Clinical Detection of Diagnostic Biomarker Panels Using Microfluidic Electrochemical Immunoarrays

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Clinical Detection of Diagnostic Biomarker Panels Using Microfluidic Electrochemical Immunoarrays

Abby Lynn Jones, Ph.D.

University of Connecticut, 2020

Cancer is a worldwide infliction. Cancer does not discriminate. Cancer does not care if you are young or old, rich or poor, disabled or in the prime of your life. It can be caused by genetics, environmental factors and/or lifestyle. The challenge, how do we diagnose and treat such a dynamic disease?

Cancer detection is expensive, invasive, inaccurate and lacks sensitivity. New methods that rely on measurements of analytes in solution for detection and quantification are promising alternatives. Panels of protein biomarkers may aid in personalized diagnosis as protein levels in patients are often upregulated or down regulated in relation to a specified disease. For the medical field this provides opportunities to bring cancer detection to clinical practice as it will enable physicians’ access to blood, saliva, or urine bioassays for screening, as well as monitoring progression and response to therapy.

The objectives of this thesis are to utilize new technology in microfluidic fabrication, 3D printing and nanomaterial synthesis for ELISA alternatives. The sensors are developed to be sensitive, rapid, inexpensive and multiplexed for point of care diagnostics. These technically facile immunoassays in human patient serum comprise a multivariable approach for statistically improving the probability of diagnosing and differentiating forms of cancer.
Clinical Detection of Diagnostic Biomarker Panels Using Microfluidic Electrochemical Immunoarrays

Abby Lynn Jones

B.S. University of New England, 2012

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut 2020
Clinical Detection of Diagnostic Biomarker Panels Using Microfluidic Electrochemical Immunoarrays

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2020
Dedicated to

My parents, Kirk Jones and Kathy Saarinen

My soulmate, Zachary Martin

My sister, Samantha Jones

My aunt and uncle, Patti Saarinen and Ray Potvin

My late grandmother, Elva Sturtevant

2020
Acknowledgements

To my parents, I can never thank you enough for all the opportunities you have provided me. Your love and support have been the backbone of where I am today. I need to thank my sister, you are my toughest critic and my greatest ally. I keep moving forward because I know you will always be there for me. Zach, you are my happily ever after. You help me every moment to be better and never give up. To my family, I love you and thank you for always being proud of who I am.

I would like to thank my major advisor, Jim. Your support and guidance have helped me grow to be a skillful researcher. Drs. Peczuh and Adamson, thank you for all our discussions and being a part of my journey. To Dan, you always make me smile. To Emilie, thank you for answering every question and concern I brought to you. To my collaborators, Dr. Dónal Leech, Dr. Norman Lee, Dr. William Watson and Dr. Dipak Dey thank you for all your time, patience, and help. To my mentor and friend, Colleen, I appreciate every moment with you. I cannot forget, my lovely friend Thaísa, to our lasting memories together.

To my lab mates, you all have become a second family. From our lab cleanings to our potlucks, I have had an amazing time with all of you. Special thanks go to Lasangi, Patty and Mohamed, you three have been there for the laughter, crying, fights and forgiveness, you will always be a part of me.
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Chapter One

Introduction

1.1 Background and Significance

Cancer morphs through many stages and forms leaving doctors and patients with a diverse array of questions. Is the cancer slow growing or is it aggressive? If it is aggressive is it localized or has it metastasized? Do we wait and see or undergo an invasive biopsy and once the results come back, what are the treatment options? The answer is, there is no straightforward answer (Figure 1.1).

![Figure 1.1. Timeline of morphological cancer stages from aggressive to indolent forms.](image)

Cancer has a major impact on society worldwide. As of last year, lung, breast, colorectal/rectum, prostate and stomach cancers had the highest number of incidences on a global
scale.\textsuperscript{1} Nationally it is estimated for the year 2019 there will be an estimated 1,762,450 new cases and 606,880 cancer related deaths.\textsuperscript{2} That correlates to 3 new cases and 1 death every minute.\textsuperscript{2} Overall cancer and the care of those who are diagnosed with cancer is a national expenditure costing over 140 billion dollars annually.\textsuperscript{3} This expense is unlikely to decrease with society aging and cancer prevalence increasing.

Current methods of detection are based on imaging techniques. These techniques might use x-rays, radioactive particles, magnetic fields or sound waves to form a picture of patients’ tissue.\textsuperscript{4,5,6} Whereupon a health care provider can determine if any changes have occurred due to disease, such as cancer. A few examples of these techniques are magnetic resonance imaging (MRI), computed tomography (CT), mammography, positron emission computed tomography (PET) and ultrasonography.\textsuperscript{7,8,9} Unfortunately, these techniques are limited in the information they can give the provider. They cannot alone say a change was due to cancer.

Further limitations include qualitative screening methods that rely on subjective results that, at times, depend on the health care providers’ training and experience. Examples in prostate cancer diagnosis include the digital rectal exam (DRE) and tissue biopsies scored by the Gleason Scale.\textsuperscript{10,11} These bias tests add to increased chances of missing small but significant cancer indicators, as well as increase the over-diagnosis and over-treatment rate.\textsuperscript{12}

Alternatively, patients can be screened for cancer without undergoing invasive procedures or bias tests by assaying bodily fluids. The biomarkers analyzed in these bodily fluids are chemical, physical or biological parameters that can be used to indicate a biological or disease state and can be grouped into a variety of categories that include proteins, glycoproteins, oncofetal antigens, hormones, receptors, DNA and RNA molecules.\textsuperscript{13,14} Despite the discovery of thousands of protein cancer biomarkers, less than two dozen have been approved for clinical use in the United
States.\textsuperscript{14-17} One potential reason is due to the fact that biomarkers can be indicative of more than one disease. Therefore, biomarker panels have been analyzed as a means of specific diagnosis, prognosis and prediction.\textsuperscript{18-22}

The goal of this dissertation was to take identified protein biomarkers to the next step; integration into an immunoassay for point-of-care diagnostics. To do this the objectives were inexpensive, requires minimal sample volume, short assay time, multiplexed, minimal expertise to operate and exhibit a wide dynamic range.\textsuperscript{14,22,23} Furthermore, device operation and assay preparation must be simplified for clinical use.\textsuperscript{24,25} To achieve these goals an ultrasensitive multiplexed electrochemical immunoassay was developed in part of a simple microfluidic system.

\textbf{1.2 Cancer Protein Biomarkers}

Unpredictable cell proliferation caused by a genetic disorder leading to the growth of a tumor is the classic description of cancer.\textsuperscript{26,27,28} A biomarker is described by the National Institute of Health (NIH) as “a characteristic used to measure and evaluate objectively normal biological processes pathogenic processes or pharmacological responses to a therapeutic intervention”.\textsuperscript{29} Therefore, to put these together, a cancer biomarker is a biological indicator of initiation, progression or spread or response to drug therapy in regards to a patients’ cancer. There are vast collections of biomarkers available that span proteomics to genomics. One important collection is proteins. Protein biomarkers are important due to the ease of use as their levels can be elevated or depressed in response to cancer.

The first protein cancer biomarker was reported in 1965 by Dr. Joseph Gold.\textsuperscript{30} He discovered carcinoembryonic antigen (CEA), a blood serum biomarker for colon cancer. Further well known biomarkers were discovered through the 1980s’ and despite increased interest in
biomarker investigation the FDA has only cleared 18 biomarkers, to date, for clinical use (Table 1.1).\textsuperscript{26,31} Reasoning behind this is partially due to the long and arduous journey a protein must take to be seen as clinically useful.

**Table 1.1.** FDA approved protein cancer biomarkers

<table>
<thead>
<tr>
<th>Source</th>
<th>Biomarker</th>
<th>Cancer Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>(\alpha)-fetoprotein (AFP)</td>
<td>Nonseminomatous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testicular</td>
</tr>
<tr>
<td></td>
<td>Human chorionic gonadotropin (hGC)</td>
<td>Testicular</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate antigen 19-9 (CA19-9)</td>
<td>Pancreatic</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate antigen 125 (CA125)</td>
<td>Ovarian</td>
</tr>
<tr>
<td></td>
<td>Thyroglobulin</td>
<td>Thyroid</td>
</tr>
<tr>
<td></td>
<td>Prostate specific antigen (PSA)</td>
<td>Prostate</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate antigen 15.3 (CA15.3)</td>
<td>Breast</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate antigen 27.29 (CA27.29)</td>
<td>Breast</td>
</tr>
<tr>
<td>Tissue</td>
<td>Carcinoembryonic antigen (CEA)</td>
<td>Colorectal</td>
</tr>
<tr>
<td></td>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>Colorectal</td>
</tr>
<tr>
<td></td>
<td>v-kit Hardy-Zuckerman 4 feline sarcoma vial oncogene homolog (KIT)</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td></td>
<td>Estrogen receptor (ER)</td>
<td>Breast</td>
</tr>
<tr>
<td></td>
<td>Progesterone receptor (PR)</td>
<td>Breast</td>
</tr>
<tr>
<td>Urine</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER2-neu)</td>
<td>Breast</td>
</tr>
<tr>
<td></td>
<td>Nuclear matrix protein 22 (NMP-22)</td>
<td>Bladder</td>
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<tr>
<td></td>
<td>Fibrin/fibrinogen degradation products (FDP)</td>
<td>Bladder</td>
</tr>
<tr>
<td></td>
<td>Bladder tumor antigen (BTA)</td>
<td>Bladder</td>
</tr>
<tr>
<td></td>
<td>High molecular CEA and mucin</td>
<td>Bladder</td>
</tr>
</tbody>
</table>

Figure 1.2 shows the major steps a protein must follow before it can be labeled as a cancer biomarker. The process begins with a number of potential analytes that will be explored for candidacy. An assay will then be developed to quantitate the promising biomarkers in clinical samples. Assay development overlaps with phase three as the samples used are retrospective validation of a large cohort stored in a tumor/blood bank.\textsuperscript{31,32} The analytes that make it through retrospective studies are then put through prospective trials determining statistical power. Once completed analyses are made of the validated biomarkers for their potential to reduce the cancer
burden on the population.\cite{31,32} Once all five stages are completed, then the protein can be declared a cancer biomarker.

Figure 1.2. Biomarker development. There are five main phases an analyte must undergo to become a biomarker. Although most phases are sequential, they are not always distinct from one another. Only analytes that successfully complete the final phase will be implemented in the clinic and categorized as a cancer biomarker.

Biomarkers can be split into five categories: screening, diagnostic, prognostic, predictive and monitoring. A screening biomarker is one that has the potential to detect cancer early and increase survival rates.\cite{31} The most well-known screening biomarker is used in prostate cancer, the Prostate Specific Antigen (PSA). Diagnostic biomarkers are characterized with the ability to diagnose a patient with cancer once symptoms have been presented.\cite{31} Many biomarkers are placed
in this category, including PSA, CA-125, Calreticulin, and Survivin. A prognostic marker is one that provides history and progression information. These biomarkers are specifically used to indicate risk and include HER-2, Carboxy terminal telopeptide of type I collagen (ICTP), Caspase-3 and Vimentin, to name a few. Predictive biomarkers are used to decide upon therapy options. There are very few predictive biomarkers that are effective at selecting patients due to disease resistance. Finally, biomarkers that are classified as monitoring, monitor a patient following therapy for recurrence such as carbohydrate antigen 19-9 (CA19-9).

Ideally these biomarkers would have 100% sensitivity and specificity when guiding a physicians’ actions for a patients’ cancer. Unfortunately, no single biomarker has the ability to do this. Moreover, no single biomarker can differentiate forms of cancer, diagnose early and advanced stages and monitor treatment. So, why are current tests relying on single biomarkers to provide actionable information? Instead, to have a suitable amount of evidence to take action, panels of multiple biomarkers are needed. Multiplexed platforms contain more information with the ability to provide the discriminatory evidence required to make clinically informed decisions.

1.3 Point of Care Diagnostics

Point of care (POC) diagnostics is not a modern notion. Point of care began at the earth’s conception with at home doctors’ visits. As technology developed so did patient testing at centralized hospitals to reduce health care costs and laboratory effectiveness. These technologies have improved greatly the care and outcomes of patients. In exchange, laboratory tests require specialized training and expensive equipment with a lengthy turnaround time. To combat this, the World Health Organization has called for new clinical diagnostic tools that can be performed at or near the site of patient care (Scheme 1.1). These tools must be reduced in
size so they can be handheld and portable, require minimal sample and reagent volumes all the while maintaining high-throughput with statistically acceptable sensitivity and specificity.\textsuperscript{34,35,37}

\textbf{Scheme 1.1.} POC testing objective.

With these specifications come limitations and challenges. A major concern in regards to POC testing is reliability. Can a test be made so it is accurate and consistent from person to person? Furthermore, depending on the development of the test how much training is required to perform it? If the test is made for an at home setting, it needs to be simplistic enough to be completed in a “do this, then this” format, whereas in a hospital or physician’s office a member of staff can be trained to complete the POC with minimal oversight. Another limiting factor for POC has been data management, developing a secure network for patient information transmission ensuring health care continuity and protection.\textsuperscript{38}

Despite these limitations, POC testing has been developed both for personal use and in the medical field. The first POC test was developed in the 1960s to measure blood glucose levels based on electrochemical detection.\textsuperscript{35,39} Followed by development of the rapid pregnancy test in the late 1970s.\textsuperscript{35} The pregnancy test is a lateral flow assay based on enzyme-linked immunosorbent assay (ELISA) chemistry. Today the POC testing market is worth over 23 billion dollars in the US alone with an expected compound annual growth rate of 14.2% globally over the next five years.\textsuperscript{40}
1.3.1 ELISA

For over 40 years ELISA has been the gold standard in quantitation of pathogens, proteins and other biological molecules. The majority of commercial ELISAs’ are designed to detect one biological marker at a time using a colored enzyme reaction with detection limits in the pg mL⁻¹ range. The technique can be broken into various types based on binding of the analyte and matching antibody. The four types of ELISA are direct, sandwich, competitive and reverse with sandwich ELISA being the most well-known.

In a Sandwich ELISA primary antibodies (Ab₁), specific to the target analyte, are attached to the surface of a 96 well plate (Scheme 1.2). Following immobilization of the primary antibody, antigen (Ag) is introduced and allowed to incubate to form an Ab₁-Ag bond. Secondary enzyme labeled antibodies are then added and bind selectively to the Ag bound on the surface. Once the secondary antibodies are bound a substrate chemical is added that causes a color change of the solution that can then be detected optically. ELISA has a long history of being a successful detection method with sensitivity and specificity to match. However, with labor and time intensive procedures ELISA does not meet the current POC demand.

**Scheme 1.2** Antigen is added to an ELISA plate coated with primary antibody followed by the addition of secondary antibody conjugated with an enzyme label. Signal is generated by addition of a substrate that producing a colored product measure by an optical reader.
1.3.2 Lateral Flow Immunoassays

Leading the charge in POC diagnostics are lateral flow assays (LFAs). LFAs are paper-based technology used for detection of proteins, haptens, nucleic acids and amplicons. They are used in most professional fields including medical, veterinary, environmental, food, and agriculture. The most well-known applications of this test are the pregnancy and Influenza LFAs. Their advantages lie in cheap production, ease of use, rapid response, and versatility. This is balanced by the fundamental challenge of design and development.

Even though the concept behind LFAs is simplistic, the architecture is critical for sensitivity and specificity. LFAs generally follow ELISA sandwich chemistry with few exceptions. A typical LFA is designed with several overlapping membranes that can include a samples pad, conjugate pad, analytical membrane like nitrocellulose and an absorption pad; the biological elements are striped/sprayed onto the membranes (Figure 1.3). Through capillary action the sample containing the target analyte moves through the test strip forming bonds with specific molecules conjugated to labeled particles and lastly immobilizing on the appropriate test line. A control line is also used to indicate proper response of the flow strip itself.
Figure 1.3. (A) LFA strip components; (B) LFA test development; the orange arrow indicates addition and flow of the sample and sample-detector nanoparticle conjugate.

Conventionally, LFAs have been used for detection of single analytes with qualitative or semi-qualitative results. With the increasing demand for early detection and non-invasive diagnostics, multiplexed testing has emerged to fill this niche. Multiplexed LFAs are fundamentally the same as the singleplex LFA, but allow for detection of multiple analytes.45
Despite the many improvements LFAs have undertaken in terms of analyte detection and quantitation they are still hindered by sensitivity and specificity issues as well as matrix effects.\textsuperscript{43,44,45}

1.4 3D Printing for Medical Diagnostics

Charles W. Hull in 1986 was the first to report stereolithography\textsuperscript{46} as a tool to fabricate 3D structures. Since then, 3D printing has evolved into a multifunctional fabrication tool that offers unique advantages for biomedical applications including diagnostics,\textsuperscript{47} scaffolds for 3D implants,\textsuperscript{48} prosthesis\textsuperscript{49} and tissue engineering\textsuperscript{50}. In recent years, the ability to convert computer assisted design (CAD) files into 3D-printed pieces has sparked significant progress in the field of diagnostics.\textsuperscript{51} As an additive manufacturing technique, production costs are lower compared to traditional subtractive manufacturing techniques like milling or ablation due to reduction of the labor and material cost. Moreover, one 3D printer can be used to produce different parts without the need for pre-fabrication changes normally required in subtractive manufacturing.\textsuperscript{52,53} These criteria make 3D printing a valuable tool in prototyping, testing and production of tools and equipment for analytical and diagnostic laboratories. In principle, CAD files of previously reported devices can be downloaded and printed in any laboratory. In this way advanced diagnostic tools can be directly utilized by researchers without the need for purchase from a commercial vendor. This approach has the potential to bring advanced diagnostic tools more rapidly to the research lab.

3D printing techniques have many applications in medical diagnostics. Each technique impacts different design aspects including cost, resolution and fabrication of complex devices in a continuous process.\textsuperscript{54-56} 3D printed microfluidic devices have been used to fabricate semi and fully automated diagnostic approaches for diseases like cancer,\textsuperscript{57,58} infectious disease,\textsuperscript{59-61} and
xenobiotic genotoxicity\textsuperscript{62}. 3D printing can also make tailored supporting devices that improve performance of existing diagnostics like spectrophotometers\textsuperscript{63} and PCR devices\textsuperscript{59,64} and is used to assist with smartphone integration for remote sensing\textsuperscript{65,66}. The ability to print materials with special properties allows for the creation of new equipment that can dramatically reduce the cost of diagnostic devices like SPR\textsuperscript{67}. All these applications use 3D printing for cost-effective multifunctional production to integrate several functions in one device\textsuperscript{68}.

Fabrication of diagnostic devices with embedded electronics and circuits have also been completed by 3D printing. The ability to print different materials simultaneously permits the fabrication of electrodes that can be incorporated into the insulator plastic matrices allowing for subsequent electrochemical detection of metals,\textsuperscript{69-71} organic compounds\textsuperscript{72,73} and biologically active molecules\textsuperscript{74}. 3D printing avoids disadvantages associated with screen printing like the need for masking and drying steps all the while exhibiting better resolution and faster fabrication\textsuperscript{75}. With 3D printing there is limited need for specialized training and devices can be tailored to the users’ requirements\textsuperscript{76,77}.

Evolving applications and developments have made 3D printing the next work horse for readily available, cheap, miniaturized, multifunctional and sensitive diagnostics. Researchers from different backgrounds have developed diagnostic assays using versatile approaches due to facility granted by 3D printing technology. It has been used as a handy tool for device prototyping and development with photolithography most commonly used because of the availability of materials exhibiting different properties and high resolution. 3D printing has pushed biomedical diagnostic research toward multifunctional devices that can perform several functions like protein and metabolite extraction, fluid flow control, and photo and electrochemical signal detection.
3D printing technology still needs improvement in order to enhance current diagnostics. First, simultaneous printing of multiple materials with high resolution and good compatibility is essential, especially for functional materials like conductive inks and biomimetic substrates. Printing multiple materials with different physical properties would greatly improve the capabilities of 3D printers to produce more complex functional architectures. The ability to print active biomaterials like enzymes and proteins in 3D formats without compromising biomaterial activity is also an important requirement for better diagnostic devices.

More complex architectures are expected in the near future with the progressive nature of 3D printing. Recent research is focused on the development of microfluidic pumps, automated flow control valves, atomic force microscopy and even sophisticated scanning electron microscope sample holders. These are examples for very complex architectures that could not have been approached in the lab easily without the use of 3D printing. This implies the significance of incorporating 3D printing in bioanalytical and diagnostic research providing a platform for achieving what was believed to be imaginary in the pre-3D printing era.

1.5 Electrochemical Immunoassays

Electrochemical immunosensing was pioneered by W. Heinemann and H. Halsall in the 1980s. They utilized sandwich ELISA technique with enzyme labeled alkaline phosphatase for the production of electroactive products. The basis of electrochemical sensors are that via a reaction with the analyte of interest an electrical signal is produced proportional to the analyte concentration. Most often this is completed with a three-electrode system including a working electrode, reference electrode connected, and counter electrode for completion of the circuit. Many techniques can be used for electrochemical detection with the three most common being potentiometric, amperometric and impedimetric.
Impedimetric sensors are based on dividing AC potential across an electrical component divided by the current flowing through it.\textsuperscript{83} This results in a complex number composed of the resistive and capacitive processes. Electrochemical impedance spectroscopy (EIS) is commonly the technique used for these biosensors as they provide information about the physico-chemical changes once the analyte binds to the bioreceptor bound on the electrode.\textsuperscript{83} Amperometric biosensors incorporate a transducer in order to analyze the charge difference between the electrodes separated by an electrolyte.\textsuperscript{83,84} These sensors convert biological recognition events from electroactive species at the electrode surface into signal for quantification of the analyte. A prime example of these types of biosensors is that of the glucose monitoring device for diabetes patients.

In electrochemical immunoassays, enzyme labels are used for electron transfer with horseradish peroxidase (HRP), glucose oxidase or alkaline phosphatase. The HRP label is used most often as it can be conjugated to streptavidin resulting in specific and strong binding to biotinylated secondary antibodies. The label can be activated by hydrogen peroxide, which oxidizes the iron heme HRP enzyme (Fe\textsuperscript{IV}=O) that can then be reduced to produce an electrochemical signal (Scheme 1.3).\textsuperscript{22} Moreover the signal can be amplified 400 times by using poly-HRP. All electrochemical detection systems described within this dissertation will be carried out with this enzyme label. Various amplification strategies have been used for ultrasensitive detection. These strategies include the use of immensely decorated nanoparticles or magnetic beads, carbon nanotubes (CNTs).\textsuperscript{22,85} Nanostructured surfaces further aid in sensitivity of electrochemical arrays by providing high surface areas for antibody attachment leading to improved access for analyte immobilization.\textsuperscript{85}
Scheme 1.3 HRP activation by hydrogen peroxide. Through addition of hydrogen peroxide the iron heme of HRP is oxidized from Fe$^{III}$ to Fe$^{IV}$=O causing hydroquinone to reduce to benzoquinone, whereupon Fe$^{IV}$=O is reduced back to Fe$^{III}$. When a potential is applied there is an electron transfer causing benzoquinone to be reduced back to hydroquinone.

1.6 Microfluidic Multiplexed Detection

Microfluidics is a technology characterized by manipulating fluids below 1 mL contained within devices designed with micrometer channels. These devices are commonly referred to as miniaturized total analysis systems (µTASs) or lab-on-chip (LoC). The initial focus of many microfluidic devices was with the miniaturization of existing laboratory experiments, resulting in rapid analysis and minimized sample and reagent consumption. Many new approaches have integrated these devices with microanalytical parts such as valves, pumps and stir plates to create a complete micro-analysis system. These systems focus on comprehensive solutions to address biological and clinical research issues; specifically in the POC setting.

At the onset, microfluidic devices were fabricated by glass or silicon. As the technology developed so did the fabrication materials. Polymers such as polymethylmethacrylate
(PMMA), polystyrene and polydimethylsiloxane (PDMS) have emerged as alternatives for facile processing. PDMS is one of the most popular polymers due to its user-friendliness, optical transparency, biocompatibility and flexibility.\textsuperscript{89,90}

For the past decade, a lot of research has been focused on electrochemical-based microfluidic devices. Li et al developed an electrochemical ELISA with a paper-based microfluidic device by patterning chromatography paper.\textsuperscript{91} Pingarrón and Bartosik developed a novel screen printed carbon electrode immunoarray for detection of microRNAs (miRNAs) associated with cervical cancer.\textsuperscript{92} Magnetic bead-antibodies conjugates were used to capture miRNAs hybridized with a DNA probe which is then bound to two biotin-labeled auxiliary DNA probes via a hybridization chain reaction (HCR). Biotin units bind streptavidin-peroxidase labels for amperometric detection upon adding $\text{H}_2\text{O}_2$ and hydroquinone mediator. In our group graphene oxide has been used for protein detection by making composites of $\text{Fe}_3\text{O}_4$ nanoparticles loaded onto graphene oxide nano-sheets ($\text{Fe}_3\text{O}_4@\text{GO}$).\textsuperscript{93} Antibodies were attached onto the paramagnetic $\text{Fe}_3\text{O}_4@\text{GO}$, specifically captured their binding partner and delivered to the screen printed carbon array. This system enabled simultaneous ultrasensitive mediator-free electrochemical detection of PSA and PSMA.

Research has developed for protein use in microfluidic devices as they are crucial elements to biological structure and function.\textsuperscript{87} Their interactions are central to understanding the complex pathways diseases follow. The pathobiology of prostate cancer is an excellent example of the complex behavior of proteins that will be discussed in later chapters of this thesis. Single protein measurements are not advantageous to understanding the pathological state of a disease as proteins seldom function within an individual network and so research efforts in the field of electrochemical protein measurements have shifted to multiplexed detection.\textsuperscript{87,94}
Our labs first microfluidic electrochemical system was fabricated with a machine molded PDMS channel sandwiched between 2 hard PMMA plates connected to a pump and sample injector by PEEK tubing (Figure 1.3). The detection chamber housed an 8-electrode screen printed carbon array as well as a platinum (Pt) wire counter electrode and silver/silver chloride (Ag/AgCl) reference electrode.

![Microfluidic Electrochemical System Diagram]

**Figure 1.4.** Components of the microfluidic device composed of micro-machined PMMA, PDMS microfluidic channel and screen printed 8 electrode carbon array.

Each individual electrode was first modified with sequential layers of PDDA and GSH-AuNPs followed by attachment of primary antibodies through their amine groups. The modified array was then stored overnight. On the day of the experiment an array is inserted into the microfluidic device whereupon protein captured magnetic beads were injected into the system. Protein analytes are captured offline in a microcentrifuge tube using antibody conjugated tosyl
magnetic beads. Using this system interleukin-6 and PSA were detected in the sub pg mL$^{-1}$ range within 1 hour. This system has been further extended to online multiplex detection of parathyroid hormone-related peptides (PTHrP, Figure 1.4). PTHrP is recognized as the major causative agent of humoral hypercalcemia of malignancy (HHM) and has also been implicated in tumor progression and metastasis of many human cancers. Using this system intact PTHrP isoform 1-173 as well as circulating N-terminal and C-terminal peptide fragments were detected for the first time with limits of detection of 3 fg mL$^{-1}$.

**Figure 1.5. Microfluidic system for PTHrP peptide online capture**

To further modernize our system we have moved toward automization for electrochemical detection. We have also designed many 3D printed devices for miniaturization, simplification and decreased cost. These new designs enable the immunoassay to be completed without manual
loading by the operator. Further application of this modular microfluidic device will be discussed in later chapters of this thesis.

1.7 Overview of the Dissertation

To date, no reliable blood test has been devised to distinguish between varying forms of cancer. There is a pressing need to identify novel strategies to stage and grade cancer so necessary interventions are made while minimizing over-diagnosis and over-treatment. The immunoassay platforms described within this dissertation will be developed to address the aforementioned need. This will assist not only in distinguishing patients who need to be monitored versus those who need immediate surgery or therapy, but will also help relieve the healthcare system of patients who would be wrongly diagnosed and over treated.

Chapter 1 provides an introduction to protein cancer biomarkers and point of care diagnostics. This chapter focuses on electrochemical based detection strategies and the incorporation of these platforms into microfluidic systems. Furthermore, a summary of the research background and significance of various projects are included as concrete foundations to accurately rationalize the work demonstrated.

Chapter 2 focuses on patient samples analysis and validation of a multiplexed biomarker panel for prostate cancer diagnosis. Starting with a number of prostate cancer biomarker proteins reported in the literature, an 8-biomarker panel was chosen that includes both general prostate biomarkers, as well as proteins that are reliably specific for aggressive and metastatic forms of cancer. A protocol was developed for the consecutive assay of this 8-protein panel in serum that features online capture and detection with a semi-automated multiple protein immunoarray. Ultra-low limits of detection (LOD) in the sub fg ml\(^{-1}\) range were achieved in 5 µL of serum for the
proteins in the panel. The results from cancer patient sample analysis supports the potential diagnostic utility of this assay to aid doctors in their screening decisions by analyzing not only the change in PSA levels, but of Insulin-like Growth Factor-1 (IGF-1), Pigment Epithelial Derived Factor (PEDF) and Serum Monocyte Differentiation Antigen CD-14 (CD-14) as well.

Chapter 3 expands on an already incorporated project by Otieno et al.\textsuperscript{96} Described is the first application of the on-line modular microfluidic device for detection of a peptide, parathyroid hormone-related peptide (PTHrP). PTHrP is recognized as the major causative agent of humoral hypercalcemia of malignancy (HHM). It has also been implicated in tumor progression and metastasis of many human cancers, including prostate cancer. The first ultrasensitive multiplexed assay to measure intact PTHrP 1-173 as well as circulating N-terminal and C-terminal peptide fragments is described in this chapter. Using ink-jet printed gold nanoparticle immunoarrays limits of detection (LODs) of 3 fg mL\textsuperscript{-1} were achieved for simultaneous detection of PTHrP isoforms and fragments in 30 minutes. Analysis by ROC gave area under the curves of 0.96, 80-83\% clinical sensitivity and 96-100\% clinical specificity. The second ultrasensitive multiplexed assay described in this chapter simplifies and condenses the first described assay to 15 minutes with LODs of 0.2 fg mL\textsuperscript{-1}. Herein cell lysates and human serum samples were evaluated to determine feasibility of detecting PTHrP isoforms in correlation to prostate cancer. These new assays for PTHrP isoforms and fragments show them to be promising biomarkers for clinical diagnosis, prognosis and therapeutic monitoring from early to advanced stages of cancer.

Lastly, Chapter 4 describes the work undertaken to make the electrochemical system described in Chapters 2 and 3 towards point-of-care. According to the World Health Organization, the ideal characteristics for a POC test is one that is Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-free and Deliverable. In order to fall more in line with these desirable
characteristics the old system was modeled based on LFAs, most importantly in terms of array modification. Instead of spotting each individual layer followed by incubation at 4°C, the layer was spotted and dried down in an oven at 40°C. This not only streamlined the modification procedure and enabled same day testing, but also opens the doors for mass manufacturing and a global reach.
1.8 References


4. He, C.; Lin, W. Hybrid nanoparticles for cancer imaging and therapy. Cancer Treat Res. 2015, 166, 173-192


Chapter 2

Clinical Detection of Prostate Cancer with a New Diagnostic Biomarker Panel

2.1 ABSTRACT

Prostate cancers can be diagnosed at varying degrees of concern with only a small fraction of those cases determined to be aggressive disease needing urgent treatment. This situation is not addressed by existing diagnostic methods, including the Prostate Specific Antigen (PSA) blood test, which cannot differentiate prostate cancers. Current clinical practice thus leads to unnecessary treatments including surgery on indolent prostate cancer patients that can adversely affect their quality of life. Measurements of small panels of molecular biomarkers holds tremendous potential for cancer diagnostics and personalized therapy guidance, but biomarker combinations with high diagnostic value for aggressive prostate cancer are currently unknown. This paper describes evaluation of two new protein panels for prostate cancer that promise to be indicative of aggressive prostate cancer and its progression, and alert physicians of cancer reoccurrence. We designed a fast multiplexed microfluidic immunoarray that features miniaturization, low sample volume, sensitivity tailorable to sample characteristics, and low reagent consumption. Assays of the protein panels feature online capture and detection with a semi-automated multiple protein immunoarray. Ultra-low limits of detection (LOD) in the sub fg ml⁻¹ range were achieved in 5 μL of serum. Statistical analysis by receiver operating characteristics (ROC) and principal component analysis (PCA) revealed that a normalized function of levels of 4 biomarkers in the panel, IGF-1, PEDF, CD-14, and PSA, can identify aggressive forms of prostate cancer with high diagnostic accuracy. These new multiplexed immunoassays represent promising and effective approaches for clinical diagnosis that can identify indolent to aggressive prostate cancer patients for the first time.
2.2 INTRODUCTION

Current clinical practice in prostate cancer screening suffers an inability to distinguish aggressive from indolent disease, often leading to unnecessary treatments that adversely affect a patient’s quality of life.\textsuperscript{1-4} Indolent forms of this non-cutaneous cancer are featured in the majority of confirmed diagnoses, but are slow growing and never result in metastasis during a patient’s lifetime. This has led many doctors to recommend a modern approach of watchful waiting or active surveillance.\textsuperscript{5} In this strategy, patients are monitored closely by either observation of symptoms and/or periodic screening tests.

Localized forms of prostate cancer have a 5-year survival rate of 99\%, while for aggressive prostate cancers this drops to 28\% once the cancer has metastasized.\textsuperscript{6} To date, no reliable blood test exists to distinguish between aggressive prostate cancers that will metastasize versus slow growing indolent cancers. This uncertainty leads to over-diagnosis and over-treatment, e.g. unnecessary surgeries, and is a major clinical challenge. There is a pressing need to identify novel strategies to stage and grade prostate cancer so that necessary interventions can be made and over-diagnoses and over-treatment minimized.

Clinical patient screening currently includes the Prostate Specific Antigen (PSA biomarker) blood test and a Digital Rectal Exam (DRE), usually completed annually for male patients over the age of fifty. The PSA test is a measurement of PSA level in a patient’s blood with concentrations above 4 ng mL\textsuperscript{-1} considered abnormal and possibly indicative of cancer.\textsuperscript{7} The DRE is a physical check for abnormalities on the prostate, but is dependent on the skill of the examiner.\textsuperscript{7,8} Should a patient’s screening detect abnormalities, the patient may be recommended to undergo an invasive core needle biopsy.\textsuperscript{9}
Current screening techniques have significant limitations.\textsuperscript{7,10,11} PSA is currently the only biomarker approved for prostate cancer diagnostics, but suffers from specificity errors with 75% of men undergoing unnecessary biopsies because of elevated PSA.\textsuperscript{11,12} This high biopsy rate is related to the fact that PSA is prostate tissue specific, not prostate cancer specific. Elevated levels occur for most abnormalities that occur in the prostate including inflammation, infection, trauma, and benign prostatic hyperplasia (BPH), which are non-cancerous.\textsuperscript{13} Biopsies use multiple needles, but can only acquire limited tissue samples, resulting in a chance of missing small but significant tumors. It is apparent that a more effective diagnostic approach is needed in patient selection for tissue biopsy and identification of prognostic factors for cancer aggressiveness.\textsuperscript{14-16}

The past 20 years of research have shown that measurements of serum biomarker panels offer early cancer detection that can improve therapy outcomes and decrease mortality.\textsuperscript{17-26} Panels of cancer biomarker proteins, as opposed to single biomarkers, are required for highly reliable cancer diagnostics.\textsuperscript{26-28} Numerous tumor biomarkers have been evaluated for facilitating early diagnosis and monitoring treatment outcomes.\textsuperscript{29-31} Unfortunately, diagnostic biomarker panels have been slow in finding their way into clinical practice.

In this paper, we first describe a new blood test using a microfluidic immunoarray to reliably identify aggressive forms of prostate cancer. Starting with a list of prostate cancer biomarker proteins reported in the literature, we identified an 8-biomarker panel that includes both general prostate cancer biomarkers such as PSA, as well as proteins that were reported to be reliably specific for aggressive and metastatic forms. The eight biomarker panel (Table 2.1) includes PSA as a benchmark\textsuperscript{10,31}, vascular endothelial growth factor-D (VEGF-D)\textsuperscript{32,33}, gene fusion proteins ETS related gene (ERG)\textsuperscript{34,35}, insulin-like growth factor-1 (IGF-1)\textsuperscript{36,37}, insulin-like Growth Factor Binding Protein 3 (IGFBP-3)\textsuperscript{37-39}, Golgi membrane protein 1 (GOLM-1)\textsuperscript{40}, pigment
epithelial derived factor (PEDF)\(^{16}\), and serum monocyte differentiation antigen CD-14 (CD-14)\(^{41,42}\). IGF-1 and IGFBP-3 are implicated in antiapoptotic properties.\(^{37}\) ERG and GOLM-1 are biomarker candidates expressed by fused prostate cancer genes.\(^{35,36,39}\) CD-14 is a general cancer biomarker associated with inflammation, thought to reflect immune response to tumors.\(^{42,43}\)

Finally, VEGF-D and PEDF are associated with angiogenesis and known to be correlated with the current prostate tissue staging method, the Gleason score.\(^{16,33,34}\)

**Table 2.1.** Biomarker Panel Normal and Cancer serum levels for early cancer detection, accurate pretreatment staging, monitoring of the disease progression and response to cancer therapy.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Normal Serum Levels</th>
<th>Cancer Serum Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA(^{32})</td>
<td>0.5-2 ng/mL</td>
<td>4-10 ng/mL</td>
</tr>
<tr>
<td>VEGF-D(^{51})</td>
<td>392±95 pg/mL</td>
<td>≤ 297 pg/mL</td>
</tr>
<tr>
<td>ERG(^{35,52})</td>
<td>Not known</td>
<td>Upregulated</td>
</tr>
<tr>
<td>IGF-1(^{40})</td>
<td>248.9±7.5 ng/mL</td>
<td>269.4±8.08 ng/mL</td>
</tr>
<tr>
<td>GOLM-1(^{39,53})</td>
<td>54.3±6.5 ng/mL</td>
<td>Upregulated</td>
</tr>
<tr>
<td>PEDF(^{54})</td>
<td>12.93±3.8 μg/mL</td>
<td>≤ 9 μg/mL</td>
</tr>
<tr>
<td>IGFBP-3(^{55})</td>
<td>3210±843 ng/mL</td>
<td>3101±924 ng/mL</td>
</tr>
<tr>
<td>CD-14(^{56})</td>
<td>4.89±0.82 μg/mL</td>
<td>6.69±0.75 μg/mL or sepsis</td>
</tr>
</tbody>
</table>

The ultrasensitive microfluidic assay simultaneously detected prostate biomarkers over three orders of magnitude clinically relevant concentrations. Excellent sensitivities and detection limits in the low fg mL\(^{-1}\) range were obtained. Analysis of the patient samples returned exceptional correlations with the PSA blood test results confirming accuracy. Statistical analysis of 66 serum samples from prostate cancer patients and 64 benign prostate cancer patients indicated clinical specificity of 56% and sensitivity of 83% for aggressive prostate cancer detection based on normalized means of the protein panel. Further studies predict good aggressive cancer diagnostic potential by combined assay of PSA, IGF-1, PEDF and CD-14. This protein panel measured in
serum in a single fast immunoassay represents a new multivariate approach for reliable diagnosis and identification of aggressive forms of prostate cancer.

Secondly, we studied the limits of detection of this microfluidic system. The majority of commercially analyzed biomarkers are naturally occurring in low ng mL\(^{-1}\) concentrations in human serum. Most diagnostic assays are therefore tailored to detect between upper fg mL\(^{-1}\) to mid to upper ng mL\(^{-1}\). This is not necessarily the detection limit of these assays though and physicians are potentially missing valuable medical information for accurate diagnosis and treatment plans.

To determine the limit of detection for the described electrochemical assay, four of the lowest abundant biomarkers from the 8-protein biomarker panel were chosen; they included PSA, VEGF-D, ERG and IGF-1. Detection limits of 0.034 to 0.341 zmol were obtained. This approach opens avenues for new biomarker discoveries and biomarker options not previously analyzed due to detection hindrances. For prostate cancer specifically, this assay can provide options for surveillance after a prostatectomy especially in regards to prostate cancer reoccurrence.

2.3 EXPERIMENTAL
2.3.1 Chemicals and Materials

Screen-printed 8-electrode carbon array (700 µm diameter) sensors were purchased from Kanichi Research Services Ltd (Manchester, England). L-glutathione reduced (GSH, ≥98%), gold (III) chloride trihydrate (HAuCl\(_4\)·3H\(_2\)O, ≥99.9%), sodium borohydride (NaBH\(_4\), 99%), poly(diallyldimethylammonium chloride) (PDDA, MW 200,000-300,000, 20% in water), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), bovine serum albumin (BSA), calf serum, Tween-20, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na\(_2\)HPO\(_4\), ≥98%), sodium phosphate monobasic monohydrate (NaH\(_2\)PO\(_4\)·H\(_2\)O, ≥98%), hydroquinone (HQ, ≥99%), hydrogen peroxide (H\(_2\)O\(_2\),
30% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pierce Streptavidin Poly-HRP was purchased from ThermoFisher Scientific. The polydimethylsiloxane (PDMS) kit was obtained from Dow Corning (Auburn, MI, USA). All solutions were prepared using 18 MΩ·cm water purified by passing house distilled water through a Hydro Service and Supplies purification system (Durham, NC, USA).

Human Kallikrein 3/PSA DuoSet ELISA (DY1344), Human VEGF-D DuoSet ELISA (DY622), Human IGF-1/IGF-1 DuoSet ELISA (DY291), Human Serpin F1/PEDF DuoSet ELISA, Human IGFBP-3 DuoSet ELISA (DY675), Human CD14 DuoSet ELISA (DY383) were purchased from R&D Systems (Minneapolis, MN, USA). GOLM1 mouse monoclonal antibody clone OT12F3 (TA600480), GOLM-1 detection antibody clone 2D6 (TA700480), GOLM-1 (NM_016548) purified human protein (TP314745), ERG Capture antibody clone 8A9 (TA600177), ERG Detection antibody clone 5F12 (TA700176), ERG (NM_182918) purified human protein (TP308093) were purchased from OriGene Technologies, Inc (Rockville, MD USA). CHI 1040C multipotentiostat (Texas) was used for 8-channel amperometric detection.

### 2.3.2 Fabrication of Immunoarray Sensor

Screen-printed 8-electrode carbon array sensors were fabricated as previously described. Briefly, arrays were coated with consecutive layers of polycation PDDA and negatively charged 5 nm glutathione-coated gold nanoparticles (GSH-AuNPs) using layer-by-layer (LbL) electrostatic adsorption for 20 min each as previously reported. Incubating a freshly prepared crosslinking solution of EDC and Sulfo-NHS for 10 min activated terminal surface carboxyl groups on the GSH-AuNP layer. The electrode sensor arrays were subsequently spotted with primary antibody (Ab1), which was allowed to incubate overnight at 4°C resulting in Ab1 immobilization via an
amidation reaction.\textsuperscript{49,50} Furthermore, prior to use, the arrays were blocked with 2\% BSA in PBS for 1 hour at 4\°C to inhibit non-specific binding (NSB).

2.3.3 Microfluidic System 1: 8-Protein Biomarker Panel for Aggressive Prostate Cancer Detection

2.3.3.1 Assay Protocol

The semi-automated microfluidic system was constructed and electrochemical measurements were all completed at room temperature with a CHI 1040C eight-channel potentiostat at conditions optimized for high sensitivity and low S/N. The detection chamber was first connected to the microfluidic system and subjected to a flow of PBS-T20 to block for NSB. 150 \( \mu \)L Ag-Ab\textsubscript{2}-HRP conjugate for Chip 1 was mixed and loaded into the 100 \( \mu \)L sample loop and injected into the capture chamber at 100 \( \mu \)L/min flow rate. Flow was then stopped and incubation was allowed for 10 minutes. Switch direction of the valve, wait 5 minutes and repeat with Ag-Ab\textsubscript{2}-HRP conjugate for Chip 2, stop flow and incubate for 20 minutes. Upon completion of incubation, chip 1 was washed by flushing with PBS-T20. To perform amperometric measurements, the array was further subjected to a flow of buffer containing 1 mM hydroquinone in PBS for 3 mins. The 8 electrodes of the array were then connected to the working electrode leads of a CHI 1040C multi-potentiostat, and the Pt and Ag/AgCl wires were connected to the counter and reference leads, respectively. Amperometric detection was performed at -0.2 V vs Ag/AgCl by injecting a mixture of 1 mM hydroquinone and 0.1 mM hydrogen peroxide into the detection chamber via the sample loop at 100 \( \mu \)L/min. Hydrogen peroxide activates HRP on the peptide-Ab\textsubscript{2}-MB-HRP conjugates to ferryloxy-HRP, which, in turn oxidizes hydroquinone to benzoquinone. Signal is generated as benzoquinone is reduced through a 2-electron transfer at the
electrode surface. Upon completion of detection of Chip 1 the solution was changed back to PBS-T20 and the procedure was repeated for Chip 2.

2.3.3.2 Study Design

The overall goal of the project was to assess these protein biomarkers ability to distinguish aggressive prostate cancer from indolent forms. To target the candidate biomarkers a microfluidic immunoarray featuring a chamber for online capture of specific target analytes from serum and enzyme labels. The chamber housed an antibody-modified 8-electrode array fabricated for multiplexing. Protein biomarkers are measured simultaneously by activation of enzyme labels and electrochemical detection. One hundred twenty nine male serum samples were analyzed in triplicate. Prospective males with elevated PSA levels, abnormal digital rectal exam (DRE) or both were identified during routine screening campaigns or by primary care physicians and recruited as study participants by George Washington University. Serum was separated from whole blood in Vacutainer tubes by spinning tubes at room temperature at 1000 to 1300 x g for 10 minutes. Isolated serum from the top phase was transferred into a microcentrifuge tube and stored at -80°C until delivery to University of Connecticut. Inclusion criteria consisted of prostate screen subjects must be ambulatory, without a history of prostate cancer between the ages of 40 and 80 years of age. Patients must have a positive prostate cancer screen test of either a serum PSA level > 7 ng/ml or abnormal digital rectal exam (estimated 50% likelihood of positive biopsy), performance status of 0, based on the criteria of the Eastern Cooperative Oncology Group and the life expectancy for the patients must exceed 5 years. Patients were excluded from the study if the patient had previous radiation to the pelvic area or prostate volume >200 grams. These protocols have been ethically approved by the relevant institutional review boards for George Washington University (IRB #091437) and University of Connecticut (IRB #HI 4-003).
2.3.3.3 Statistical Analysis

Patient sample data was first analyzed using MedCalc software. Calculated protein concentrations from the assays were first classified as Benign (0) or Cancer (1) based on Gleason score and then reassessed as Indolent (0, Gleason score 6) or Aggressive (1, Gleason score 8 or 9). Results were then input into MedCalc to complete ROC analyses and graph Box and Whisker plots. Minitab software was used for principal component analysis to analyze the variance contributed by each biomarker and for Power calculations to determine whether our sample size was statistically diverse.

2.3.4 Microfluidic System 2: Ultra-ultrasensitive Detection of a 4-Protein Panel for Prostate Cancer

2.3.4.1 Assay Protocol

The assay protocol is similar to that of the first assay. The semi-automated microfluidic system was constructed and electrochemical measurements were all completed at room temperature with a CHI 1040C eight-channel potentiostat at conditions optimized for high sensitivity and low S/N. The detection chamber housing the 8-electrode modified array was first connected to the microfluidic system and subjected to a flow of PBS-T20 to block for NSB. 150 μL Ag-Ab$_2$-HRP conjugate was mixed and loaded into the 100 μL sample loop and injected into the capture chamber at 100 μL/min flow rate. Flow was then stopped and incubation was allowed for 30 minutes. Upon completion of incubation, the chamber was washed by flushing with PBS-T20. Amperometric measurements were performed as described for Assay 1 (2.3.3.1).
2.4 RESULTS

Microfluidic System 1: 8-Protein Biomarker Panel for Aggressive Prostate Cancer Detection

2.4.1 Microfluidic Immunoarray

We designed a bead-free microfluidic immunoarray evolved from an earlier bead-based array. The immunoassay system features a syringe pump, 4-port switching valve, injector valve, and dual channel detection chamber (Figure 2.1A&B).
Figure 2.1. Dual chip electrochemical immunoassay: (A) Microfluidic immunoassay system, (B) dual chip microfluidic device, (C) 8-electrode screen printed carbon array, sensors at the top and rectangular electrical contacts at bottom, (D) Detection pathway for the immunoassay.
A top PMMA plate features four female ports (4 mm diameter) to accommodate male plastic fittings (1.5 mm i.d.) for attaching 0.2 mm i.d. polyether ether ketone (PEEK) tubing to serve as inlet and outlet (Figure 2.2). The top plate also has four holes to accommodate reference Ag/AgCl and counter platinum electrode wires in each channel (0.2 mm). The two PMMA plates enclose flexible channels molded from polydimethylsiloxane (PDMS) forming two sealed channels (1.5 mm x 2.8 cm) with volumes of 63 μL, each to encompass two 8-sensor detection chips.

![Components of the dual channel microfluidic device made of micro-machined PMMA, PDMS microfluidic channels and screen-printed carbon electrode arrays.](image)

**Figure 2.2.** Components of the dual channel microfluidic device made of micro-machined PMMA, PDMS microfluidic channels and screen-printed carbon electrode arrays.

The protocol was designed to assay the panel simultaneously in two groups, (i) to group biomarkers with similar clinical concentration ranges in serum and (ii) to minimize cross reactivity (Figure 2.3). Cross reactivity is especially important for IGFBP-3 and IGF-1, because IGFBP-3
has high affinity for IGF-1 so these proteins are assayed separately to minimize cross-talk. There are two 8-sensor chips in the PDMS detection channels (Figure 2.1). Chip 1 assays proteins GOLM-1, PEDF, IGFBP-3 and CD-14 that are at high ng/mL to μg/mL levels in serum, except GOLM-1 found at low ng/mL levels. Chip 2 assays proteins PSA, VEGF-D, ERG and IGF-1 found in pg/mL to low ng/mL levels in serum.

**Figure 2.3.** Cross reactivity of the system with representative amperometric peaks from Chip 2. Microfluidic arrays were modified as described in the main text and were allowed to capture only one type of analyte, in this representation 0.13 ng mL^{-1} PSA.

Screen printed carbon sensor electrodes (Figure 2.1C) were coated with a layer of polycation followed by a dense layer of 5 nm glutathione-gold nanoparticles to increase sensor surface area; capture antibodies were chemically attached to glutathione carboxylates as reported above. Arrays were then incubated with 2% BSA at 4°C for 1 hr to minimize non-specific binding (NSB). The two arrays were then sealed into the detection chamber and washed with
Tween-20 in PBS buffer (PBS-T20). We next injected a mixture of biotinylated detection antibodies (corresponding to the capture antibody immobilized in Chip 1), streptavidin poly-HRP, and either a standard protein set or 100 times diluted patient sample and incubated for 10 minutes (Figure 2.1D). This was followed by injection into chip 2 of a mixture containing corresponding detection antibodies with a 20 minute incubation. Following each incubation the arrays were washed with 0.05% PBS-T20. A solution of 1 mM hydroquinone was then flowed over the array which with mediation of electron flow will reduce hydrogen peroxide and oxidize HRP which in turn oxidizes hydroquinone to benzoquinone (Figure 2.1D). Amperometric signals were then recorded by CHI 1040C eight-channel electrochemical workstation.

Assay performance began with optimization to improve both the sensitivity and signal to noise ratio (Figure 2.4). Optimization included concentrations of capture as well as detection antibodies, and streptavidin poly-HRP. We started by assessing detection antibody (Ab₂) concentrations using a fixed capture antibody (Ab₁) concentration of 100 μg mL⁻¹ and varied standard concentrations for each standard protein. Optimal concentration was obtained when the signal difference was greatest between control and sample concentrations. Optimized Ab₂ concentrations were 1 μg mL⁻¹ for IGFBP-3, CD-14, PSA, VEGF-D and IGF-1 and 1.5 μg mL⁻¹ for GOLM-1, PEDF and ERG. We then assessed Ab₁ concentrations using the optimized Ab₂ and same varied standards. Capture antibody concentration were optimized at 22.5 μg mL⁻¹ for PSA and IGF-1, 25 μg mL⁻¹ for ERG and GOLM-1 and 45 μg mL⁻¹ for VEGF-D, PEDF, IGFBP-3 and CD-14. Lastly, an optimized concentration of 2 μg mL⁻¹ poly-HRP in 0.01% BSA was determined using optimized concentrations for Ab₁ and Ab₂.
Figure 2.4. Optimization results for sandwich immunoarray performance upon employing standard concentrations. First, optimized outcomes for Chip 1: (A) Detection antibody (Ab$_2$), (B) Capture antibody (Ab$_1$) and (C) Streptavidin poly-HRP. Followed by optimization results for Chip 2: (D) Detection antibody (Ab$_2$), (E) Capture antibody (Ab$_1$) and (F) Streptavidin poly-HRP.
We next aimed to detect signals of clinical interest by establishing calibration curves of protein standards in five times diluted calf serum in PBS buffer. Calf serum is an acknowledged surrogate for human serum as they have similar total protein concentration (4.0-9.0%), as reported by the manufacturer (Sigma-Aldrich). Calibrations for protein biomarkers are shown in Figure 2.5 generating dynamic ranges spanning 5- to 6-fold log decades from low fg mL\(^{-1}\) to high pg mL\(^{-1}\). Detection limits for chip 1 ranged from 0.32-3.1 fg mL\(^{-1}\) with sensitivities ranging from 2.4-5.3 μA cm\(^{-2}\) [log C]\(^{-1}\). Detection limits for chip 2 ranged from 0.27-2.7 fg mL\(^{-1}\) and sensitivities from 3.7-6.1 μA cm\(^{-2}\) [log C]\(^{-1}\).
Figure 2.5. Immunoarray Calibrations of protein standards in 5-fold diluted calf serum give peaks in the protocol by injecting a mixture of 1 mM hydroquinone and 0.1 mM H₂O₂ at -0.2 V vs Ag/AgCl for Chip 1 (A & B) and Chip 2 (C & D) after analyte protein and antibody binding. Associated calibration graphs with 6-fold log-linear dynamic ranges also shown.
We assessed accuracy using spike-recovery assays in commercially available human serum (Table 2.2). Samples were prepared by spiking five standard concentrations of proteins for both chips. Recoveries obtained ranged from 83-128% in good agreement with the analytically acceptable range. This demonstrates the microfluidic immunoarray accurately and reliably measures concentrations of proteins in the clinical range without interference from matrix effects.

**Table 2.2.** Spiked human serum percent recovery for all protein biomarkers. A recovery range of 81-128% was achieved, demonstrating the ability to accurately and reliably measure the panel without matrix interference effects.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low concentration</td>
</tr>
<tr>
<td></td>
<td>(8.1E-05-0.0028 ng/mL)</td>
</tr>
<tr>
<td>PSA</td>
<td>92</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>105</td>
</tr>
<tr>
<td>ERG</td>
<td>81</td>
</tr>
<tr>
<td>IGF-1</td>
<td>113</td>
</tr>
<tr>
<td>GOLM-1</td>
<td>122</td>
</tr>
<tr>
<td>PEDF</td>
<td>118</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>101</td>
</tr>
<tr>
<td>CD-14</td>
<td>96</td>
</tr>
</tbody>
</table>

During the period when we were analyzing samples, we monitored reproducibility of the assay for quality control samples to ensure reliability. We periodically analyzed pooled patient serum provided by the Katzen Cancer Research Center, George Washington University School of Medicine & Health Sciences from male patients of European and African American decent. We observed minimal changes in reproducibility for multiple assays with the average signals remaining well within ±10% over 560 days (Figure 2.6).
Figure 2.6. Quality control reproducibility data of the assay system using 2 pooled human serum samples of African American (includes two outliers) and European descent.

We further validated the assay by monitoring the inter- and intra-day stability. Inter-day stability consisted of analyzing identical samples on three separate days on six separate arrays; three for chip one and three for chip two. Intra-day stability on the other hand consisted of evaluating identical samples consecutively on the same day with again six separate arrays; three for chip 1 and three for chip 2. The relative precision of inter-day assay reproducibility was < 7% and intra-day reproducibility was < 5% demonstrating the consistency of the system.

2.4.2 Analysis of Clinical Patient Samples

The 8 biomarkers were measured in prostate cancer patient’s serum to explore the diagnostic value of the panel for identifying aggressive cancers. We analyzed a cohort of samples collected at George Washington University (GWU) Hospital from 129 males identified during routine prostate cancer screening by their primary care physician as having elevated PSA levels,
abnormal DRE or both. Upon acceptance to the study, biopsy grades analyzed by Gleason score were included as a basis set for aggressiveness.

Upon receipt frozen on dry ice from GWU, samples were thawed and ten, 5 uL aliquots were taken from each sample and separately placed into microcentrifuge tubes. All samples and aliquots were then refrozen at -80°C. On the day of analysis, one aliquot was removed from each sample to be analyzed and diluted with PBS buffer 100 times and analyzed on the immunoarray. For statistical analysis of the results, patients were segregated based on Gleason score to determine the predictive power of the biomarker panel for differentiation of benign, indolent (Gleason score of 6), significant (Gleason 7) and aggressive (Gleason ≥8) forms of prostate cancer (Table 2.3). Forty nine percent of the population were benign, 25% were Indolent, 17% were significant and 9% were aggressive.

Table 2.3. Clinical Characteristics of prostate patient samples.

<table>
<thead>
<tr>
<th></th>
<th>Benign</th>
<th>Indolent</th>
<th>Significant</th>
<th>Aggressive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Patients</strong></td>
<td>n=64 (49%)</td>
<td>n=32 (25%)</td>
<td>n=22 (17%)</td>
<td>n=12 (9%)</td>
</tr>
<tr>
<td><strong>Age (average, years)</strong></td>
<td>63</td>
<td>67</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td><strong>Patients with [PSA]≤4.0 ng/mL</strong></td>
<td>50 (78%)</td>
<td>21 (66%)</td>
<td>12 (55%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td><strong>Patients with [PSA]&gt;4.0 ng/mL</strong></td>
<td>14 (22%)</td>
<td>11 (34%)</td>
<td>10 (45%)</td>
<td>8 (67%)</td>
</tr>
</tbody>
</table>

Statistical power estimates based on data set characteristics gave 93-100% power for all biomarker proteins confirmed a high statistical relevance in the diverse sample set (Table 2.4).
Table 2.4. Power calculation results for each individual biomarker.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Benign vs Cancer</th>
<th>Indolent vs Aggressive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>97%</td>
<td>97%</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>95%</td>
<td>97%</td>
</tr>
<tr>
<td>ERG</td>
<td>94%</td>
<td>100%</td>
</tr>
<tr>
<td>IGF-1</td>
<td>99%</td>
<td>100%</td>
</tr>
<tr>
<td>GOLM-1</td>
<td>98%</td>
<td>93%</td>
</tr>
<tr>
<td>PEDF</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>CD-14</td>
<td>97%</td>
<td>96%</td>
</tr>
</tbody>
</table>

Results obtained from assaying all the samples were compared to the existing clinical standard for prostate cancer detection, the PSA blood test. A correlation plot of initial PSA by GWU vs. our PSA results gave a linear plot with slope close to 1, intercept close to zero, and correlation coefficient of 0.88, again demonstrating the accuracy of the immunoassay (Figure 2.7).

Figure 2.7. Correlation plot of the microfluidic immunoarray results for the human serum samples against the results from GWU for the same samples.
Box and whisker plots were utilized to visually represent data graphically through their quartiles. Figure 2.8 displays the majority of the population for each set of data residing in the central box of the plots indicating minimal variability within each of the populations. When we observe the plots as a whole there is no well-defined distinction between benign and cancer populations, but there was when analyzing indolent and aggressive data sets. Therefore, clear predictability is not available by box and whisker plot analysis for benign and cancer populations. There is however evidence based on indolent and aggressive populations that distinguishing characteristics and predictability can be made.
**Figure 2.8.** Box plots presenting distribution of protein biomarker levels in male patient serum samples (excluding outliers): Benign vs Cancer (A) Chip 1 and (B) Chip 2 and Indolent vs Aggressive (C) Chip 1 and (D) Chip 2 (Indolent- Samples with Gleason score of 6, Aggressive-Samples with Gleason score of 8). Markers represent individual patient sample concentrations and error bars are plotted at 95% confidence interval.

Receiver operating characteristic (ROC) analysis and principle component analysis (PCA) were done to assess the protein biomarkers ability to distinguish forms of prostate cancer. AUC values are a measurement of how well a biomarker (in this case) can distinguish between two diagnostic groups. The closer the AUC is to 1, the more the biomarker can distinguish one group.
from another. Sensitivity is the probability the test will be positive when the disease is present and specificity is the probability the results are negative when disease is not present. Using ROC analysis both testing sets were assessed for their ability to distinguish benign vs cancer and indolent vs aggressive cancers (Figure 2.9). For benign vs. cancer, IGF-1, PEDF and CD-14 had the largest areas under the curve (AUC) while for indolent vs. aggressive cancer, PEDF, IGFBP-3 and CD-14 had the largest AUC values (Table 2.5).

**Figure 2.9.** ROC statistical analysis of Patient Samples comparing Benign vs Cancer (Gleason score of 6, 7, 8, or 9), for (A) Chip 1 and (B) Chip 2 and Indolent (Gleason score of 6) vs Aggressive (Gleason score of 8 or 9) for (C) Chip 1 and (D) Chip 2.
Table 2.5. ROC statistical results: (A) AUC, sensitivity and specificity for Indolent vs Aggressive patient samples, (B) AUC, sensitivity and specificity for Benign vs Cancer patient samples, best 4 biomarkers highlighted under each category.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>0.73 ± 0.08</td>
<td>57.1</td>
<td>83.3</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>0.73 ± 0.08</td>
<td>62.9</td>
<td>75.0</td>
</tr>
<tr>
<td>ERG</td>
<td>0.74 ± 0.08</td>
<td>57.1</td>
<td>84.7</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.73 ± 0.08</td>
<td>54.3</td>
<td>81.9</td>
</tr>
<tr>
<td>GOLM-1</td>
<td>0.73 ± 0.08</td>
<td>68.6</td>
<td>69.4</td>
</tr>
<tr>
<td>PEDF</td>
<td>0.81 ± 0.07</td>
<td>71.4</td>
<td>81.9</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.82 ± 0.07</td>
<td>68.6</td>
<td>84.7</td>
</tr>
<tr>
<td>CD-14</td>
<td>0.81 ± 0.07</td>
<td>85.7</td>
<td>66.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>0.67 ± 0.08</td>
<td>86.9</td>
<td>41.2</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>0.65 ± 0.08</td>
<td>31.8</td>
<td>100</td>
</tr>
<tr>
<td>ERG</td>
<td>0.66 ± 0.08</td>
<td>71.0</td>
<td>58.8</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.71 ± 0.08</td>
<td>86.0</td>
<td>52.9</td>
</tr>
<tr>
<td>GOLM-1</td>
<td>0.63 ± 0.08</td>
<td>50.5</td>
<td>76.5</td>
</tr>
<tr>
<td>PEDF</td>
<td>0.70 ± 0.09</td>
<td>64.5</td>
<td>70.6</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.62 ± 0.09</td>
<td>24.3</td>
<td>100</td>
</tr>
<tr>
<td>CD-14</td>
<td>0.73 ± 0.08</td>
<td>58.9</td>
<td>82.3</td>
</tr>
</tbody>
</table>

We further differentiated the biomarkers ability to distinguish the different populations by PCA (Figure 2.10, Table 2.6). Principal Component Analysis enables transformation of a complex data set into a more manageable set by identifying key relationships between component variables.
Figure 2.10. PCA of the 8-protein biomarker panel was utilized to compare proportions of variance in regards to the sample populations: Benign (Black), Cancer (Blue), Indolent (Green) and Aggressive (Red).

For benign patient samples the largest principle components were obtained by IGF-1 and ERG. This indicates these biomarkers were the most influential at analyzing the population. For indolent patient samples (Gleason score of 6) the largest principal components were obtained by PSA, VEGF-D and ERG. In data sets cancer (Gleason score of 6, 7, 8 or 9) and aggressive (Gleason score of 8 or 9), the same five biomarkers were the most effective at analyzing the populations. PSA was the most significant followed by equal variance of all biomarkers in Chip 1: GOLM-1, PEDF, IGFBP-3 and CD-14.
Table 2.6. PCA variances of each biomarker for each of the populations. The greater the variance is, the more effective the biomarker is at analyzing that population (best biomarkers highlighted).

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Benign</th>
<th>Cancer</th>
<th>Indolent</th>
<th>Aggressive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>0.13</td>
<td>0.28</td>
<td>0.28</td>
<td>0.29</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>0.0030</td>
<td>0.050</td>
<td>0.13</td>
<td>0.060</td>
</tr>
<tr>
<td>ERG</td>
<td>0.25</td>
<td>0.049</td>
<td>0.28</td>
<td>0.063</td>
</tr>
<tr>
<td>IGF-1</td>
<td>0.30</td>
<td>0.048</td>
<td>0.011</td>
<td>0.061</td>
</tr>
<tr>
<td>GOLM-1</td>
<td>0.077</td>
<td>0.25</td>
<td>0.038</td>
<td>0.24</td>
</tr>
<tr>
<td>PEDF</td>
<td>0.066</td>
<td>0.25</td>
<td>0.083</td>
<td>0.24</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.0010</td>
<td>0.25</td>
<td>0.081</td>
<td>0.24</td>
</tr>
<tr>
<td>CD-14</td>
<td>0.14</td>
<td>0.25</td>
<td>0.086</td>
<td>0.24</td>
</tr>
</tbody>
</table>

2.4.3 Statistical Analysis using Normalized Concentrations

Protein concentrations determined in the assays were converted to normalized values by dividing biomarker protein concentrations for each sample by the respective sample averages. These values were then averaged for each sample to give a single normalized parameter characteristic of levels of all measured proteins. One biomarker was removed sequentially from the analysis based on individual AUC values to determine a significant panel. The normalized means with classification status for cancer (1) or benign (0) and aggressive (1) or indolent (0) were input to MedCalc.

Using the normalized mean function of the proteins, the ROC results for indolent vs cancer returned the highest AUC value of 0.67 at the removal of three biomarkers with remaining biomarkers PSA, ERG, PEDF, IGFBP-3 and CD-14 (Table 2.7A). For benign vs cancer, a panel of three biomarkers including PEDF, IGF-1 and CD-14 was determined to have the highest AUC of 0.66 indicating the best diagnostic ability (Table 2.7B).
Table 2.7. Normalized ROC analysis for (A) Indolent vs Aggressive and (B) Benign vs cancer, best inclusive panels highlighted for each category.

<table>
<thead>
<tr>
<th>A</th>
<th>Biomarkers Used</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full 8-protein panel</td>
<td>0.67</td>
<td>83.3</td>
<td>56.3</td>
<td></td>
</tr>
<tr>
<td>PSA, ERG, IGF-1, GOLM-1, PEDF, IGFBP-3, CD-14</td>
<td>0.67</td>
<td>83.3</td>
<td>56.3</td>
<td></td>
</tr>
<tr>
<td>PSA, ERG, IGF-1, PEDF, IGFBP-3, CD-14</td>
<td>0.65</td>
<td>83.3</td>
<td>56.3</td>
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</tr>
<tr>
<td>PSA, ERG, PEDF, IGFBP-3, CD-14</td>
<td>0.67</td>
<td>83.3</td>
<td>56.3</td>
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<tr>
<td>ERG, PEDF, IGFBP-3, CD-14</td>
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<td>75.0</td>
<td>62.5</td>
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<tr>
<td>PEDF, IGFBP-3, CD-14</td>
<td>0.61</td>
<td>75.0</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>IGFBP-3, CD-14</td>
<td>0.61</td>
<td>75.0</td>
<td>62.5</td>
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<table>
<thead>
<tr>
<th>B</th>
<th>Biomarkers Used</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full 8-protein panel</td>
<td>0.58</td>
<td>69.7</td>
<td>65.6</td>
<td></td>
</tr>
<tr>
<td>PSA, VEGF-D, ERG, IGF-1, GOLM-1, PEDF, CD-14</td>
<td>0.58</td>
<td>69.7</td>
<td>65.6</td>
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<tr>
<td>PSA, VEGF-D, ERG, IGF-1, PEDF, CD-14</td>
<td>0.58</td>
<td>69.7</td>
<td>65.6</td>
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<tr>
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<td>71.2</td>
<td>64.1</td>
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<tr>
<td>PSA, IGF-1, PEDF, CD-14</td>
<td>0.59</td>
<td>71.2</td>
<td>609</td>
<td></td>
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<tr>
<td>IGF-1, PEDF, CD-14</td>
<td>0.66</td>
<td>83.3</td>
<td>68.7</td>
<td></td>
</tr>
<tr>
<td>IGF-1, CD-14</td>
<td>0.66</td>
<td>83.3</td>
<td>68.7</td>
<td></td>
</tr>
</tbody>
</table>

Microfluidic system 2: Ultra-ultrasensitive Detection of a 4-Protein Panel for Prostate Cancer

2.4.4 Ultra-ultrasensitive Microfluidic Immunoarray

We further simplified the above described electrochemical microfluidic system by condensing the protein panel and changing to a programmable syringe pump (Fusion 100) (Figure 2.11A). The device itself contains the same top PMMA plate featuring 2 female ports to serve as an inlet and outlet for attaching male plastic fittings and polyether ether ketone (PEEK) tubing (Figure 2.11B). The top plate also has four holes to accommodate reference Ag/AgCl and counter platinum electrode wires (0.2 mm). The PMMA plate encloses a flexible channel molded from polydimethylsiloxane (PDMS) forming a sealed channel (1.5 mm x 2.8 cm) with volume of 63 μL to encompass a 8-sensor detection chip.
Figure 2.11. Ultra-ultrasensitive electrochemical immunoassay: (A) Microfluidic immunoassay system, (B) Microfluidic device, (C) Screen printed carbon array.

The protocol was designed to assay only the biomarkers from Chip 2, described above, in a simplified design with minimized cross reactivity. The screen printed carbon sensor electrode was modified as described above (Figure 2.1D) following the same general assay procedure with optimized conditions. Capture and detection antibody concentrations remained constant to those mentioned previously for all biomarkers. Assay improvement was initiated with optimization of the incubation time. To allow sufficient time for antigen to bind at ultralow concentrations, incubation times of 15, 20, 25 and 30 minutes were investigated. Using concentrations of 0.025 fg mL\(^{-1}\) for IGF-1, 0.076 fg mL\(^{-1}\) ERG, 0.18 fg mL\(^{-1}\) VEGF-D and 0.26 fg mL\(^{-1}\) PSA, a 30 minute incubation was chosen as the optimal incubation time as it gave the largest signal difference between the sample and control (Figure 2.12).
Figure 2.12. Optimization of incubation time for the 4-protein biomarkers. Incubation times were 15 to 30 minutes with the optimal time being that with the largest difference between the sample and control.

We next established calibration curves of protein standards in five times diluted calf serum in PBS buffer. Calibrations for protein biomarkers are shown in Figure 2.13 generating dynamic ranges from high ag mL\(^{-1}\) to low ng mL\(^{-1}\). Detection limits for PSA, VEGF-D, ERG and IGF-1 were 127 ± 7, 88 ± 10, 36 ± 5 and 13 ± 3 ag mL\(^{-1}\), respectively. Sensitivities ranged from 9.9-13.6 μA cm\(^{-2}\) [log C]\(^{-1}\). These detection limits correlate to the detection of 21 ERG molecules, 50 IGF-1 molecules, 122 PSA molecules and 205 VEGF-D molecules.
Figure 2.13. (A) Calibration curves and (B) Associated amperometric curves of protein standards in 5-fold diluted calf serum.

We also assessed the accuracy of the assay by spike-recovery assays in commercially available human serum (Table 2.8). Samples were prepared by spiking 5 standard concentrations of proteins. Recoveries obtained were within the analytically acceptable range with an average recovery of 102% and a range of 82-123%. This demonstrates the assays ability to accurately and reliably measure the four proteins without matrix interference.

Table 2.8. Spiked human serum percent recovery.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>[Low] (0.025-0.35 fg mL(^{-1}))</th>
<th>[Mid] (0.20-1.2 fg mL(^{-1}))</th>
<th>[High] (13-65 fg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>91%</td>
<td>102%</td>
<td>115%</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>85%</td>
<td>123%</td>
<td>87%</td>
</tr>
<tr>
<td>ERG</td>
<td>90%</td>
<td>119%</td>
<td>119%</td>
</tr>
<tr>
<td>IGF-1</td>
<td>111%</td>
<td>98%</td>
<td>82%</td>
</tr>
</tbody>
</table>

2.5 DISCUSSION

We describe the first diagnostic panel and immunoassay platform with the capability to stage and grade prostate cancer with respect to its aggressive nature. Multiplex measurements of
the candidate biomarkers were evaluated on an electrochemical immunoarray offering scalability, low cost, speed, and high sensitivity and specificity, and detection limits as low as 0.27 fg mL\(^{-1}\).

Patient sample analysis revealed significant differences between the levels of the biomarkers in benign, indolent, and aggressive samples. The distinction between indolent and aggressive sample concentrations by individual biomarkers was first observed in assessment of box and whisker plots. ROC analysis further revealed the panels’ ability to distinguish stages of prostate cancer. AUC indicated four biomarkers that were able to distinguish benign from cancer patient samples and indolent from aggressive samples. These biomarkers were IGF-1, PEDF, IGFBP-3 and CD-14. Further statistical analysis was performed to determine the extent to which each biomarker impacted the detection of the three populations. Using PCA, three of the four biomarkers from the ROC analysis reappeared as having the highest proportions of variance. These biomarkers were IGF-1, PEDF and CD-14. PSA was also determined to be an influential biomarker by PCA analysis as it appeared in both indolent and aggressive data sets with the highest proportion of variance.

One of the main advantages of this study is the assays capability to significantly improve on the prediction of a positive cancer biopsy when using the panel of biomarkers together opposed to PSA alone based on statistical analysis of the cohort. We are also able to capture clinically relevant ranges for each of the targeted biomarkers with high sensitivity. Multiplex detection resulted in sensitivities ranging from 2.4-6.1 μA cm\(^{-2}\) [log C]^\(^{-1}\) with the highest of those obtained by biomarkers PSA and CD-14. The ability to measure these biomarkers with high sensitivity and accomplish a distinction of aggressive cancer forms marks a new beginning for prostate cancer diagnosis as it can aid physicians to appropriate treatment options.
One limitation we observe for the dual sensor chip is the current measurements for control output have slight variations from day to day. We believe that this can be overcome with assaying standard protein concentrations and running appropriate controls along with test samples. Given information collected for the sample cohort we are unable to perform risk calculations based on age, family history and digital rectal exam. Further optimization in automization protocols are being further pursued to minimize user intervention. While a larger cohort of samples is needed for confirmation, the results suggest a high potential of the panel for aggressive prostate cancer diagnosis.

Overall, the study suggests that while the entire eight protein biomarker panel is capable of detecting aggressive forms of prostate cancer, they do not act equally and are not all required. Therefore, the final panel can be reduced to four biomarkers PSA, IGF-1, PEDF and CD-14 as they are fundamental to detecting and distinguishing forms of prostate cancer. The results from cancer patient sample analysis supports the potential diagnostic utility of this assay to aid doctors in their screening decisions by analyzing not only the change in PSA levels, but of all four biomarkers.

We also describe the first diagnostic panel and immunoassay with the capability to detect attogram levels of protein biomarkers in a diagnostic platform. Reasons for the ability to detect the proteins at such high sensitivity can be related to the prevention of non-specific binding via incubation with BSA, immobilization with glutathione gold nanoparticles which enhances the electron transfer between the redox pair and the electrode surface, increased electrode surface area due to the LbL modification, and the optimized incubation time. One of the possible reasons for the large difference observed in detection limits could be the quality of the antibodies and their storage conditions.
To determine the feasibility of using these biomarkers for monitoring prostate cancer reoccurrence after prostatectomy we will be analyzing patient samples from patients post-surgery. These assays will assist not only in classifying patients who need to be monitored versus those who need immediate surgery or therapy, but will also relieve the healthcare system of patients who would be wrongly diagnosed and over-treated.
2.6 REFERENCES


Chapter 3
Cancer Diagnostics with Semi-Automated Electrochemical Microfluidic Immunoarrays for Parathyroid Hormone-Related Peptide Detection

3.1 ABSTRACT

Parathyroid hormone-related peptide (PTHrP) is recognized as the major a causative agent of humoral hypercalcemia of malignancy (HHM). The paraneoplastic PTHrP has also been implicated in tumor progression and metastasis of many human cancers. Conventional PTHrP detection methods like immunoradiometric assay (IRMA) lack the sensitivity required to measure levels prior to the development of hypercalcemia and pose potential health hazards due to radioactive labels. We describe the first ultrasensitive multiplexed assay to measure intact PTHrP 1-173 as well as circulating N-terminal and C-terminal peptide fragments. A microfluidic immunoarray was employed featuring a microfluidic chamber for on-line capture of the peptides from serum onto magnetic beads with multiple copies of peptide-specific antibodies and enzyme labels. Limits of detection (LOD) of 3 fg mL⁻¹ (~1000 fold lower than IRMA) were achieved for simultaneous detection of PTHrP isoforms and fragments in 30 min. Good correlation for patient samples was found with IRMA (n=57); \( r^2 = 0.99 \) assaying PTHrP 1-173 and 1-86 fragment. Analysis by ROC gave area under the curve of 0.96, 80-83% clinical sensitivity and 96-100% clinical specificity. Secondly, we developed an immunoarray featuring online capture of the isoforms in prostate cancer cell lines and patient samples indicating proof of concept that the new assay will be able to identify and stage patients aiding as prognostic indicators as disease progresses. Limits of detection of 0.05-0.2 fg/mL were achieved in 15 minutes. Concentrations of the biomarkers were determined in conditioned media and cell extracts. Analysis with prostate cancer patient samples showed a strong correlation with increasing Gleason score. The new
ultrasensitive, multiplexed assays for PTHrP and fragments is promising for clinical diagnosis, prognosis and therapeutic monitoring from early to advanced stage cancer patients and to examine underlying mechanisms of PTHrP overproduction.

3.2 INTRODUCTION

Parathyroid hormone (PTH)-like factor was first postulated by Albright over 60 years ago\(^1\) as a humoral factor responsible for development of hypercalcemia in cancer patients, and later described as humoral hypercalcemia of malignancy (HHM).\(^2\) The true nature of this PTH-like factor remained elusive since it escaped detection by immunoassays using antibodies raised against PTH\(^3,4\) but could be detected in bioassays using PTH receptor.\(^5,6\) This led to cloning and characterization of this PTH-like factor now known as parathyroid hormone-related peptide (PTHrP)\(^7,8\) and to development of specific immunoassays.\(^9,10\)

Human PTHrP has three isoforms of 139, 141 and 173 amino acids, and is widely expressed in normal and cancerous human tissues.\(^11,12\) All isoforms have identical sequences through residue 139 and undergo posttranslational cleavage generating N-terminal, mid-region and C-terminal peptides with distinct physiological functions. PTHrP exerts PTH-like actions in bone and kidney by binding and activating the guanylyl nucleotide-binding (G) protein-linked receptor (PTH1R) causing hypercalcemia.\(^13,14\)

Distinct from PTH, PTHrP acts as endocrine, autocrine, paracrine, or intracrine factor in a vast range of important physiological roles including skeletal development, placental calcium transport, muscle relaxation, and mammary gland development.\(^15,16\) Circulating levels of PTHrP correlate with disease progression in cancers including breast, prostate, melanoma\(^17-20\) and bone metastasis.\(^21-23\) Currently PTHrP can only be detected in the blood when hypercalcemia develops\(^9,10\), when there is \(\sim50\%\) chance of mortality in 30 days.\(^24\) Thus, existing PTHrP assays
are limited to confirming the humoral origin of hypercalcemia, but cannot provide early detection of PTHrP-producing tumors, which requires assays with much higher sensitivity.

In addition, radioimmunoassay (RIA) and immunoradiometric assays (IRMA)\textsuperscript{9,10,25} for PTHrP use high energy isotopes such as \textsuperscript{125}I that pose a health hazard and have short shelf-lives.\textsuperscript{26,27} Enzyme linked immunosorbent assay (ELISA)\textsuperscript{28}, immunofluorometric assays\textsuperscript{29} and mass spectrometry\textsuperscript{26,30} lack the sensitivity required to measure PTHrP in early stage cancers. None of these assays can measure specific PTHrP isoforms and in particular the human specific PTHrP 1-173 isoform.

Herein, we describe the first ultrasensitive method to detect PTHrP 1-173 and smaller peptide fragments using a novel semi-automated modular microfluidic device.\textsuperscript{31} The first microfluidic system delivers sample to a capture chamber where enzyme-labeled magnetic beads equipped with antibodies capture target peptides. These beads are washed and delivered to 8-sensor gold immunoarray\textsuperscript{32} decorated with a second set of antibodies that recognize and bind bead-bound target peptides. Peptides are measured simultaneously by activation of enzyme labels and electrochemical detection. Intact PTHrP isoforms as well as N- and C-terminal fragments were detected simultaneously in serum with limit of detection (LOD) of 3 fg mL\textsuperscript{-1}, 1000-fold lower than commercial PTHrP assays. Good correlation between microfluidic immunoarray and IRMA results in cancer patient serum was obtained. The second microfluidic system removes the need for processing magnetic beads, simplifying the assay featuring automation, miniaturisation, sensitivity adjustable to the sample characteristics, rapid analysis, and low reagent consumption for the detection of PTHrP and PSA in human cancer patient serum and cell conditioned media. Incubation times were tailored to obtain limits of detection (LOD) in sub-fg mL\textsuperscript{-1} without a significant compromise in sensitivity leading to a simple, quick cancer diagnostic test and
personalized cancer therapy strategy that may be able to transform the current methods of prostate
cancer diagnosis. This system will be utilized to evaluate PTHrP peptides as aggressive prostate
cancer biomarkers by analysis of human serum samples from patients.

3.3 EXPERIMENTAL

3.3.1 Chemicals and Materials

Horseradish peroxidase (HRP), sterile filtered bovine calf serum, Tween-20, bovine serum albumin (BSA), L-glutathione reduced (GSH, ≥98%), gold (III) chloride trihydrate (HAuCl₄·3H₂O, 99.9%), sodium borohydride (99%), tetraoctylammonium bromide, 3-mercaptopropionic acid (MPA), 1dodecane thiol, poly(amic acid), poly(diallyldimethylammonium chloride) (PDDA, MW 200,000-300,000, 20% in water), 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), Tween-20, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na₂HPO₄, ≥98%), sodium phosphate monobasic monohydrate (NaH₂PO₄·3H₂O, ≥98%), hydroquinone (HQ, ≥ 99%) and hydrogen peroxide (H₂O₂, 30%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pierce Streptavidin Poly-HRP was purchased from ThermoFisher Scientific. Kapton FPC film (127 m thick) was purchased from American Durafilm. The poly(dimethoxy)silane (PDMS) kit was purchased from Dow Corning. MyOne tosylactivated beads (1 µm diameter, Dynabeads) and were from Invitrogen. Screen-printed 8-electrode carbon array (700 µm diameter) sensors were purchased from Kanichi Research Services Ltd (Manchester, England). Immunoreagents (monoclonal antibodies, polyclonal antibodies, BSA) were dissolved in pH 7.4 phosphate saline buffer (PBS, 5.9 mM Na₂HPO₄, 3.9 mM NaH₂PO₄, 2.7 mM KCl, 120 mM NaCl). 400 mM EDC and 100 mM NHSS were dissolved in water immediately before use. All solutions were prepared
with 18 MΩ·cm water purified through use of a Hydro water purification system (Durham, NC, USA).

3.3.2 PTHrP Peptides and Antibodies

Intact PTHrP 1-173 was produced from cDNA encoding the PTHrP 1-173 isoform. Human PTHrP fragments 1-33, 151-169, 140-173 were purchased from Sheldon Biotechnology Center (McGill University). Human recombinant PTHrP 1-86 Human recombinant PTHrP 1-86 was from Bachem (Torrance, CA, cat # H-9815). Monoclonal antibodies M45 (IgM) and PA158 (IgG) were raised against PTHrP 1-33; monoclonal antibody PA104 (IgG) was raised against PTHrP 140-173; monoclonal antibody PA6 (IgG) was raised against PTHrP 151-169, PA104, PA158. All monoclonal antibodies were purified by affinity chromatography (Medilabs, Quebec) and found highly specific with no cross reactivity with PTH and other unrelated peptides. The bioactivity of these monoclonal antibodies was tested previously both in vitro and in vivo. Polyclonal antibodies against human PTHrP 1-173 (IgY lots 3103 and 3104 were raised in chicken and purified commercially (Genway Biotech, San Diego, CA). Stock concentration of peptide standards (200-500 ng for 1-33, 151-169, 140-173 and 1173 from Sheldon Biotechnology Center, McGill University and PTHrP 1-86 was from Bachem (Torrance, CA, cat # H-9815) were first diluted in water or PBS buffer pH 7.4 to 50 pmol/L (1 ng/mL) and stored at -80 °C (according to the manufacturer’s specifications). The antibodies were reconstituted in PBS buffer pH 7.4 down to the working concentration and stored at -80 °C. The peptides and antibodies were stable for 12 months. On assay days, one vial of 50 pmol/L peptide standard was then diluted to 1 pmol/L followed by serial dilution in 5x diluted calf serum in PBS buffer pH 7.4. The diluted standards were used the same day they were prepared and any left-over standards were discarded. In all the calibration curves 5x diluted calf serum was employed as assay diluent for serial dilutions.
3.3.3 Microfluidic System 1: Inkjet Printed Gold Arrays

3.3.3.1 Human Serum Samples

Human serum samples from 22 healthy subjects and 37 cancer patients with solid tumors were obtained from McGill University Health Center Biorepository. Blood was drawn in regular tubes, tubes put on ice immediately and separated within 60 min, aliquoted and stored at -80 °C prior to assay. No degradation of the serum samples was observed by assaying the samples after 6 months interval (Table 3.1). All samples used in this study were acquired under an institutional review board–approved protocol, and informed consent was obtained from all study participants.

Table 3.1. Stability of the serum samples after 6 months.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>February 2015 IRMA PTHrP Level (pg/mL)</th>
<th>August 2015 IRMA PTHrP Level (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3244</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>3267</td>
<td>18</td>
<td>21</td>
</tr>
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<td>3530</td>
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<td>15</td>
</tr>
<tr>
<td>3659</td>
<td>24</td>
<td>30</td>
</tr>
</tbody>
</table>

3.3.3.2 Immunoradiometric assay (IRMA) for PTHrP

We used a commercial PTHrP assay for correlation purposes (PTHrP RUO, Active® IRMA catalog # DSL8100 Beckman Coulter Canada Inc, Montreal, Canada). This IRMA has been previously described to measure PTHrP in various cancer stages and to establish normal control values. It uses an N-terminal monoclonal antibody raised against PTHrP1-40 and a midregion monoclonal antibody raised against PTHrP 57-80. It has a sensitivity of 0.3 pmol/L (3 pg/mL) and linearity up to 212 pmol/L (2100 pg/mL). Internal controls were made using pooled samples and conditioned media from PTHrP producing cells lines. The inter-assay variability was 4.4% and the
intra-assay variability was 4.7%, according to the manufacturer’s specifications. Normal values obtained from 40 healthy volunteers range from 0-15 pg mL\(^{-1}\) (0-1.5 pmol L\(^{-1}\)).

3.3.3.3 Microfluidic Device

As previously reported by Otieno et al the microfluidic system featured two devices; detection chamber and capture chamber.\(^3\) The detection chamber (Figure 3.1A) features a molded, soft PDMS 1.5 mm wide rectangular channel placed on top of the electrode array. The microfluidic channel was supported by two flat poly(methylmethacrylate) (PMMA) plates manufactured to fit on both sides of the PDMS slab and bolted together to seal the microfluidic channel 1.5 mm wide, 2.8 cm long, and 63 μL in volume. The top PMMA plate contained female ports (4 mm diameter) for screwing in male plastic fittings to hold 0.2 mm i.d. PEEK tubing for inlet and outlet purposes. The top PMMA plate also contained 0.6 mm holes to hold Ag/AgCl and 0.2 mm holes for Pt wire electrodes. The capture chamber (Figure 3.1B), on the other hand, features a PDMS channel with an oval cylindrical channel housing a tiny magnetic stir bar. The PDMS channel is sandwiched between two PMMA plates to form a channel that is 100 μL in volume.
3.3.3.4 Production of Gold Immunoarrays

Gold Array Fabrication followed previously established protocols by Jensen et al. Gold nanoparticle ink was prepared at 100 mg mL\(^{-1}\) in toluene and filtered using a 0.2 μm cutoff PTFE filter. The ink was then injected into a Dimatix cartridge for use in the Dimatix Inkjet materials printer. Upon printing the gold, the arrays were sintered for 15 minutes at 200°C. The arrays lightened in color indicating the loss of the dodecane thiol layer and Au cores coalescing. Immediately after being sintered the arrays were returned to the Dimatix Inkjet materials printer to print the poly(amic acid) insulation layer. The poly(amic acid) ink was prepared, as previously reported, by diluting the 10% (m/m) poly(amic acid) solution in pure N-methyl-2-pyrrolidone (NMP) to 1% (m/m) and the adding the solution to a liquid crystal Dimatix printer cartridge immediately prior to use. Electrode surface area was calculated by cycling the gold arrays in 0.18 M H\(_2\)SO\(_4\) between 1.5 V and -0.2 V at 100 mV s\(^{-1}\). Current density was used for quantitation of the standards. The peak height (I, nA) was divided by the surface area of the electrode to yield the
current density that was plotted against the concentration of the peptide fragments. Log fitting was used to plot the data.

3.3.3.5 Array Fabrication

Immunoarrays were fabricated from 4 nm dodecanethiol decorated gold nanoparticles (AuNPs) on Kapton sheet as previously described using Dimatix inkjet materials printer.\textsuperscript{32,34} The inkjet-printed electrode arrays were annealed to drive off the thiol layer and then insulated with poly(amic) acid, a Kapton precursor layer. The electrode arrays were then cleaned in 0.18 M sulfuric acid, by cycling potential between 1.5 and -0.2 V vs. saturated calomel electrode (SCE) to remove gold oxide from the surface. The electrode arrays were coated with self-assembled monolayer (SAM) of mercaptopropionic acid (MPA) to introduce carboxyl groups on the surface of the array. The surface carboxyl groups were activated by freshly prepared EDC and NHSS to attach monoclonal antibodies (Ab\textsubscript{1}) to array elements through amidization overnight. The arrays modified with Ab\textsubscript{1} were then washed with PBS-T20 to remove excess unbound Ab\textsubscript{1} and incubated with 2\% BSA for 1 hr to minimize non-specific binding (NSB). The Ab\textsubscript{1}-modified arrays were then fitted into the detection chamber for amperometric measurement. Multiple horseradish peroxidase (HRP) and antibodies (Ab\textsubscript{2}) were attached onto 1 \( \mu \)m magnetic beads (HRP-MB-Ab\textsubscript{2}) as previously described.\textsuperscript{34}

3.3.3.6 Derivatization of Magnetic Beads

Magnetic bead bioconjugates [tosyl-activated magnetic beads (MBs)-Horseradish peroxidase (HRP)-Antibody (Ab\textsubscript{2})] were prepared using as previously described\textsuperscript{34} with slight modification to reduce the bead conjugate preparation time from 42 to 24 hr. Briefly, tosylactivated magnetic beads (MB) (0.2 mg) were washed 3x with sodium borate buffer (pH 9.5), then reconstituted in 3 M ammonium sulfate + 0.1 M sodium borate buffer (volume 1:1). Horseradish
peroxidase (HRP) (3 mg) and polyclonal antibody (Ab$_2$) (0.8 mg) were then simultaneously added to the dispersion and incubated at 37 °C for 18 hrs. The magnetic bead bioconjugates (HRP-MB-Ab$_2$) were then washed with PBS-T20 and reconstituted in 0.5% BSA at 37°C for 6 hrs to block NSB. The resulting beads were washed 3x with 0.1% BSA, reconstituted in 600 µL of 0.1% BSA and stored at 4°C. The Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, IL, USA) was used to assess the number of Ab$_2$ molecules per MB, estimated from the amount left in solution after MB-Ab$_2$ conjugation and compared to a standard curve of Ab$_2$ (Table 3.2).$^{35,36}$ The number of Ab$_2$ per MB was estimated to be (4–11) x 10$^4$ for different polyclonal antibodies for PTHrP. The number of horseradish peroxidase labels per magnetic beads determined by measuring enzyme activity using 2,2'-Azino-bis(3-Ethylbenzthiazoline-6 Sulfonic Acid) (ABTS) as reactant (Table 3.2).$^{37}$ Activity assays showed that the number of HRP per MB was (1.3-2.6) x 10$^5$.

### Table 3.2. Characterization of Magnetic Bead Conjugate.

<table>
<thead>
<tr>
<th>Secondary Antibody (Ab$_2$)</th>
<th>BCA (Ab$_2$ per bead)</th>
<th>ABTS (HRP per bead)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA45</td>
<td>40,000 ± 6,000</td>
<td>256,000 ± 20,000</td>
</tr>
<tr>
<td>IgY 3103</td>
<td>96,000 ± 7,000</td>
<td>165,000 ± 19,000</td>
</tr>
<tr>
<td>IgY 3104</td>
<td>114,000 ± 11,000</td>
<td>127,000 ± 5,000</td>
</tr>
</tbody>
</table>

#### 3.3.3.7 Detection of PTHrP Isoforms and Fragments

The semi-automated microfluidic system was constructed as previously reported$^{31}$ (Figure 3.2) and electrochemical measurements were all completed at room temperature with a CHI 1040B eight-channel potentiostat at conditions optimized for high sensitivity and low S/N. The capture and detection chamber were first connected to the microfluidic system and subjected to a flow of PBS-T20 to block for NSB.$^{31}$ 30 µL of HRP-MB-Ab2 conjugate was reconstituted in 130 µL of 20 mM PBS buffer (pH 7.4), loaded into the 100 µL sample loop and injected into the capture chamber at 100 µL/min flow rate. 5 µL of PTHrP standard reconstituted in 5x diluted calf serum was loaded into the sample loop and injected into the capture chamber. HRP-MB-Ab$_2$ conjugate
were held in the capture chamber, through use of a neodymium magnet positioned above the top PMMA plate, as the peptide standard or serum/plasma sample was injected.

Figure 3.2. Immunoarray with online peptide capture: (A) microfluidic device and (B) detection pathway.
For simultaneous detection of N-terminal (1-33 or 1-86), and C-terminal peptides (151-169, 140173) or intact 1-173 isoform, 10 µL of HRP-MB-Ab\textsubscript{2} conjugate for each peptide was reconstituted in 130 µL of PBS buffer, loaded into the sample loop and injected into the capture chamber followed by injection of a mixture of the three standard peptides diluted in calf serum. Flow was then stopped, magnet removed and incubation was allowed for 30 mins with stirring for the peptide to be captured by HRP-MB-Ab\textsubscript{2} conjugate. The resulting peptide-Ab\textsubscript{2}-MB-HRP conjugates were washed by flushing the capture chamber with PBS-T20 while holding the magnet bar on top of the PMMA plate and then re-dispersed in PBS-T20. The direction of flow was changed and the peptide-Ab\textsubscript{2}-MB-HRP conjugates were transported into the detection chamber housing the Ab\textsubscript{1}-modified 8-electrode array. After peptide-Ab\textsubscript{2}-MB-HRP conjugates filled the detection chamber, flow was stopped and incubation was allowed for 15 mins for Ab\textsubscript{1} on the array to capture the bead conjugate. Unbound bead conjugate was then washed off by resuming buffer flow.

To perform amperometric measurements, the arrays were further subjected to a flow of buffer containing 1 mM hydroquinone in PBS for 4 mins. The 8 electrodes of the array were then connected to the working electrode leads of a CHI 1040 multi-potentiostat, and the Pt and Ag/AgCl wires were connected to the counter and reference leads, respectively. Amperometric detection was performed at -0.3 V vs Ag/AgCl by injecting a mixture of 1 mM hydroquinone and 0.1 mM hydrogen peroxide into the detection chamber via the sample loop at 100 µL/min. Hydrogen peroxide activates HRP on the peptide-Ab\textsubscript{2}-MB-HRP conjugates to ferryloxy-HRP, which, in turn oxidizes hydroquinone to benzoquinone. Signal is generated as benzoquinone is reduced through a 2-electron transfer at the electrode surface.
3.3.4 Microfluidic system 2: Screen-Printed Carbon Arrays

3.3.4.1 Prostate Specific Antigen Antibodies

Human Kallikrein 3/PSA DuoSet ELISA (DY1344) was purchased from R&D Systems (Minneapolis, MN, USA). CHI 1040C multipotentiosstat (Texas) was used for 8-channel amperometric detection.

3.3.4.2 Human Serum Samples

Prospective males with elevated PSA levels, abnormal digital rectal exam (DRE) or both identified during routine screening campaigns or by primary care physicians were recruited as study participants by George Washington University. Serum was separated from whole blood in Vacutainer tubes by spinning tubes at room temperature at 1000 to 1300 x g for 10 minutes. Isolated serum from the top phase was transferred into a microcentrifuge tube and stored at -80°C until delivery to University of Connecticut. Inclusion criteria consisted of prostate screen subjects must be ambulatory, without a history of prostate cancer between the ages of 40 and 80 years of age. Patients must have a positive prostate cancer screen test of either a serum PSA level > 7 ng/ml or abnormal digital rectal exam (estimated 50% likelihood of positive biopsy), performance status of 0, based on the criteria of the Eastern Cooperative Oncology Group and the life expectancy for the patients must exceed 5 years. Patients were excluded from the study if the patient had previous radiation to the pelvic area or prostate volume >200 grams. These protocols have been ethically approved by the relevant institutional review boards for George Washington University (IRB #091437) and University of Connecticut (IRB #Hl 4-003).

3.3.4.3 Cell Extracts and Conditioned Media

Protein cell lines were lysed and measured following the protocol described by Yu et al from McGill University Health Center Biorepository.39 Protein cell extracts from human prostate
cell lines (PC-3, RWPE1 and RWPE2) were collected at 72h post-seeding and extracted in RIPA buffer. Conditioned media for each cell line was collected at 72h post-seeding. Regular RPMI media + 10% FBS (for PC-3 cell line) and Gibco Keratinocyte-SFM + EGF & BPE (for RWPE1 and RWPE2 cell lines) were used as controls for the experiments.

3.3.4.4 Microfluidic System

We designed a bead-free microfluidic immunoarray evolved from an earlier bead-based assay (Figure 3.3). The system contains a programable infusion pump (Fusion 100) to deliver buffer and the reagents, manual sample injector (Rheodyne™ 7725/7725i) and 100 μL PEEK sample loop. The detection chamber features a molded PDMS channel supported by two flat poly(methylmethacrylate) (PMMA) plates manufactured to fit on both sides of the PDMS slab and bolted together to seal the microfluidic channel 1.5 mm wide, 2.8 cm long, and 63 μL in volume. The top PMMA plate contained female ports (4 mm diameter) for screwing in male plastic fittings to hold tubing for inlet and outlet purposes. The top PMMA plate also contained 0.6 mm holes to hold Ag/AgCl and 0.2 mm holes for Pt wire electrodes. The syringe pump, manual injector and detection chamber were connected using 0.2 mm i.d. PEEK tubing.

Figure 3.3. Magnetic bead-free microfluidic system: (A) Microfluidic system, (B) Microfluidic device.
3.3.4.5 Fabrication of Immunoarray Sensor

Screen-printed 8-electrode carbon array sensors were fabricated as previously described.\textsuperscript{31,34,37} Briefly, arrays were coated with successive layers of polycation PDDA and negatively charged 5 nm glutathione-coated gold nanoparticles (GSH-AuNPs) using layer-by-layer (LbL) electrostatic adsorption for 20 min each as previously reported.\textsuperscript{38} Incubating a freshly prepared crosslinking solution of EDC and NHSS for 10 min activated terminal surface carboxyl groups on the GSH-AuNP layer. The electrode sensor arrays were subsequently spotted with primary antibody (Ab\textsubscript{1}), which was allowed to incubate overnight at 4°C resulting in Ab\textsubscript{1} immobilization via an amidation reaction.\textsuperscript{37} Furthermore, prior to utilizing, the arrays were blocked with 2\% BSA in PBS for 1 hour at 4°C to inhibit non-specific binding (NSB).

3.3.4.6 Detection of PSA and PTHrP Peptides

Following fabrication and blocking of the immunoarray sensor, one array was inserted into the detection chamber and connected to the system. The system was conditioned by flowing PBS-T20 for 3 minutes to minimize NSB. 150 μL mixture containing a ratio of 1:1:1 Ab\textsubscript{2}:Sample:poly-HRP was injected into the detection chamber for 1 minute, flow was stopped and incubated for 15 minutes for peptide and protein capture. For simultaneous multiplexed detection of the protein and peptides, mixed Ab\textsubscript{2} or Ag for each protein and peptide were injected into the detection chamber. After washing, amperometric detection at -0.2 V vs Ag/AgCl was enabled by flowing 1 mM hydroquinone + 0.1 mM H\textsubscript{2}O\textsubscript{2} through the detection chamber. All electrochemical measurements were completed at room temperature with a CHI 1040C eight-channel potentiostat.
3.4 RESULTS

Microfluidic system 1: Inkjet Printed Gold Arrays

3.4.1 Optimization of Polyclonal and Monoclonal Antibodies

PTHrP undergoes post-translational cleavage at lysine or arginine to N-terminal, mid-region and C-terminal peptide fragments.\(^{37}\) To establish optimal conditions for the assays as well as to improve on both the signal to noise ratio and sensitivity, polyclonal antibodies on the surface of the arrays and monoclonal antibodies on the conjugate magnetic beads were optimized. N-terminal fragments (1-33 & 1-86) were paired with antibodies PA158 and MA45, while the C-terminal fragments (151-169 & 140173) were paired with antibodies (PA6 & PA104) and (IgY3103 & IgY3104). We began with optimizing the antibody concentrations on the magnetic beads keeping a consistent Ab\(_1\) antibody concentration of 100 µg/mL, and employing standard concentration of 0, 2.5, and 5 pg/mL for standard peptide fragments (of 151-169, 140-173, and 1-33). The optimal secondary antibody concentration was determined from the greatest signal difference between the control and sample concentration for IgY3103 50 µg/mL, for IgY3104 50 µg/mL, and for M45 20 µg/mL (Figure 3.4 A,B,C). Once the secondary antibody concentration was confirmed for the conjugate magnetic beads the optimal concentration of primary antibody on the surface of the arrays was established using a consistent optimized secondary antibody concentration (IgY3103 50 µg/mL, IgY3104 50 µg/mL, and M45 20 µg/mL) again employing standard concentrations of 0, 2.5, and 5 pg/mL for standard peptide fragments (of 151-169, 140-173, and 1-33). The greatest signal difference between control and sample concentration indicated the optimal primary antibody concentration to be 100 µg/mL for all polyclonal antibodies (Figure 3.4 D,E,F).
Figure 3.4. Optimization of polyclonal antibody (Ab₁) and monoclonal antibody (Ab₂) concentration using a control and standard concentrations of 2.5, 5 pg/mL for (A) PA104, (B) PA6, and (C) PA158, (D) MA3103, (E) MA3104, and (F) MA45. Optimal concentrations for both Ab₁ and Ab₂ are circled.

3.4.2 Single Peptide Detection

We first designed single peptide assays for intact PTHrP 1-173, N-terminal (1-33 & 1-86) and C-terminal fragments (151-169 & 140-173). PA158, PA6 & PA104 were employed as capture antibodies on the sensors while M45, IgY3103 & IgY3104 were attached onto magnetic beads as detection antibodies (Table 3.2, Figure 3.4). Peptide standards were dissolved in 5x diluted calf serum. 39 Calf serum diluted 5x in PBS pH 7.4, a good surrogate for human serum in immunoassays, 39 was employed as peptide standards diluent. Calf serum as reported by the
manufacturer (Sigma-Aldrich) has a total protein concentration of 5.5–8.0%, which is similar to human serum (4.0–9.0%).

Analytical performance of the immunoassay was evaluated including its linearity, precision (intra-assay and inter-assay), sensitivity, specificity, limit of detection, carry-over effect, and method comparison using IRMA. Inter-assay precision was determined by running the same concentration of two standards on different batches of arrays and intra-assay variation was determined by measuring the variation within the 8-electrode array on same batch. In each case, samples were run in triplicate for each run, with two separate runs each day for 5 days. The inter-assay relative precision was <7% and intra-assay precision was <5%. Analytical recovery of the assay was also evaluated by spiking the diluted calf serum with peptide standards. Analytical recovery above 80% was obtained.

Calibrations for peptide fragments are shown in Figure 3.5. Signals for peptide-free controls result from residual NSB of magnetic beads and direct reduction of H₂O₂. Peaks increased linearly with log C for peptide fragments from 150 aM to 7 pM. LODs measured as 3-SDs above control were 150-170 aM (3-5 fg mL⁻¹) for all peptides. LODs were 1000-fold better than commercial IRMA and ELISA kits (3-6 pg mL⁻¹). Sensitivities (as slopes of calibration plots) were 1.98 – 2.12 μA cm⁻² [log C]⁻¹ for 1-33, 140-173 and 151-169, but increased to 3.55 – 4.98 μA cm⁻² [log C]⁻¹ for larger peptides (1-86 and 1-173) (Figure 3.5, Table 3.3).
Figure 3.5. Calibrations for peptide fragments. (A) for 1-33 at -0.3 V vs Ag/AgCl. Calibrations for PTHrP fragments in 5x diluted calf serum (n=8): (B) 1-33, (C) 1-86, (D) 151-169, (E) 140-173 and (F) intact PTHrP 1-173.

Table 3.3. Detection limit and sensitivity from single peptide detection

<table>
<thead>
<tr>
<th>Peptide Fragments</th>
<th>Fragment size (# amino acids)</th>
<th>Detection Limit (fg/mL)</th>
<th>Sensitivity (μA/cm²)/(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-33</td>
<td>33</td>
<td>5 (1.3 fM)</td>
<td>2.22</td>
</tr>
<tr>
<td>151-169</td>
<td>19</td>
<td>4 (1.7 fM)</td>
<td>2.12</td>
</tr>
<tr>
<td>140-173</td>
<td>33</td>
<td>3 (0.72 fM)</td>
<td>1.98</td>
</tr>
<tr>
<td>1-86</td>
<td>86</td>
<td>4 (0.44 fM)</td>
<td>4.89</td>
</tr>
<tr>
<td>1-173</td>
<td>173</td>
<td>3 (0.15 fM)</td>
<td>3.55</td>
</tr>
</tbody>
</table>
3.4.3 Multiplexed Peptide Detection

Based on the sensitivities observed during single peptide detection, the larger peptide fragments (1-86 & 1-173) were selected for multiplexed detection. Employing the 8 electrode array, a sandwich assay was build up on the first three electrodes for detection of 1-173 using PA104 and IgY3103, the next three electrodes for detection of 1-86 using PA158 and MA45 and the last two electrodes for detection of 1-173 using PA6 and IgY3104 (Figure 3.6). Magnetic bead conjugate (10 µL each) with MA45, IgY3103 and IgY3104 were combined, dispersed in PBS and injected into the capture chamber via the sample loop. A mixture of standards for 1-86 and 1-173 (volume ratio 1:2) was then introduced into the capture chamber with the aid of the magnet bar positioned above the capture chamber. All steps, from washing the resulting peptide-bead conjugate to amperometric detection, were performed as illustrated above for single peptide detection. Prior to obtaining calibration plots, binding studies were done to determine the specificity of the antibodies towards the peptide fragments (Figure 3.6B). Minimal cross-reactivity was observed between the antibodies for 1-173 and 1-86 peptide analytes.

Figure 3.6. A) Multiplexing strategy for the peptide fragments on a single 8-electrode inkjetprinted AuNPs array. B) Representative amperometric response for detection of a mixture of 1-173 and 1-86 on a single 8-electrode array.
Calibrations for detection of mixtures are shown in Figure 3.7. Linear dynamic range was 3 fg mL\(^{-1}\) to 12 pg mL\(^{-1}\). LODs were 4 fg mL\(^{-1}\) for 1-86, 6 fg mL\(^{-1}\) for 1-173 using PA104, and 3 fg mL\(^{-1}\) for 1-173 using PA6. Good reproducibility is illustrated by small error bars. Combination of PA104 and IgY3104 and PA6 & IgY3103 gave similar sensitivity towards detection of intact PTHrP1-173 isoform (4.49 vs 4.48 \(\mu\)A cm\(^{-2}\) [log C]\(^{-1}\)).

**Figure 3.7.** Array results for standard peptide mixtures in 5x diluted calf serum at -0.3 V vs Ag/AgCl for (A) intact PTHrP 1-173 using PA104, (C) 1-86 peptide fragment, (E) intact PTHrP 1-173 using PA6, and calibration plots for intact PTHrP 1-173 (B and F) and (D) 1-86 fragment (n = 3).
3.4.4 Validation of Accuracy

Serum samples from cancer patients with solid tumors and cancer-free individuals were assayed and compared with IRMA results. The antibody used for 1-86 peptide binds all three PTHrP isoforms and their N-terminal fragments. The antibody used for 1-173 binds PTHrP 1-173 and shorter C-terminal fragments including 140-173 and 151-169.\textsuperscript{37} Significant differences in PTHrP levels between cancer patients and cancer-free controls were observed. Cancer patient samples had larger amounts of PTHrP up to 9 pM compared to healthy individuals (<1 pM, Figure 8A,B), with statistical difference between means confirmed by t tests (P < 0.001).

Assays by the immunoarray (1-86) and IRMA gave similar levels of PTHrP and t tests (P < 0.001) confirmed no significant difference between the two methods (Figure 3.8C). The immunoarray detected PTHrP in all samples including 4 samples with PTHrP levels that were too low to be measured by IRMA. Immunoarray results for 1-86 also gave good linear correlation with IRMA for 57 samples (22 controls and 35 cancer subjects) with slopes close to 1 (0.90 ± 0.02), intercepts near 0 (1.33 ± 0.51) and r\textsuperscript{2} = 0.99 (Figure 3.8D). These results confirm the accuracy of our immunoarray protocol. Values obtained with microfluidic assays measuring intact PTHrP 1-173 and its (C-terminal) fragments (red) were only slightly lower than those from the 186 assay (blue) recognizing all three isoforms and short N-terminal fragments, suggesting that PTHrP 1-173 and its short C-terminal fragments are the major forms of PTHrP in serum (Figure 3.8C).
Figure 3.8. Distributions of PTHrP levels in cancer patient serum (37) and cancer-free individuals (22) for (A) 1-86; (B) PTHrP 1-173; (C) bar graph comparing IRMA and immunoarray (1-86 and 1-173) results for PTHrP (n = 12) and (D) correlation plot of IRMA and immunoarray data (1-86 and 1-173) (n = 57). Asterisk (*) denotes value below IRMA LOD.

Data were also analyzed using receiver operating characteristic (ROC) plots to predict diagnostic accuracy. Here, sensitivity (true positive rate) is plotted against 100-specificity (false positive rate) for different cutoff points. A test with perfect discrimination has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). The area under a ROC curve (AUC) quantifies the overall ability of the test to discriminate between individuals with and without the disease. Data with zero false positives and zero false negatives has an AUC of 1.00.
For PTHrP (n = 57) the ROC plot had AUC 0.96 for the 1-86 fragment assay and 0.94 for PTHrP 1-173. The 1-86 fragments gave 80% sensitivity and 100% specificity while intact PTHrP 1-173 gave 82.9% sensitivity and 95.5% specificity. The cancer vs noncancer cutoff PTHrP was 1.1 pM using the 1-86 assay, in agreement with IRMA results. Curves for individual peptides (Figure 3.9A) gave relatively similar sensitivity and specificity when using normalized, mean values of the two peptides (Figure 3.9B).

**Figure 3.9.** Receiver operating characteristic (ROC) curves for (A) serum assays for 1-173 (red) with AUC 0.94, 95.5% specificity and 82.9% sensitivity and 1-86 (blue) with AUC 0.96, 100% specificity and 80% sensitivity and (B) normalized value for both 1-86 and 1-173 with AUC 0.96, 100% specificity and 80% sensitivity.

### Microfluidic system 2: Screen-Printed Carbon Arrays

#### 3.4.5 Multiplexed Analysis

Optimization was studied again as analysis would be completed on a new sensor with a different set and variety of proteins and peptides. We first optimized secondary antibody
concentrations by keeping a consistent Ab\(_1\) antibody concentration of 100 μg mL\(^{-1}\). The optimal secondary antibody concentration was determined from the greatest signal difference between the control and sample concentration. For IgY and M45 this was 5 μg mL\(^{-1}\) and was 1 μg mL\(^{-1}\) for PSA (Figure 3.10 A,B,C). We then optimized the primary antibody on the surface of the arrays using the optimized Ab\(_2\) concentrations. For the primary antibody a concentration of 25 μg mL\(^{-1}\) was optimal for PTHrP antibodies PA104 and PA158 and was 22.5 μg mL\(^{-1}\) for PSA (Figure 3.10 D,E,F). Streptavidin poly-HRP has been optimized previously at 2 μg mL\(^{-1}\) and was used for these assays.

**Figure 3.10.** Optimization of monoclonal antibody (Ab\(_2\)): (A) IgY, (B) M45, (C) PSA Ab\(_2\) and polyclonal antibody (Ab\(_1\)): (D) PA104, (E) PA158, and (F) PSA Ab\(_1\) using a control and standard concentrations of 1.25 and 2.5 pg/mL.

Prior to obtaining calibration plots, cross reactivity studies were completed to determine the highest specificity of each antibody for the PTHrP peptides and PSA protein. Analysis was completed on multiple layouts to observe that which minimized the cross-reactivity between the
antibodies and peptide analytes. Employing the 8 electrode array, a layout of PSA, PA158 and PA104 were used for multiplexed detection (Figure 3.11).

![Graphs showing amperometric response for detection of PSA, PTHrP 1-33 and PTHrP 140-173 using the optimized sensor Ab1 layout of PSA, PA158 and PA104.]

**Figure 3.11.** Representative amperometric response for detection of a mixture of PSA, PTHrP 1-33 and PTHrP 140-173 using the optimized sensor Ab1 layout of PSA, PA158 and PA104.

Calibration curves for multiplexed detection were obtained for PTHrP peptide fragments and PSA (Figure 3.12). The linear dynamic range was 0.20 fg mL⁻¹ to 1.4 pg mL⁻¹ and LODs were 0.68 fg mL⁻¹ for PSA, 0.20 fg mL⁻¹ for PTHrP peptides. Sensitivities were similar for all markers ranging from 10.3-12.5 µA cm⁻² [log C]⁻¹, except for peptide fragment 151-169 which had a lower sensitivity at 7.69 µA cm⁻² [log C]⁻¹.
Figure 3.12. Array results for standard peptide mixtures in 5x diluted calf serum at −0.2 V vs Ag/AgCl for (A) PSA, (B) 1-33 using M45 and PA158, (C) 1-86 using M45 and PA158, (D) 140-173 using IgY and PA104, (E) 151-169 using IgY and PA104 and (F) intact PTHrP 1-173 using IgY and PA104. Their corresponding amperometric responses (G-M).
3.4.6 Validation of Accuracy

Cell lysates and serum samples from prostate cancer patients were tested to determine PTHrP peptide response. The PTHrP peptide fragments used for this analysis were 1-33 and 140-173 as they bind different antibodies specifically and gave consistent responses when running previous experiments described above. Three prostate cell lines (PC-3, RWPE1 and RWPE2) were used and the cell extracts and conditioned media were tested with regular media controls. Each sample was run in triplicate. Significant differences were observed between PSA and PTHrP peptide fragment concentrations between the cell extracts and conditioned media (Table 3.4).

**Table 3.4.** Analysis of PSA and PTHrP peptide fragments 1-33 and 140-173 in (A) cell extracts and (B) cell conditioned media

<table>
<thead>
<tr>
<th>Human Cell Line</th>
<th>PSA, pg mL(^{-1})</th>
<th>PTHrP 1-33, pg mL(^{-1})</th>
<th>PTHrP 140-173, pg mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RWPE1</td>
<td>0.086</td>
<td>0.046</td>
<td>0.096</td>
</tr>
<tr>
<td>RWPE2</td>
<td>0.0061</td>
<td>0.074</td>
<td>0.0093</td>
</tr>
<tr>
<td>PC3</td>
<td>0.0013</td>
<td>5.1E-05</td>
<td>0.00012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human Cell Line</th>
<th>PSA, pg mL(^{-1})</th>
<th>PTHrP 1-33, pg mL(^{-1})</th>
<th>PTHrP 140-173, pg mL(^{-1})</th>
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</thead>
<tbody>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RWPE1</td>
<td>2.0</td>
<td>0.58</td>
<td>0.19</td>
</tr>
<tr>
<td>RWPE2</td>
<td>240</td>
<td>0.15</td>
<td>1.1</td>
</tr>
<tr>
<td>PC3</td>
<td>6.4</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Human serum samples were also analyzed. Nine prostate cancer patient samples were used to determine if the PTHrP peptide fragment concentrations were correlated with increasing Gleason score (3 sample each). Gleason score is used by pathologists to score prostate tissue samples to determine severity of prostate cancer.\(^{41}\) Samples with known Gleason scores of 6 (indolent), 7 (significant) and 9 (aggressive) were chosen for comparison (n=3). Analysis confirmed positive correlation of PSA and the two PTHrP peptide fragments with increasing Gleason score (Figure 3.13).
Figure 3.13. Prostate cancer patient serum analysis of PSA and PTHrP peptide fragments 1-33 and 140-173. Samples tested fell into three categories based on Gleason score: Indolent (Gleason 6), Significant (Gleason 7) and Aggressive (Gleason 9).

3.5 DISCUSSION

Results described above demonstrate the first assays for simultaneous detection of PTHrP, its peptide fragments and PSA in serum and cell lysates with ultralow LODs of 0.2 fg mL\(^{-1}\). In the first microfluidic system, 1 µm superparamagnetic beads with 250,000 HRP labels and 120,000 antibodies (Ab\(_2\)) per bead enabled high capture efficiency and ultrahigh sensitivity in a 30 min assay. 1-86 peptide assays measured the level of all three PTHrP isoforms (PTHrP1-139, 1-141 and 1-173) and their fragments containing N-terminal end of the peptides while assay using 1-173 measured the long isoform PTHrP1-173 and shorter fragments in serum. A novel and interesting observation is that circulating concentration of the PTHrP 1-173 isoform and its fragments was
only slightly lower that the concentrations of all three isoforms and their fragments suggesting that PTHrP 1-173 is the predominant circulating form of PTHrP. This finding requires further study in larger cohorts.

Sensitivities of single-detection assays ranged from 2–5 µA cm⁻² [log C]⁻¹. The highest sensitivities were obtained for peptide fragments 1-86 & 1-173. Assay results revealed a significant difference between the levels of PTHrP in healthy individuals (<10 pg mL⁻¹) compared to cancer patients and our assays could also detect PTHrP levels in all the samples (Figure 3.4). In agreement, ROC analyses (Figure 3.5) gave 80-83% sensitivity and 96-100% specificity for clinical detection of cancer. While more samples need to be analyzed, results suggest a high potential of PTHrP 1-86, 1-173 immunoassay for early stage cancer diagnostics.

In the second microfluidic system, screen printed carbon sensors were used for the investigation of PTHrP in prostate cancer. PSA was included in this investigation as it is the only biomarker currently approved for prostate cancer diagnosis. Unlike in the first assay, this system was simplified to reduce time, complexity, and cost. Magnetic beads were replaced with streptavidin poly-HRP and a programmable pump was used for reduced consumer interface. Assay results showed an increase in concentration of PSA and PTHrP peptide fragments in cell conditioned media of prostate cell lines. Confirming the feasibility of quantitating PTHrP peptides in cell lines, specifically prostate cell lines, and can be further investigated for intra-cellular levels (e.g. circulating tumor cells). Moreover, analysis of prostate cancer serum samples positively correlated PSA and PTHrP peptides with increasing Gleason score. Indicating that PTHrP and its peptides can be used as potential biomarkers in staging and determining progression of prostate cancer. To confirm diagnostic potential in prostate cancer a larger cohort of patient samples will need to be run.
The microfluidic immunoarray offers a simple, rapid, low cost way to simultaneously detect PTHrP peptide fragments and PSA. Inkjet printing technology offers both a simple and elegant way to fabricate disposable low-cost sensor electronics for the immunoarray. A single 8-electrode array cost ≈$0.2, and up to 56 arrays can be printed in a single run. Thus, ease of fabrication and utilization of commercial components makes this approach accessible to virtually any biomedical laboratory at low cost. Capture and detection chambers are made by templating PDMS channels on machined aluminum molds to avoid lithography, and mounted on hard plastic PMMA housings with inlet and outlet lines. The microfluidic devices require only small sample volumes (5 µL) and offer options of automation and reliability to enhance reproducibility and throughput. These advantages make the microfluidic immunoarrays a promising tool for development of sensitive, integrated, portable, clinical diagnostic devices in a short time with minimal sample and reagent requirements.

In summary, we describe two novel approaches for simultaneous detection of isoforms of PTHrP and PSA in assay formats suitable for comparing circulating forms. These assays provides accurate analysis of normal and pathological clinical samples with numerous potential applications in pathologies and physiological conditions in which PTHrP has been implicated. Results of cancer patient samples support the diagnostic utility of such assays.
3.6 REFERENCES


23. Miki T, Yano S, Hanibuchi M, Kanematsu T, Muguruma H, Sone S. Parathyroid hormone-related protein (pthrp) is responsible for production of bone metastasis, but not visceral
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Chapter 4

3D Printed Electrochemical Biosensor Array Towards Point-of-Care

Prostate Cancer Detection

4.1 ABSTRACT

Over 3 million men living in the United States have been diagnosed with prostate cancer. It is the second most diagnosed cancer for men throughout the US and has worldwide implications. Current practices for detection and diagnosis of prostate cancer are often limited in terms of sensitivity and specificity. Moreover these practices are unable to distinguish between aggressive and indolent forms of the cancer. Such limitations have put a financial burden on the healthcare system and have caused significant harm to patients who have undergone unnecessary treatments. Measurements of small panels of protein biomarkers in serum hold immense potential for cancer diagnostics and personalized treatment plans. Here we describe the development of a simple, low-cost, mass producible microfluidic system for automated capture and detection of prostate cancer biomarkers. The protein panel includes Prostate specific antigen (PSA), vascular endothelial growth factor-D (VEGF-D), pigment epithelial derived factor (PEDF), insulin growth factor-1 (IGF-1), insulin growth factor binding protein-3 (IGFBP-3), monocyte differentiation antigen CD-14 (CD-14), gene fusion proteins ETS related gene (ERG) and Golgi membrane protein 1 (GOLM-1). This panel includes protein biomarkers that are thought to be both general prostate cancer biomarkers as well as proteins that were reported to be reliably specific for aggressive and metastatic forms. The system is being developed with a 3D printed device designed with multiple channels for reagents to assist with automated detection. This device will be integrated with a modified electrode sensor to achieve high sensitivity. Ultralow detection limits in sub fg mL\(^{-1}\) range were achieved in a 15 min assay for multiplexed detection of the biomarker protein panel.
4.2 INTRODUCTION

Prostate cancer is the second most common cause of cancer-related death and is the most common male non-cutaneous malignancy in the United States.\textsuperscript{1} In the US alone 3.1 million men live with prostate cancer, 174,650 new cancer cases are diagnosed every year and approximately 31,620 die each year.\textsuperscript{2} The lifetime risk of men developing prostate cancer is 1 in 9. In a population with increased longevity, it is likely that prostate cancer will become even more clinically prevalent in future. This projected increase is a major public health concern, especially when there are dilemmas associated with both detection and treatment.\textsuperscript{3}

Current practices for initial assessment of prostate cancer include the prostate specific antigen (PSA) blood test and digital rectal exam (DRE).\textsuperscript{4,5} If PSA levels are above 4 ng mL\textsuperscript{-1} and the DRE test is abnormal, then the patient will undergo a transrectal ultrasound (TRUS) biopsy. These practices have led to early detection of prostate cancer and improved the patient survival rate and treatment outcomes. However, they have also led to inaccurate assessments often resulting in unnecessary or unwarranted treatments that adversely affect patient quality of life with minimal gain.\textsuperscript{6-9} PSA, for example, has low specificity to prostate cancer as the biomarker is also elevated in conditions such as benign prostatic hypertrophy (BPH), prostatitis, and catheterization. In addition, PSA has limited predictive power with an inability to clearly distinguish aggressive forms of prostate cancer from indolent forms.\textsuperscript{2}

Localized forms of prostate cancer have a 5-year survival rate of 99\%, while for aggressive prostate cancers the 5-year survival rate drops to 28\% once the cancer has metastasized.\textsuperscript{10} To date, no reliable blood test has been devised to distinguish between aggressive prostate cancers that will metastasis versus slow growing non-metastatic cancers. This uncertainty leads to over-diagnosis and over-treatment, e.g. unnecessary surgeries, and is an overarching clinical challenge. There is
a pressing need to identify novel strategies to stage and grade prostate cancer so that needed interventions are made while minimizing over-diagnosis and over-treatment.

One format being used to fill the niche of cancer detection is immunoassays. Immunoassays make use of highly specific antigen-antibody binding and provide sensitive ways to detect a wide range of biomacromolecules, bacteria, viruses, and small molecules. There are a range of types of immunoassay systems including single analyte sensors, 96-well plate formats, arrays, microfluidic sensors, microfluidic arrays, etc. A big target is encompassed by medical diagnostic biomarkers, which are "molecules that can be measured objectively as indicators of normal or disease processes and responses to therapeutic intervention".11 Accurate, low cost measurements of multiple proteins are major applications of immunoarrays that are critical for future early detection and monitoring of cancer and other diseases. Multiplexing is very important, since panels of biomarker proteins, as opposed to single biomarkers, are required to provide sufficient information content for reliable disease diagnostics.

Development of new immunosensors and immunoarrays face stiff competition from the many available commercial immunoassays. Enzyme-linked immunosorbent assay (ELISA) is the gold standard with limits of detection (LODs) of 3-10 pg mL\(^{-1}\) for many proteins,\(^{11-13}\) but has well-known limitations in sensitivity, analysis time, and multiplexing. However, the newer commercial multi-protein immunoassays can be expensive, technically demanding, and usually do not achieve detection below pg mL\(^{-1}\) levels.\(^{14}\) Many commercial methods feature microbead technologies with optical or ECL readout and 1-10 pg mL\(^{-1}\) LODs,\(^{11,15}\) including Mesoscale electrochemiluminescence (ECL)\(^{16}\) and Luminex\(^{17}\) fluorescent bead systems, Quansys Q-Plex\(^{TM}\) multiplexed ELISA.\(^{18}\) An exception is the newer Simoa HD-1\(^{19}\) protein counting system from Quanterix that has achieved protein LODs 4-200 fg mL\(^{-1}\).\(^{20,21,22}\) Simoa-HD-1 detected PSA down
to 14 fg mL\(^{-1}\) (0.4 fM) in serum of prostate cancer patients after prostate removal, illustrating an important application of low abundance protein detection, since PSA increases after surgery when cancer returns. Despite major advances, fast, cost effective immunoassay measurements of multiple proteins below \(~4\) fg mL\(^{-1}\) (~10 aM) with commercial kits and hardware remain problematic.\(^{23-25}\)

3D printing has evolved into a multifunctional fabrication tool that offers unique advantages for biomedical applications.\(^{26-29}\) As an additive manufacturing technique, production costs are lower compared to traditional subtractive manufacturing techniques due to reduction of labor and material cost. In addition, the same 3D printer can be used for multiple processes without the need for pre-fabrication changes normally required in subtractive manufacturing techniques.\(^{30,31,32}\) These criteria make 3D printing a valuable tool in prototyping, testing and production of tools and equipment for analytical and diagnostic laboratories.

The most representative use of 3D printing technology in diagnostics is the design and development of microfluidic devices. Microfluidic devices offer the most promising approach for miniature fluidic devices due to their ability to handle small sample volumes and assay reagents in a controlled manner. 3D printing has pushed prototyping and development of microfluidics forward by supporting fast and easy design with lower production costs compared to traditional microfabrication techniques. 3D printing also offers an efficient tool to generate geometrically complex microfluidic devices with the aid of 3D design software. Thus, eliminating the hassle associated with traditional manufacturing tools.

This chapter describes the work undertaken to further develop the electrochemical microfluidic immunoassay described in Chapter 2 to an automated point-of-care test. To enhance current systems to fall under the World Health Organizations guidelines for an ideal POC test we
modeled our current microfluidic systems on lateral flow assays. Instead of spotting and incubating individual layers with overnight incubation at 4°C, each layer was spotted and dried down in an oven at 40°C. We also are developing a 3D printed microfluidic device for electrochemical detection of multiplexed protein panels for prostate cancer detection. Together, this will not only streamline the modification procedure and enabled same day testing, but will also open doors for mass manufacturing and a global reach.

4.3 EXPERIMENTAL

4.3.1 Chemicals and Materials

Screen-printed 8-electrode carbon array (700 µm diameter) sensors were purchased from Kanichi Research Services Ltd (Manchester, England). L-glutathione reduced (GSH, ≥98%), gold (III) chloride trihydrate (HAuCl₄·3H₂O, ≥99.9%), sodium borohydride (NaBH₄, 99%), poly(diallyldimethylammonium chloride) (PDDA, MW 200,000-300,000, 20% in water), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), bovine serum albumin (BSA), calf serum, Tween-20, sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na₂HPO₄, ≥98%), sodium phosphate monobasic monohydrate (NaH₂PO₄·3H₂O, ≥98%), hydroquinone (HQ, ≥99%), hydrogen peroxide (H₂O₂, 30%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pierce Streptavidin Poly-HRP was purchased from ThermoFisher Scientific. The polydimethylsiloxane (PDMS) kit was obtained from Dow Corning (Auburn, MI, USA). All solutions were prepared using 18 MΩ·cm water purified by passing house distilled water through a Hydro Service and Supplies purification system (Durham, NC, USA).

Human Kallikrein 3/PSA DuoSet ELISA (DY1344), Human VEGF-D DuoSet ELISA (DY622), and Human IGF-1/IGF-1 DuoSet ELISA (DY291), were purchased from R&D Systems
(Minneapolis, MN, USA). ERG Capture antibody clone 8A9 (TA600177), Detection antibody clone 5F12 (TA700176), and purified human protein (TP308093) were purchased from OriGene Technologies, Inc (Rockville, MD USA). CHI 1040C multipotentiostat (Texas) was used for 8-channel amperometric detection.

4.3.2 Fabrication of Microfluidic Detection Chamber

The microfluidic detection chamber was constructed as previously reported. Briefly, in-house machine made molds were developed to form the microfluidic channel. The mold was filled with PDMS, cured and the soft PDMS microfluidic channel was formed with desired dimensions. The channel was then sandwiched between two hard flat poly(methylmethacrylate) (PMMA) plates, also machined in-house, that are then screwed together. The detection chamber (Figure 4.1) contains 4 mm diameter inlet and outlet ports to fit 1.5 mm standard plastic fittings that hold 0.2 mm i.d. PEEK tubing. The chamber also has two 0.6 mm parallel holes for Ag/AgCl reference electrode and two 0.2 mm parallel howls for platinum counter electrode.

![Figure 4.1 Components of the microfluidic device made of micro-machined PMMA, PDMS microfluidic channel and screen-printed carbon electrode arrays.](image-url)
4.3.3 Formation of Immunoarray Sensor by Immobilization of Proteins

Screen-printed 8-electrode carbon array sensors were fabricated following architecture previously described\textsuperscript{33-36} with changes in the incubation of materials. The arrays were first coated with a layer of polycation PDDA and placed in an oven at 40°C for 1 min 40 sec whereupon the arrays were washed with water and dried under nitrogen gas. Successive layers of negatively charged 5 nm glutathione-coated gold nanoparticles (GSH-AuNPs), freshly prepared crosslinking solution of EDC and NHSS and primary antibody were completed following the same process described with PDDA (Figure 4.2). A layer of 25 M Trehalose was then spotted for preservation and increased stability by protecting the proteins from inactivation or denaturation.\textsuperscript{37} At this point the arrays can either be placed in a desiccator, vacuum sealed and stored overnight or used immediately. For immediate use the arrays are blocked with 2% BSA (minimizes non-specific binding), placed in the oven until dry, washed with PBS-T20 and dried again.

4.3.4 On-line Capture and Detection Protocol

The immunoassay capture strategy is illustrated in Figure 4.2. An on-line protein capture microfluidic system was employed providing a semi-automated methodology for protein detection. Details on the assays performance will be briefly described.
The system was first subjected to a flow of PBS-Tween 20 using a programmable infusion syringe pump (Fusion 100). The detergent solution was used to minimize adhesion and NSB of undesirable molecules. Once the system was washed, a mixture of secondary antibody (Ab$_2$), antigen (Ag), and streptavidin poly-HRP (poly-HRP) was prepared in an equal volume ratio. The mixture was then loaded into a 100 μL sample loop, injected at 100 μL min$^{-1}$ and allowed to fill the detection chamber housing the Ab$_1$ modified sensor. Upon filling the chamber, the mixture was incubated for 15 minutes to facilitate protein capture and completion of the immunoarray sandwich. PBS-T20 flow was resumed to remove unbound bioconjugates followed by further washing with hydroquinone (HQ) for electrochemical background signal production.

Amperometric detection was completed at -0.2 V vs Ag/AgCl by injecting a mixture of 1 mM HQ mediator and 0.1 mM hydrogen peroxide at 100 μL min$^{-1}$ via the sample loop to activate the HRP labels. Prior to detection, the sensor array, counter electrode and reference electrode were connected to a CHI 1040C multipotentiostat. Whereupon, an electrochemical redox cycle
Amperometric signals are proportional to concentration of protein analyte. Once detection was completed, a fresh modified sensor array was inserted into the detection chamber for analysis of the remaining samples.

4.4 RESULTS

4.4.1 Development of an Electrochemical POC Platform

Optimization of the multiplexed system was completed previously and conditions were used for this system (Figure 4.3). Briefly, detection antibody (Ab$_2$) concentrations were assessed first using a fixed capture antibody (Ab$_1$) concentration of 100 μg mL$^{-1}$ and varied standard concentrations for each standard protein. Optimal concentration was obtained when the signal difference was greatest between control and sample concentrations. Optimized Ab$_2$ concentrations were 1 μg mL$^{-1}$ for PSA, VEGF-D and IGF-1 and 1.5 μg mL$^{-1}$ for ERG. We then assessed Ab$_1$ concentrations using the optimized Ab$_2$ and same varied standards. Capture antibody concentration were optimized at 22.5 μg mL$^{-1}$ for PSA and IGF-1, 25 μg mL$^{-1}$ for ERG and 45 μg mL$^{-1}$ for VEGF-D. Lastly, an optimized concentration of 2 μg mL$^{-1}$ poly-HRP in 0.01% BSA was determined using optimized concentrations for Ab$_1$ and Ab$_2$. 
Figure 4.3 Optimization results for sandwich immunoarray performance upon employing standard concentrations. (A) Optimized results for Ab$_2$, (B) Optimized outcomes for Ab$_1$, (C) Optimized concentration of streptavidin-HRP label.

When deciding to further develop the system towards point of care, three dry down methods were investigated. They were 40°C oven, 37°C incubator and vacuum sealed with a desiccator. The temperatures of 37°C and 40°C were chosen as they are below the temperature that could cause irreversible unfolding and aggregation. The three methods produced classic representative cyclic voltammograms (CVs) for each layer dried down on the sensor surface versus when then were incubated wet as described previously. The best method of the three was determined to be drying in the oven at 40°C with washes after each layer (Figure 4.4). This method took 1 min 40 sec while the incubator took almost 8 min and the desiccator 15 min.
Figure 4.4 To wash or not to wash between modification layers? (A) No wash and (B) Wash. A wash is needed after drying down each layer to remove any unbound components.

We next aimed to detect signals of clinical interest by establishing calibration curves of multiplexed protein standards in five times diluted calf serum in PBS buffer. Calf serum is an acknowledged surrogate for human serum as they have similar total protein concentration (4.0-9.0%), as reported by the manufacturer (Sigma-Aldrich). The arrays were first prepared by modifying the electrodes with layers of PDDA/GSH-AuNPs/EDC&Sulfo-NHS/Ab1/Trehalose. Upon completing the modification procedure each of the arrays was placed into separate petri dishes and all were placed in a desiccator, vacuum sealed and left overnight (16-20 hrs). The following morning Trehalose was washed off with PBS-T20 and the electrodes were blocked with 2% BSA for 1 hour. Following this incubation period the 2% BSA was washed with PBS-T20 and the arrays were once again dried off in the oven at 40°C. Control and standards were then run for calibration generation. The calibrations for protein biomarkers are shown in Figure 4.5 generating dynamic ranges spanning 5- to 6-fold log decades from low fg mL\(^{-1}\) to high pg mL\(^{-1}\). Detection limits ranged from 3.24 fg mL\(^{-1}\) to 259 fg mL\(^{-1}\) with sensitivities ranging from 3.8-6.3 μA cm\(^{-2}\) [log C]\(^{-1}\).
Figure 4.5 Immunoarray Calibrations of protein standards in 5-fold diluted calf serum gives peaks by injecting a mixture of 1 mM hydroquinone and 0.1 mM H$_2$O$_2$ at -0.2 V vs Ag/AgCl for (A) PSA, (B) VEGF-D, (C) ERG, and (D) IGF-1 after analyte protein and antibody binding.

We further simplified this protocol by examining a complete dry down procedure of each solution. Following the same procedure just described we dried down both 2% BSA and PBS-T20 on the second day instead of a wet incubation of 2% BSA for 1 hour. Using this method we tested the entire 8 protein biomarker panel. The calibrations for protein biomarkers are shown in
Figure 4.6 generating dynamic ranges spanning 5- to 6-fold log decades from low fg mL$^{-1}$ to high pg mL$^{-1}$. Detection limits for PSA, VEGF-D, ERG and IGF-1 ranged from 3.24 fg mL$^{-1}$ to 32.4 fg mL$^{-1}$ with sensitivities ranging from 3.9-4.5 μA cm$^{-2}$ [log C]$^{-1}$. Detection limits for GOLM-1, PEDF, IGFBP-3 and CD-14 ranged from 7.63 fg mL$^{-1}$ to 62.0 fg mL$^{-1}$ with sensitivities ranging from 6.8-8.1 μA cm$^{-2}$ [log C]$^{-1}$.

![Figure 4.6 Immunoarray Calibrations of protein standards in 5-fold diluted calf serum, all components of electrode modification dried down at 40°C.](image)

Following confirmation of dried down capabilities we assessed accuracy of the assay using spiked recovery experiments in commercially available human serum (Table 4.1). Samples were prepared by spiking five standard concentrations of the four lowest abundant protein biomarkers. Recoveries obtained ranged from 87-120% in good agreement with the analytically acceptable range. This experiment aids in demonstrating the accuracy and reliability of the immunoarray for measuring concentrations of the proteins in clinical ranges without interference from matrix effects.
Table 4.1. Spiked human serum percent recovery for the lowest abundant biomarkers.

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Low Concentration (6.48E-6 – 6.48E-5)</th>
<th>Mid Concentration (2.08E-4 – 5.19E-4)</th>
<th>High Concentration (6.25E-2 - 0.531)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>86.5%</td>
<td>120%</td>
<td>94.2%</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>99.0%</td>
<td>90.8%</td>
<td>116%</td>
</tr>
<tr>
<td>ERG</td>
<td>99.2%</td>
<td>101%</td>
<td>98.8%</td>
</tr>
<tr>
<td>IGF-1</td>
<td>117%</td>
<td>119%</td>
<td>101%</td>
</tr>
</tbody>
</table>

One of the longest portions of the original protocol is overnight incubation of the primary antibody. This not only ensures maximum binding, but also ensures all samples are run over the same time period. In order to make this protocol POC and mass producible, an overnight incubation needs to be reduced or completely eliminated. We tested whether an overnight incubation was required for sensitivity with our new protocol. Calibration curves for the lowest abundant biomarkers are shown in Figure 4.7 generating dynamic ranges spanning 4 to 5-fold log decades from low fg mL⁻¹ to high pg mL⁻¹. Detection limits ranged from 1.06-32.5 fg mL⁻¹ with sensitivities ranging from 4.5-7.0 μA cm⁻² [log C]⁻¹. These detection limits and sensitivities are comparable to previous validated systems. Thus enabling us to prepare and test assays within one day; reducing the processing and analysis time.
We designed the microfluidic devices in 123D design software (Autodesk) and transferred them to a Form2 stereolithographic 3D printer where they were printed using Formlabs clear resin (Figure 4.8). The device has four main components: the syringe inlet, snake channels for reagents, detection chamber and outlet. The inlet has an outer diameter of 3 mm and 2 mm length, suitable for connecting tubing for a 1 mL syringe and syringe pump connection. Each of the reagent channels were 20 mm in length by 3 mm wide for total fill volume of approximately 120 μL. The
detection chamber was designed for attachment of our sensor array with holes for the reference and counter electrode wires. The chamber was 20 mm long by 3 mm wide by 1.5 mm high for an approximate fill volume of 90 μL. The holes for the counter and reference electrodes were 0.6 mm in diameter. Finally, the outlet was made with to be 0.5 mm high by 1.6 mm wide by 4.5 mm long for reagents to pass through and leave the detection chamber.

Figure 4.8 3D design of the electrochemical microfluidic device.

The next step for our device is integration with the dried down assay to determine if improvements need to be made to the design. We also must insure that reliable amperometric peaks and clinically relevant calibrations are obtained. The combination of the new dried down assay with the 3D printed device will not only create a system that is inexpensive and mass producible, but also one that is easy to operate and does not require cold-room storage qualifying it for point-of-care settings.

4.5 DISCUSSION

Results described above demonstrate the initial steps taken to further develop an electrochemical system that can accurately detect a panel of protein biomarkers. Development of
a 3D printed device enhances the automation capabilities of the entire microfluidic system. Only addition of the protein sample (standard or patient sample) is required by the operator. The system utilizes poly-HRP providing efficient capture of secondary antibody and enzyme label for signal amplification. Sensitivities of the immunoarray obtained from the slope of the calibration curves ranged from 3.8-8.1 μA cm\(^{-2}\) [log C]\(^{-1}\) for multiplexed detection. The decrease in sensitivity was partly due to minimal cross-reactivity observed between the proteins of the biomarker panel. A wide dynamic range was also obtained from 1.06 fg mL\(^{-1}\) to 0.71 ng mL\(^{-1}\) enabling detection of clinically relevant concentrations for future patient serum sample analysis.

One of the main advantages of this study is the assays capability to detect clinically relevant ranges of proteins without interference from matrix effects. The ability to dry down individual components for modification of the array enables testing of samples quicker than previous assays. The 3D printed device enables options for mass scalability, automation and low cost. Further studies need to be completed in terms of stability and patient sample analysis to support the potential diagnostic utility of this assay to aid doctors in their screening decisions. This assay will not only assist classifying patients and help relieve the healthcare system, but will also provide the first point-of-care diagnostic test for prostate cancer detection.
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