the liquid volume so that a substantial proportion of it will reside within the evanescent field volume. Therefore, enhancement of the fluorescent output of homogeneous assays requires integration of the nanostructured surface and a fluid channel with a submicron height dimension.

A variety of approaches for creating microfluidic channels with submicron depth have been demonstrated through the application of photolithography [127, 128], sacrificial etching [129-132], and PDMS collapse [133-135]. However, these techniques either are low throughput, offer poor surface adhesion strength, or
poor control of channel dimensions. The electrohydrodynamic jet (e-jet) printing approach used here is cost-effective, highly controllable and very simple. E-jet printing [136-138] is a nano-manufacturing process that uses electric field-induced fluid jet printing through micron-scale nozzles to achieve nanometer to micrometer-scale droplet placement accuracy and accurate control of ~0.5 pl dispense volumes. The printed fluid used in our approach is UV-curable optical adhesive (NOA74, Norland Products Inc., Cranbury, NJ). Assembly of the flow channel requires printing the adhesive pattern upon the PC substrate, attaching a glass cover with pre-drilled inlet/outlet holes to the PC surface, and curing by a short exposure to UV light. The approach can produce channels of arbitrary shape with micrometer-scale precision of lateral dimensions.

In this discussion, we demonstrate, to our knowledge, the first reported instance of fluorescence enhancement of a homogeneous assay using a nanostructured surface. We utilize a one-dimensional PC slab fabricated upon a silicon substrate as the enhancement surface that is designed to resonantly couple with a $\lambda = 637$ nm laser when the PC is covered in aqueous media. In order to achieve a submicron-height microfluidic channel that can be easily integrated with the PC, we demonstrate a new approach for creating a fluid channel, in which we print a pattern of droplets of light-curable adhesive using electrohydrodynamic jet (e-jet) printing. Rigorous Coupled Wave Analysis (RCWA) computer simulations were used to visualize the extent of resonant PC evanescent field into the volume of the flow channel, and to predict the electric field enhancement experienced by fluorophores extending through the channel
volume. As an exemplary demonstration of homogeneous PCEF, we performed a FRET-based molecular beacon assay for detection of a specific miRNA sequence. Using a liquid volume of 20 nL to fill the nanochannel, we demonstrated up to 8 x increase in fluorescence intensity by exciting the PC under resonant coupling conditions and detected the miRNA at a concentration as low as 62 nM.

5.3 Methods

5.3.1 Device Structure and Detection Principle

The sensor structure is comprised of a transparent glass layer, the microfluidic channel region, and a PC surface for fluorescence enhancement (Figure 5.1a). As liquid flows through the channel, the fluorescent emitters will be excited by the laser illumination, with fluorophores closest to the PC surface experiencing electric fields that are amplified with respect to the incident laser intensity. Fluorescent emission is detected through the transparent glass cover.

The structure and fabrication of PC was described previously in Chapter 2. Resonant modes of the PC can be excited at a specific incident angle for a given laser illumination wavelength [53]. At the resonance coupling condition of the PC, the enhanced electric fields are confined to the surface of the device, and extend into the adjacent media with exponentially decaying intensity as one moves away from the PC surface. By confining the assay solution to the evanescent field region of the PC, one can achieve an increase in the emission intensity of the dye throughout the channel.
A commercially available simulation tool for RCWA (DIFFRACTMOD, RSoft) was used to characterize the device response to an incident transverse magnetic (TM) illumination source at $\lambda = 637$ nm. In the model, refractive indices were assigned using previously measured values for each material ($n_{\text{TiO}_2} = 2.35$, $n_{\text{SiO}_2} = 1.46$, $n_{\text{Glass}} = 1.52$, $n_{\text{Water}} = 1.33$) and device dimensions were used as specified in our previous description. The upper surface of the microfluidic channel was placed at a height of $t = 830$ nm, using values obtained from fabricated devices (Figure 5.2f).

We first calculated combinations of incident angle and wavelength that result in resonant coupling to the PC, at which electric field enhancement occurs [139]. Simulation predicts that, for a laser source of $\lambda = 637$ nm, the reflectance is maximized for an incident angle of $\theta = 1.55$ degrees with respect to normal. By Snell’s Law, the corresponding resonant angle for illuminating the upper surface of the glass window, which should be used to compare with the experimental results, is $\theta_{\text{air}} = 2.39$ degrees. Simulation of the local electric field distribution at the resonant condition (Figure 5.1c), predicts that the maximum electric field enhancement is $225 \times$ the incident electric field magnitude of the laser, and shows that the greatest field enhancements occur within the TiO$_2$ film and in the regions in direct contact with the TiO$_2$. The simulations show that the field enhancement decays rapidly with increasing distance, $d$, from the PC, and that some regions on the PC surface experience greater electric field magnitude than others. To quantify the field enhancement as a function of $d$, taking into account the electric field distribution, we define the average enhancement factor $F_{\text{average}}$. 
as the average intensity \(|E|^2\) enhancement of all the points in the simulation with a distance \(d\) from the PC surface. Mathematically, the averaged enhancement factor is defined as

\[
F_{\text{average}}(d) = \frac{1}{\Lambda} \int_0^\Lambda |E_{\text{local}}(x, d)|^2 \, dx ,
\]

where \(E_{\text{local}}(x, d)\) is the electric field enhancement at location \((x, d)\). \(F_{\text{average}}(d)\) is plotted with \(d\) ranging from 5 nm to 700 nm (Figure 5.1d), showing that the field enhancement decreases rapidly as one extends the liquid volume further from the PC surface. The zoom-in plot (inset of Figure 5.1d) suggests that no significant enhancement of the local intensity is observed when a fluorescent molecule is placed at a distance of \(~500\) nm from the PC surface, implying that the fluorescent molecules should be confined within the 500 nm volume above the PC in order to obtain a substantially enhanced fluorescence signal.

5.3.2 E-Jet Printing of the Submicron-Channel

A novel fabrication method based upon e-jet printing was developed to create the submicron channels. Micron-size droplets of NOA74 were printed upon the PC substrate to form a channel pattern. Separately, a glass cover was prepared by drilling two inlets holes and one outlet hole. The glass cover and the epoxy-patterned PC were subsequently aligned and bonded by squeezing them together, followed by curing the adhesive by exposure to UV illumination.

The e-jet printing approach uses an electric field to induce droplet release flow from a micro capillary nozzle to the substrate, eliminating the need of a mask, while allowing precise printing of any desired pattern [137]. The microfluidic channel is 500 \(\mu\)m wide and 13.6 mm long, with a central detection
region with a diameter of 2 mm. The assay region width was selected to fit the extent of the focused laser illumination line provided by the detection instrument. The channel lateral dimensions were defined by filling the regions surrounding the channel with printed droplets of adhesive, as shown in Figure 5.2a. In order to achieve a channel thickness as small as possible, NOA74 was chosen as the optical adhesive due to its low viscosity (80-95 cps) at room temperature. The droplet size and droplet density were selected to achieve a continuous adhesive film after the PC-glass attachment step, while avoiding the use of excessive material that would result in lateral flow, which would reduce the channel width.

We used a gold/palladium (Au/Pd) coated nozzle with a tip diameter of 5 µm that was placed 30 µm from the continuously moving PC surface (v = 1 mm/s). The NOA74 was extracted [138] from the nozzle to the PC substrate to form discrete droplets, under a periodically modulated voltage (280 V high and 220 V low, 20 Hz) at room temperature and 30% humidity. The printing process required ~20 minutes to produce the pattern for one microfluidic channel. Each droplet occupies an area of ~20 X 30 µm^2, with 10 - 30 µm spacing between droplets (Figure 5.2d). The vertical height of the droplets before being squeezed was measured to be 1.2 ± 0.1 µm using a stylus contact profilometer (Alpha-Step IQ, KLA-Tencor Inc., CA), and the squeezing process is intended to reduce the adhesive height as the droplets spread to fill their surrounding volume.

To complete device fabrication, a glass cover was prepared with three holes (diameter = 1mm) to match the inlets/outlet locations of the channel. An evenly distributed force of ~100N was applied at room temperature by a 20 lb
Teflon-wrapped steel handheld roller. An optical image of the NOA74 after attachment to the glass cover is shown in Figure 5.2e, demonstrating that the droplets spread out and overlap with each other after squeezing. Finally, the adhesive was cured by exposing the assembly through the glass cover to a high intensity UV lamp (Xenon) for 50 seconds. Plastic tubing with inner diameter of 0.75 mm and outer diameter of 1 mm were inserted into the inlets and outlet and sealed with optical adhesive (NOA62, Norland Products Inc., Cranbury, NJ) that was cured by a second exposure to the UV lamp.

A completed microfluidic assembly and a magnified view of the observation window are shown in Figure 5.2b-c. The two inlets are provided to enable separate introduction of the test sample and the assay label reagent, where they are allowed to mix within the central flow channel and assay region. For this work, measurements are performed while no flow occurs within the device. Between assays, buffer is introduced through both inlets, and waste is collected through the outlet port. Visual observation shows that smooth channel walls are obtained after droplet inter-diffusion along the straight sections of the microfluidic channel, but that the channel edges are irregular surrounding the rounded detection region. Because the PC grating lines are oriented parallel to the microfluidic channel, we hypothesize that the compressed adhesive is able to preferentially flow along the low portions of the grating, and is less able to flow in the direction perpendicular to the grating. Cross section SEM images of the channel (Figure 5.2f) were used to measure a fluid channel height of 830 nm.
5.4 Results

5.4.1 Fluorescence Enhancement Characterization

Using the custom reflection setup mentioned in Chapter 1, we measured the resonant angle of PC in water is 2.4 degree, which matches the simulation prediction. The fluorescence intensity enhancement provided by the PC can be determined by comparing the fluorescent emission intensities from a channel with the PC surface illuminated at the resonant coupling condition (on-resonance) to measurement from the same channel when the PC is illuminated at an angle that does not satisfy the resonant coupling condition (off-resonance). The enhancement factor (EF) is defined as

\[
EF = \frac{I_{\text{fluo\_on}} - I_{\text{bg\_on}}}{I_{\text{fluo\_off}} - I_{\text{bg\_off}}},
\]

where \(I_{\text{fluo\_on}}\), \(I_{\text{bg\_on}}\), \(I_{\text{fluo\_off}}\) and \(I_{\text{bg\_off}}\) represent the fluorescence from a bulk dye solution and background fluorescence from buffer when the illumination of PC is on-resonance and off-resonance respectively. The EF was initially characterized by filling the fluid channel with a fluorescent dye (LD700, Exciton Inc., Dayton, OH, USA) dissolved in water at a concentration of 540 ng/ml, and measuring the fluorescent intensity \(I_{\text{fluo\_on}}\) and \(I_{\text{fluo\_off}}\) via illumination on-resonance (\(\theta = 2.4\) degrees) or off-resonance (\(\theta = 0.1\) degrees). The background fluorescence intensities \(I_{\text{bg\_on}}\) and \(I_{\text{bg\_off}}\) were determined by detecting the fluorescence from the device filled with DI water. The fluorescence intensity for each measurement is the sum of the fluorescence from the center of the illuminated area of 8 \(\mu\)m X 1mm. All of the fluorescence intensities were detected under the same
instrument settings (laser current = 70 mA, and CCD gain = 1, sensitivity = 0, exposure time = 0.04 ms).

As shown in the angle spectrum (Figure 5.3), the on-resonance fluorescence signal is $I_{\text{fluo on}} = 207584$ counts while the off-resonance fluorescence signal is $I_{\text{fluo off}} = 39472$ counts. The background fluorescence were measured as $I_{\text{bg on}} = 57840$ counts and $I_{\text{bg off}} = 21200$ counts for the respective off-resonance and on-resonance conditions. An enhancement factor of 8.2 can be calculated by applying the EF definition, representing the gain supplied by the enhanced excitation effect, indicating the potential to achieve an order of magnitude increase in fluorescence signal, compared to performing the same assay without a PC.

5.4.2 Bio-Detection Demonstration by Molecular Beacon Assay

A homogeneous fluorescent assay using molecular beacon probes for detection of a specific miRNA sequence was performed by flowing solutions containing the molecular beacon probe and a sample containing the target miRNA simultaneously through the two inlets. We utilized a previously reported molecular beacon probe and miRNA pair as an initial demonstration of PC enhanced fluorescence of a homogenous assay [140]. The molecule miR-21(5'-ACTCC TACG GGACGCAGC-3') was the target while a mutant molecule miR-21 with a single base mismatch (5'-ACTCC TACG CGACGCAGC-3', mismatched base is marked in bold) was used as a negative control for demonstration of selectivity. Both of the mutant miR-21 and the mature miR-21 were diluted by
phosphate buffer sodium (PBS, pH = 7.4) into five concentrations ranging from 62 nM to 1 µM. The molecular beacon was designed to be complementary to the mature sequence of miR-21 (Cy5-5’-CATCC GCTGC CTCC CGTA GGAG T G-3’-BHQ2, the complementary region is indicated in bold). Each beacon consists of a 5’ Cy5 fluorophore, a 3’ BHQ2 quencher and a hairpin stem-loop sequence that is complementary to the mature miRNA flanked by residues necessary to form a beacon stem. The beacon solution was diluted to a concentration of 1 µM. The mature miR-21, mutant mature miR-21, and DNA molecular beacons were synthesized by Integrated DNA Technologies (Coralville, Iowa).

The performance of sensor and detection instrument were studied in the context of a molecular beacon assay for miR21. A 1 µM DNA molecule beacon solution and test sample containing mature miR21 were pumped into two separate inlets by a syringe pump (PHD2000 series, Harvard Apparatus) at a pumping rate of 0.5 µL/min for ~5 minutes. Waste liquid was collected at the outlet. The total volume inside the channel was calculated to be ~20 nL. After performing a fluorescence intensity measurement and flushing the system completely with deionized water, the detection experiment was repeated, using a new miR21 concentration. The concentrations of miR21 were 31 nM, 62 nM, 125 nM, 250 nM, 500 nM after dilution with the molecular beacon. Based upon previous studies of intermixing between laminar flows adjacent introduced into the same flow channel, we expect complete intermixing between the two reagents to occur through the 10 mm channel length, resulting in a mixed sample reaching the circular measurement zone. Fluorescence intensities from the
center of the observation window were collected over a range of incident angles for each concentration. The donor fluorophore of molecular beacon probe is not completely quenched when the probe is in its native unreacted state, resulting in the presence of “background” fluorescence, even if no target is present. The background fluorescence is also amplified by PCEF, thus we expect to observe higher fluorescence intensity even for zero concentration of the analyte. We define the noise of the fluorescence measurement to be the standard deviation of the fluorescent intensity when no target molecule is present, in which the standard deviation is obtained by measuring the same zero concentration sample three times in a row. The lowest detectable fluorescence intensity is defined as the mean of these three measurements, plus 3 x the standard deviation. The background fluorescence level was measured by this approach for the case when the PC is illuminated in the on-resonance state, and again with the PC illuminated at the off-resonant state. The off-resonance illumination is obtained by de-tuning the illumination angle by 2 degrees from the on-resonant angle. In order to demonstrate the selectivity of the assay, a concentration series of mutant mature miR21 (62 nM, 125 nM, 250 nM, 500 nM, 1 uM) were added into the 100 µL beacon solution as controls. The fluorescence intensities were obtained with the PC illuminated on-resonance and off-resonance for each concentration, and compared with that from the mature miR21. Hybridization of beacon to miRNA was performed for 3 min, as verified by performing the assay in a conventional 100 µL cuvette, and measuring the output with a conventional fluorometer (Varian Cary Eclipse fluorometer, Agilent). Detection instrument
settings for measuring fluorescence output from the PC-integrated flow channel were the same for all measurements. (laser current = 80 mA, CCD camera sensitivity = 70, gain = 1 and integration time = 0.04 ms).

Dose-response curves for detection of mature miR21 (target, black lines) and mismatched mutant miR21 (control, blue lines), obtained from the device illuminated using on-resonance (solid lines) and off-resonance (dashed lines) conditions are plotted together in Figure 5.4(a). The comparison between fluorescence intensities at the same concentration both on-resonance and off-resonance clearly shows that much stronger fluorescence were produced by the PC surface. The enhancement was calculated to be ~8x for the miRNA mixture with concentrations higher than 62 nM. The figure also demonstrated the selectivity of the assay. As shown in Figure 5.4(a), the fluorescence from the mutant mature miR21 with the highest concentration (500 nM) is only 1/7 of that from the mature miR21 at the on-resonance condition and no fluorescence was detectable in samples with concentration lower than 250 nM, demonstrating excellent selectivity.

To determine the detection limit, the fluorescent intensities of the hybridized target and the background are plotted together in Figure 3.4b. The solid black represents the on-resonance fluorescence intensities of the hybridized targets at different concentrations. The error bars represent the standard deviation of three separate fluorescence measurements for each concentration. The on-resonance lowest detectable fluorescence signal $M_l = 77200$ (the solid red line in Figure 3.4b), which was calculated by adding the
background fluorescence $\text{Mb} = 74800$ and the noise $S_d = 800$, is slightly higher than the fluorescence from the 31 nM hybridized target. Therefore the detection limit for on-resonance PC is 62 nM. The result demonstrates the high sensitivity of the biosensor, considering only $\sim0.02 \mu$L sample solution was used to fill the channel, representing $\sim7.4 \times 10^6$ molecular beacon probes for the assay. By comparing the fluorescence from the samples (dashed black line) and the lowest detectable fluorescence (dashed red line), the detection limit of the off-resonance PC is 125 nM. Although the background fluorescence was slightly enhanced, the sensitivity was still substantially improved utilizing the PC resonance effect.

5.5 Discussion and Conclusion

We have demonstrated an 8x fluorescence intensity increase for detection of miRNA in a homogeneous molecular beacon assay by a PC as one of the internal surfaces of a microfluidic channel. The increased sensitivity enables detection of 62 nM miRNA in a 20 nL sample. In order to confine the liquid volume within the evanescent field region of the PC, we developed an e-jet printing method to inexpensively fabricate fluid channels with $<1 \mu$m channel height that utilizes the PC as one internal surface.

The approached demonstrated here may be applied to any homogeneous fluorescent assay, including FRET, that is typically performed in a cuvette and measured by a conventional fluorometer, with the benefit of consuming a $\sim3$-orders of magnitude lower sample volume. Another fluorescent assay that may potentially benefit from this approach is polymerase chain reaction (PCR), which requires several thermal cycles to create enough fluorescently tagged products.
to generate a measurable signal from sample volumes in excess of 20 µL. The enhanced fluorescence provided by a PC integrated within a fluid channel may enable detection of PCR products with fewer cycles of amplification and thus a shorter detection time.

An important aspect of this research is that the fluorescence enhancement was achieved in a microfluidic format that permits the integration of functional components and allows high throughput detection. By multiplexing the operation of several parallel microfluidic chambers, the presence of multiple miRNA sequences may be probed at the same time.

To conclude, we have presented a novel method, supported by experimental demonstrations and numerical modeling, to enhance the fluorescence of a homogeneous molecular beacon assay, in which the analyte solution is confined to the evanescent field region of a PC surface by a submicron-height microfluidic channel. A simple and inexpensive fabrication technique based on e-jet printing was developed to create the shallow channel. The approach yields excellent sensitivity while consuming only nL reagent volumes per assay. In light of the simplicity of the detection instrument and the compatibility with existing biodetection tools, this approach may be broadly applied in disease diagnostics and other fluorescence-based sensing applications.
5.6 Figures

**Figure 5.1.** (a) Schematic diagram of the device structure, comprised of a transparent glass layer, a microfluidic region and a PC surface for fluorescence enhancement. (b) SEM image of the one-dimensional PC with a period of 360 nm and duty cycle of 36%. (c) Evanescent electric field enhancement distribution at the resonant coupling condition of the PC. (d) The average enhancement of field intensity as the function of distance, \( d \), above the top TiO\(_2\) layer of the PC.
Figure 5.2. (a) Schematic diagram of the fabrication process: I. The channel pattern was e-jet printed on a silicon PC (Si-PC); II. The Si-PC was carefully aligned with a glass slide and pressed against each other by a roller to squeeze the droplets. The layout of the channel consisting of two inlets with diameters $D_1 = 1\, \text{mm}$ (left), one observation window with a diameter $D_R = 2\, \text{mm}$ (middle), and one outlet with a diameter $D_1 = 1\, \text{mm}$ (right). The total length and the width of the channel are $L = 13.6\, \text{mm}$ and $W = 0.5\, \text{mm}$. (b) Photograph of the PC integrated into the submicron flow channel. (c) Optical image of the observation window. (d) Optical image of the printed NOA74 droplets. (e) Optical image of the continuous NOA74 film after squeezing the droplets. (f) SEM image of cross section of a completed device, showing the thickness of the channel is $\sim 830\, \text{nm}$. 
Figure 5.3. Fluorescence intensities obtained from 50 µg/mL LD700 solution filled within the channel when excited over a range of illumination angles.
Figure 5.4. Results of the miR21 detection by DNA molecular beacon assay. (a) Dose response curves of mature miR21 (target, black lines) and one pair mismatched mutant miR21 (control, blue lines), obtained from PC at on-resonance (solid lines) and off-resonance (dashed lines) conditions. The results confirm the selectivity of the assay as well as the PCEF enhanced excitation effect. (b) A zoom-in plot of the fluorescence intensities for the detection of mature miR21 at on-resonance (black, solid line) and off-resonance (black, dashed line) conditions. The red lines represent the lowest detectable fluorescence at on-resonance (solid line) and off-resonance (dashed line) conditions.
CHAPTER 6 CONCLUDING REMARKS

6.1 Summary of Current Work

This dissertation makes a number of contributions to advancing the PCEF technology. It expands the application of PCEF to allergy test, allows automatic assay by integrating microfluidic channels, and implements the PCEF technology to another important fluorescence assay –molecular beacon assay. The following is a summary of the main contributions.

1. Detection of low-concentration allergen-specific Immunoglobulin E (IgE) in human sera using a Photonic Crystal Enhanced Fluorescence (PCEF) microarray platform. A comparison of the lowest detectable concentration of IgE measured by the PC microarray system and a commercially available clinical analyzer demonstrated that the PCEF microarray system provides higher sensitivity. The PCEF system was able to detect low concentrations of specific IgE ~0.02 kU/L, which is 5-fold to 17-fold more sensitive than the commercially available FDA-approved analyzers. In preliminary experiments using multi-allergen arrays, we demonstrate selective simultaneous detection of IgE antibodies to multiple allergens.

2. Development of a detection platform that integrates photonic crystal surface with a microfluidic cartridge. Simultaneous goals of high analytic sensitivity (single-digit pg/mL), high selectivity, low sample volume, and assay automation were achieved. A plastic microfluidic device for holding the PC was
created using a low-cost and flexible process. An assay automation system that utilizes a leak-free fluid interface was built to introduce the correct sequence of fluids through the microfluidic device. Through the use of the assay automation system and the PC embedded within the microfluidic device, 1.59 pg/mL-level limit of detection was demonstrated by performing a representative biomarker assay (for IL3 and TNF-α). Improved intensity uniformity from spot-to-spot was achieved by the microfluidic automatic system compared to performing assays with manual manipulation of assay liquids. The system’s capabilities, when fully developed, are compatible with the goal of diagnostic instruments for point-of-care settings.

3. Enhancement of a liquid-based homogenous fluorescence assay using the resonant electric fields from a PC surface. A PC was integrated within a fluid channel with submicron height, using electrohydrodynamic jet (e-jet) printing of a light-curable epoxy adhesive to define the fluid channel pattern. Using a molecular beacon nucleic acid fluorescence resonant energy transfer (FRET) probe for a specific miRNA sequence, an 8x enhancement of the fluorescence emission signal was demonstrated, compared to performing the same assay without exciting resonance in the PC detecting a miRNA sequence at a concentration of 62 nM from a liquid volume of only ~20 nl. The approach may be utilized for any liquid-based fluorescence assay for applications in point-of-care diagnostics, environmental monitoring, or pathogen detection.
6.2 Future Work

While we have successfully demonstrated high sensitivity, multiplexed and automatic platform for biomarker detection by integrating PC surface and microfluidic channels, there are still needs to further improve the platform so that the system can be readily used in clinical environment. Our future direction will require integrating several innovations into the current platform: (1) The Si-PC integrated with Fabry-Perot optical cavity will be used to deliver ~50x greater signal enhancement than current-generation PCEF devices. (2) A laser-machined blood filter will separate plasma from a droplet of heparinized whole blood in 60 seconds. (3) Use of ultrasonic agitation to speed the immunoassay, enabling the entire assay protocol to be performed automatically in <60 minutes without user intervention.

In order to improve the sensor’s performance, we will incorporate the Si-PC with a Fabry-Perot cavity [141]. The function of the mirror is two-fold. First, it will reflect fluorescence emission of the PC surface that would normally be directed downward (away from the detection optics), increasing detected photon intensity by 2x. Second, computer models suggest that it is possible to choose the distance between the PC surface and the mirror so as to establish a Fabry-Perot optical cavity, in which constructive interference results in 3-5x greater electric field intensity at the plane of the assay surface. We will fabricate the PC using a silicon substrate, with the PC grating structure defined by deep-UV photolithography and reactive ion etching in a semiconductor foundry. This
approach provides uniform and low-cost fabrication of the PC grating together with a metal reflection mirror beneath the PC.

In a clinical laboratory setting, plasma separation from whole blood can easily be performed using centrifugation, although collection of blood and its processing remain challenging at the point of care (POC) [142-144]. Inclusion of blood cells or their components in an ELISA results in inaccurate quantification, reduced sensitivity, or even assay failure [145, 146]. Therefore, on-chip plasma separation from blood cells is required to obtain optimal assay performance. In our future work, we will focus on developing an approach to easily integrate a plastic filter with the microfluidic cartridge. Although a variety of microfluidic approaches for achieving on-chip plasma separation have been reported utilizing capillary force [147], the Zweifach-Fung effect [148] and H$_2$O$_2$-powered pumping [149], there is still an unmet need for a simple and rapid plasma separation device to facilitate point-of-care testing for ELISA applications [150]. We will collaborate with the Demirci Group at Stanford to integrate the plasma filter with the microfluidic chip. The Demirci Group recently demonstrated a microchip that can separate plasma and virus selectively from unprocessed whole blood by using an elegant approach that does not require an external pump or substantial dilution of the sample. As described in preliminary results, the approach was originally developed for separation of virus from whole blood, where up to 89.9 ± 5.0% HIV virus was recovered in the flow-through while 81.7 ± 6.7% of RBCs and 89.5 ± 2.4% of WBCs were retained by the filter. The
approach utilizes an inexpensive laser-cut polymer membrane filter that can be easily integrated with the microfluidic cartridge.

Acoustic streaming is an effective mixing technique applicable to microarrays. In this technique, an ultrasound wave passes through a liquid medium (coupling gel) and transfers the momentum to the test fluid, generating a steady circular flow occurring in a high-intensity non-linear acoustic field. Therefore, the target molecules in the sample were observed to move in both vertical and horizontal directions under the influence of acoustic streaming facilitating hybridization with capture molecules on the surface [151-153]. In the previous study, fluorescence signal from DNA hybridization was increased by 24% using acoustic streaming with applied voltage of 24 V and resonant frequency of 1.67 MHz [152]. In our future work, we will use a piezoelectric ultrasonic transducer (resonant frequency = 1 MHz, Olympus NDT Inc., MA, USA) to create the acoustic streaming during the sample incubation step. The transducer will be driven by a square wave (Vpp = 10 V) generated by a function generator (model 33210A, Agilent Technologies, CA, USA) at the applied voltage of 10 V. To avoid the heat accumulated, the transducer will be turned on and off. The on and off times for activating the disks will be varied to find an optimum condition. To minimize the reflected power, a layer of coupling gel will be used to effectively couple the acoustic wave into the sample.

Significant funds and efforts have been committed to the future of cancer diagnostics with the hope of solving several existing formidable analytical
challenges; if successful, the payoff and utility of such a detection platform will be great.
REFERENCES


proteins at subfemtomolar concentrations,” *Nature Biotechnology*, vol. 28, no. 6, pp. 595-625, Jun., 2010.


G. W. Hagy, and G. A. Settipane, “Prognosis of positive allergy skin tests in an asymptomatic population: A three year follow-up of college students,” Journal of Allergy and Clinical Immunology, vol. 48, no. 4, pp. 200-211, Oct., 1971.


