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Surface Functionalization and Analysis Thereof for an Ovarian Cancer Diagnostic Biosensor

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Surface Functionalization and Analysis Thereof for an Ovarian Cancer Diagnostic Biosensor

by

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of the requirements for the degree of
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DEDICATION

I would like to dedicate this thesis to my mother Fauzia Kausar, who has motivated and inspired me to be my best in everything I do. I would also like to dedicate it to my best friend and brother, Shan Ahmad. This research would not have been possible without their support, prayers and confidence in me throughout the years.

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ABSTRACT

Ovarian cancer is the fifth leading cause of cancer death among women in United States and has an alarming 1.4% (1 in 71) lifetime risk. The lack of overt symptoms and the absence of a reliable screening test to detect ovarian cancer result in over 70% of women being diagnosed after the disease has spread beyond the ovary resulting in a poor prognosis. A key characteristic of ovarian cancer is the ability of tumor cells to evade apoptosis, or programmed cell death contributing to the limitless replicative potential, which is a hallmark of all carcinogenesis. There is conclusive evidence that levels of bcl-2 are elevated in ovarian cancer patients' indication that this protein is an ovarian cancer biomarker. The overall goal of this thesis is to functionalize a substrate for specific, sensitive and cost-effective bcl-2 capture. This surface will ultimately be incorporated into an acoustic wave-based diagnostic device for worldwide point-of-care (POC) ovarian cancer detection.

This research looks to assess the capture of this analyte protein on a series of bioconjugated surfaces. For the research to be diagnostically applicable, certain factors reveal themselves as more important than others. Since the surface-bound capture antibody must recognize the bcl-2 protein, it is vital to ensure upright orientation of this specific antibody with high affinity for the analyte. Furthermore once integrated with a nanosensor, the surface will sense a change in the mass on the surface, which requires that the surface is highly resistant to non-specific binding. Bioconjugation techniques were employed to initiate self-assembled monolayers (SAM) of silanes, immobilize antibodies (via amine-crosslinking or direct adsorption of protein A/G) and disperse polyethylene glycol (PEG) reagents to reduce non-specific binding on the glass

substrates. 3-aminopropyltrimethoxysilane (3-APTMS) and chlorodimethyloctylsilane (ODMS) were deposited on the surface to create initial hydrophilic and hydrophobic properties on which molecular self-assembly could occur. Testing a variety of assemblies with and without the presence of silanes, amine-crosslinking and PEGylation reagents, the substrate displaying the highest efficacy of bcl-2 capture was revealed. These various surfaces were assessed through contact angle and a novel sandwich enzyme linked immunosorbent assay (ELISA) for sensitivity and specificity of bcl-2 standard capture.

The consistently low background and facile assembly of the ODMS based substrate with direct adsorption of protein A/G and the PEGylation reagent, Pluronic, was deemed the 'best' functionalized surface for non-specific recruitment of the bcl-2 protein. The substrate also consistently displayed low signal-to-noise ratio which was of extreme importance in this research to guarantee the prevention of false-positive results when detecting nascent carcinogenic behavior. Elucidation of this substrate assembly is the first step towards the long term objective of this thesis, which is to construct a cost-effective early ovarian cancer detection device which can be implemented at the point-of-care to those who need it the most. This is ultimately expected to dramatically improve health outcomes for females worldwide.

CHAPTER 1: INTRODUCTION

1.1 Motivation for Research

With the rapid advances in proteomic characterization and micro-technologies, diagnostic techniques have quickly become a catalyst for progression in long term health strategy, preventative medicine and providing timely care to patients in developed regions of the world. However the technological capabilities in high-resource settings are far superior to low-resource settings. It is because of this that the vast majority of deaths that occur annually from major infectious diseases occur in under-privileged regions of the world (1). Nevertheless, a solution lies in development of point-of-care (POC) diagnostic tests which can be easily and efficiently implemented in low resource settings improving health and general well-being of the world. The ability to produce rapid results visually, lack of sophistication in operation and low-cost in production: allow POC diagnostics to be implemented with an extremely high efficacy in high and low resource settings alike. Thus, POC diagnostic can results in a decrease of the burden of disease due to infection around the world. Infectious diseases which plague low-resource settings (acute respiratory infections, malaria, and tuberculosis) often display disease progression through the generation of biological changes in various ways such as upkeep of antigens, proteins or antibodies. There is low motivation in regions where the burden of death is not as high for infectious disease; both intuitively as well as financially, for initiatives in POC test development. However if the versatility of POC diagnostics could be applied to detect specific carcinogenic pathways and thus onset of tumor growth, it would greatly benefit humanity all around the globe. The objective of this

thesis is to quantify the specificity and selectivity of selected bioconjugated surfaces to a known ovarian cancer biomarker, the anti-apoptotic bcl-2 protein. The surface with highest efficacy can then be integrated with a novel nanosensor to create a POC diagnostic device for worldwide detection of stage I and stage II ovarian cancer.

Ovarian cancer is the fifth leading cause of death among women in United States and the disease has 1.4% (1 in 71) lifetime risk. Patients with ovarian cancer have a short median survival time after diagnosis, as the 5-year survival rate being less than 40%. Early stage ovarian cancer represents an important target for screening mediums to detect, since the cancer is lethal in most late stage cases. The lack of explicit symptoms and the absence of a reliable screening test to detect ovarian cancer results in over 70% of women being diagnosed after the disease has spread beyond the ovary, when it is too late (2). Currently, the only standardized screening procedures for ovarian cancer are pelvic examinations, ultrasounds and blood levels of CA-125 or cancer-antigen 125. However each method has significant limitations. Pelvic examinations are typically limited to the detection of advanced stages, which is thus of no consequence towards improving overall prognosis of the disease. Similarly, ultrasounds are not only incapable to distinguish between benign and malignant disease but are also subject to variation in interpretations among sonographers (3). Although CA-125 levels are useful to monitor ovarian cancer progression, they can also be elevated due to other disorders including benign gynecological disease and hepatic disease preventing accurate and conclusive identification of early ovarian carcinogenesis (4). While other screening modalities, including pap-smears for cervical cancer and mammography for breast cancer detection, respectively, have improved early detection and survival associated with those tumor types, no true screening exists for ovarian cancer. The absence of reliable screening methods to detect early ovarian cancer contributes to the overall poor prognosis of the disease (5). Therefore, this

research addresses the need for a reliable, simple, and economic testing platform to detect ovarian cancer. This master's thesis will focus around the elucidation of an elite bioconjugated surface to specifically and sensitively capture a known biomarker of ovarian cancer, bcl-2. These results can then be integrated with a novel, inexpensive nanosensor to quantify the capture creating a POC diagnostic device essentially enabling worldwide early recognition of ovarian cancer.

This research is predicated upon the use of the bcl-2 protein which can be considered a biomarker, or biological indicator corresponding to the onset of ovarian cancer. The apoptosis-regulating family of bcl-2-like proteins contain roughly 25 members and is named for the central anti-apoptotic bcl-2 protein. A great deal of research over the past two decades has indicated that bcl-2 is overexpressed in a large number of tumor growths. In 2008, the novel work of Dr. Patricia Kruk at the University of South Florida revealed that elevated levels of bcl-2 were present in urine from patients with early as well as late stage ovarian cancer (6). Utilizing these findings, the novelty of this project lies in being the first of its kind to detect the onset of ovarian cancer via elucidation of a novel, facile and economical surface functionalization technique capable of efficient bcl-2 capture. The functionalization approach investigated will focus on oxygen plasma treatment, silanization, efficient antibody immobilization and maintenance of non-fouling surface reagents to render self-assembled complexes with various properties. Subsequently, these test assemblies will be tested by novel sandwich ELISA technique to arrive at the most suitable substrate for sensitive and specific bcl-2 capture.

1.2 Significance of Research in Ovarian Cancer Diagnostics

Advancements in proteomics and nanotechnology have led to biosensors having also evolved greatly in sophistication over the past couple decades. The imperative function of a biosensor is to detect and quantify an analyte, which is the bcl-2 protein in this research. This biosensor consists of two primary parts: an inorganic platform capable of analyte recruitment and a nanosensor for concentration quantification. A nanosensor or transducer can employ a wide variety of principles of detection. For nanoscale detection an ideal transducer would possess a high electromechanical coupling coefficient along with low mechanical impedance. Current state of the art biosensors employ piezoelectric detection techniques and since this technology has been studied extensively, the behavior and limitations are well understood (7). The mechanical impedance of the piezoelectric transducers is very high compared to that needed to measure nanoscale mass loadings. Since the bandwidth of high impedance piezoelectric materials are very limited, the overall detection sensitivity of piezoelectric transducers are limited preventing suitable identification parameters. As seen in figure 1.1, the qualities of typical piezoelectric devices possess restrictions on reliable mass quantification. To overcome the aforementioned limitations there have recently been findings in an ultrasonic capacitance detection method to identify mass loading change generated by analyte adhesion on the surface. Work has been published on capacitive micromachined ultrasonic transducers (CMUTs) design for high bandwidth operation and electromechanical coupling coefficients for improved transmit and receive performance. CMUTs also offer additional advantages in ease of manufacturing, stability, low cost, high yield, and reliability (8, 9)

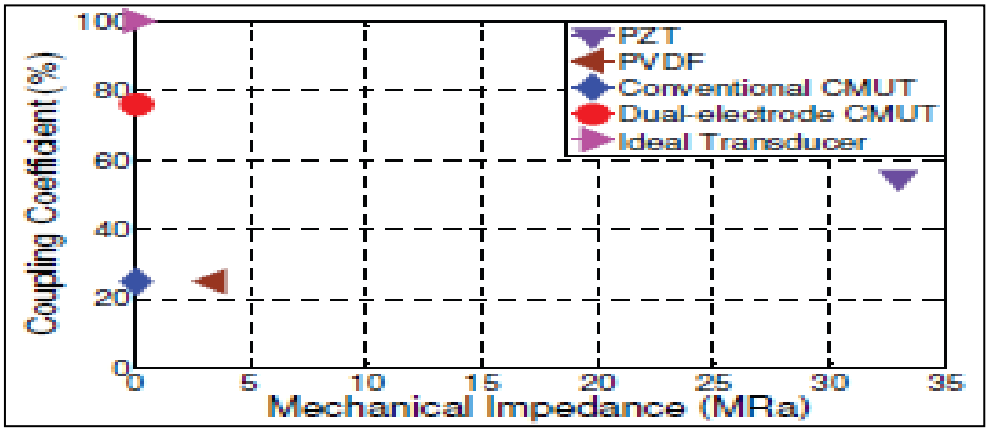

Figure 1.1 Diagnostic Performance of Various Transducers. (7)

The self-assembly that will functionalize the surface with the highest efficacy measured by bcl-2 capture can therefore be easily integrated with a variety of biosensors enabling detection worldwide. In order to validate the research it must be certain that a proper diagnostic device can be created. To do this, first and foremost, the criteria and specifications of a diagnostic device must be recognized.

The World Health Organization: Sexually Transmitted Diseases Diagnostics Initiative have coined the term 'ASSURED test' to describe the ideal characteristics of a POC diagnostic test (10), seen below. To be deemed an ideal diagnostic device the operation must be affordable by those at risk of infection, highly sensitive and specific, have few false-negative as well as false-positive results requiring minimum training and be performed without any expensive equipment., as seen in figure 1.2 on the next page.

• **Affordable by those at risk of infection**
• **Sensitive**
• **Specific**
• **User-friendly**
• **Rapid and Robust**
• **Equipment-free**
• **Deliverable to those who need it**

Figure 1.2 Characterization of the Ideal Diagnostic Test.

This promising research presented in this thesis is the first step in designing a diagnostic testing platform for urinary bcl-2 level quantification that will provide impetus towards developments of a new, accurate, safe and simple way of early ovarian cancer detection. This would undoubtedly result in recognition and treatment of thousands of previously undiagnosed cases of ovarian cancer. This thesis can result in long term beneficial impact on cost-effective early ovarian cancer detection at the POC, which can significantly improve our lives today and the health of our future generations. The proposed diagnostic device would also satisfy the benchmarks of an ASSURED test:

- Affordability: Currently employed proteomic diagnostic techniques are extremely pricey as each CA-125 enzyme linked immunosorbant assay (ELISA) test kit costs over $300 ($10 per specimen) and each bcl-2 ELISA test kit costs over $500 (approximately 18 per specimen) (6). From a standard 16 inch (approximately 41 cm) diameter silicon wafer, over 4000 nanosensors can be fabricated. The resulting cost of each nanosensor is less than $1 (8). Once the surface assembly showing the most diagnostic promise is identified, a large number of substrates can be functionalized at low costs.

- Specificity/Sensitivity: The functionalized surface will be classified as a non-fouling surface ensuring that nonspecific binding does not occur, eliminating the chance for false-positive results. It has been experimentally verified that mass-loaded nanosensors sensitivity can detect in the order of 0.15 $^{pg}/_{ml}$ truly enabling early ovarian cancer detection once a novel, facile functionalization technique can be established (7-9).
- User-Friendly: The test would not necessitate the need for trained personnel for operation. The simple procedure would employ female urine similar to that of the common pregnancy test and would engender fast, reliable results.
- Rapid/Robust: Since only urine is necessary for operation, the need for refrigeration and specimen storage is eliminated. Also, the nanosensor would give results instantaneously of a bcl-2 protein from a battery supply. Typically a standard 1.5 volt battery can supply the energy needed for mass quantification sensors. Because of the nanoscale realm of the measurements, a large number of readings can be executed without battery replacement (7).
- Equipment Free: A fully-integrated bio-functionalized nanosensor device would remove the need for any additional equipment for testing besides urine.
- Delivery: Since the device would be disposable and conveniently small, it can be deliverable to those who need it the most. Thus, because of the ease of transportation of the POC device, a large number of tests can be implemented at virtually any location.

1.3 Utilization of Biomarkers to Uncover Carcinogenic Pathways

Through a complex signal transduction cascade: lymphocytes or white blood cells maintain homeostasis by surveying a range of antigens inside the body. Upon detection of an invader antigen, secretion of antibodies to maximally eliminate the pathogen occurs. Each lymphocyte or B-cell (beta cell) has a unique receptor on its surface that will capture specific antigens and thereby monitor any irregularities. The secretion of antibodies to counter these pathogens is a staple of the immune response whose responsibilities include pathogen and toxic neutralization, innate immunity, recruitment to upregulate phagocytosis and other cellular purification processes (11). Cancer and the widespread cellular pandemonium that it causes on affected patients' immune response cannot yet be treated as it is not fully comprehended. If we target various specific deficient or disproportionate cellular-indicator proteins whose functionality is tampered with by carcinogenic signal transductions, discrete cancers can be traced at an increasingly earlier timeframe therefore ensuring much more positive prognosis for patients. This approach bodes well for the future to detect carcinogenesis as itnot only allows for a great deal of novel techniques but also employs numerous biomarkers to be elucidated for different types of cancer.

Biomarkers can be understood as an indicator of a biological state and thus can be the key to detect early stage diseases. This allow for immediate treatment and thus greatly improve patient prognosis, by understanding some of the biological mechanisms employed by the disease to override the immune system. There is no disease where the elucidation of impaired signal pathways is more needed than cancer. There has been a great deal of biomarkers uncovered in a number of cancers; bcl-2 being the most recent for ovarian cancer. In 2004, researchers from the department of Medicine and Center for Cancer Research at the University of Salamanca in Spain quantitatively demonstrated

elevated levels of bcl-2 expression in a variety of B-cell malignancies: proliferative neoplastic B-cells malignancies, B-cell acute lymphoblastic leukemia, B-cell chroniclymphomatic leukemia, diffuse large B-cell lymphoma and multiple myeloma (12). In 2008, Dr. Patricia Kruk and her colleagues at the Cellular and Molecular Biology department at the University of South Florida conclusively revealed that urinary levels of bcl-2 are elevated in ovarian cancer patients (6). Urinary levels of bcl-2, as seen in figure 1.3 were found in the study to be: 0.59 $^{ng}/_{ml}$ healthy patients, 2.60 $^{ng}/_{ml}$ early stage and 3.58 $^{ng}/_{ml}$ late stage ovarian cancer (6). Further information regarding bcl-2 as an ovarian cancer biomarker is presented in chapter 2.4. This thesis aims to evaluate the capture of the apoptosis regulator protein, bcl-2, on functionalized surface with maximum specificity and efficacy in hopes to detect ovarian cancer. This groundbreaking research is the first of its kind to implement a novel assay to detect concentrations of an ovarian cancer biomarker as an analyte.

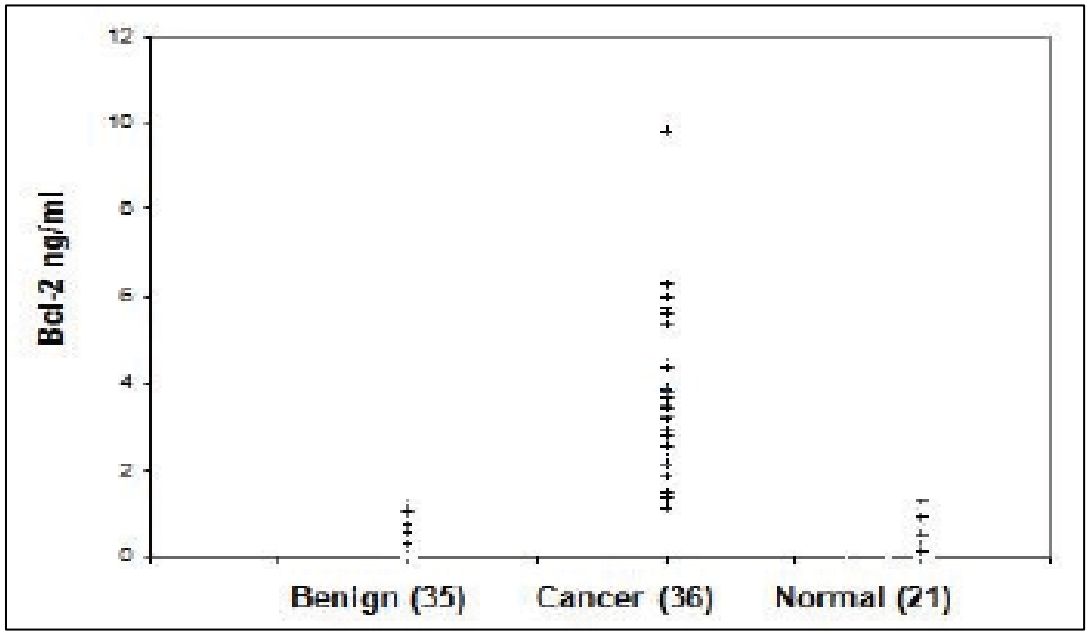

Figure 1.3 Bcl-2 Levels Elevated in Ovarian Cancer Patients. (6)

1.4 Content of Thesis

Chapter 2 provides detailed information on:

- Clinical needs for emerging testing technologies such as POC diagnostics
- Novel proteomic methods to reveal biomarkers.
- Influence of the Bcl-2 family proteins on cellular survival.
- Overview of project.

Chapter 3 provides a comprehensive look into:

- Bioconjugation techniques for reagent functionalization on self-assembled monolayers (SAM) for assays targeting immunoglobulins.
- Silanization techniques: 3-Aminoproplytrimethoxysilane (3-APTMS) and Chlorodimethyoctylsilane (ODMS).
- Crosslinking Reagents and protein A/G for immobilization of target antibodies.
- Poly (ethylene-glycol) reagents to maintain non-fouling surfaces: $MS(PEG)_4$, $BS(PEG)_5$ and Pluronic.

Chapter 4 discusses:

- Novel ELISA technique used.
- Interpreting ELISA readings.
- Selection of functionalized surface displaying the maximum bcl-2 analyte selectivity and specificity.
- Serial dilution of bcl-2 protein.

Chapter 5 provides a brief synopsis of the research and the future considerations needed.

Appendix A and B provide a review of POC tests towards primary infectious diseases in low-resource settings and proteomic techniques, respectively. Appendix C contains contact angle measurements and appendix D contains selected ELISA results.

CHAPTER 2: CANCER BIOMARKERS IN POINT-OF-CARE DIAGNOSTICS

2.1 Impact of POC Testing on Infectious Disease Detection

Point-of-care (POC) tests can be defined as laboratory tests which are intended for the purpose to be used directly at the site of patient care resulting in quicker delivery of results, thus inducing faster clinical decisions. It is because of this that POC tests are highly advantageous towards the management of infectious diseases. As a result of the undeniable benefits of POC testing it has quickly become an indispensable part of intensive-care units, emergency rooms, laboratories of hospital, physicians' offices, outpatient clinics and recently even patients' homes. Such tests have become the fundamental of care for critically ill-patients allowing for evaluation of critical parameters such as blood or glycaemic control at the patients' bedside (13). Therefore, immediate identification of a disease state allows the use of adequate pragmatic therapy, which has been shown to improve the outcome in critically ill patients. In the last 15 years, the availability and use of POC tests have greatly increased and can be found in use all around the world. Novel engineering of POC testing platforms have resulted in a significant proportion of laboratory testing currently being conducted at the point of care. In the setting of infectious diseases, most existing POC tests consist of immunoassays, namely agglutination, immunochromatographic and immunofiltration tests (14).

Ideal POC tests need to rely on easily accessible mediums such as urine, blood, saliva, or nasopharyngeal (throat) swabs. Although the use of these tests does not require laboratory expertise to conduct, inevitably performance is clearly linked to the experience of the operator and this factor can influence the overall validity of the results.

POC tests are usually assessed in comparison with standard microbiological procedures, sometimes being performed at the same time consequently allowing for the POC test to be quantifiably measured. As biomedical engineers, developing technologies in response to the needs of the always transforming world's medical community is a primary way to have a large impact on society in a highly benevolent manner. In this way the most basic medium for disease detection techniques lie in POC diagnostics (15).

2.1.1 Impact of POC Testing in Low-Resource Settings

The 'global burden' of disease is a comprehensive regional and global assessment of the mortality and disability caused by various diseases. As a means of comparing the burdens of mortality produced by distinctive diseases, the World Health Organization (WHO), has proposed the use of DALYs or disability-adjusted life-years as a quantifiable measure of the severity of a disease, as seen in figure 2.1 (16). Not surprisingly, the majority of deaths that are caused by infectious diseases occur in third world countries or low-resource settings. As a matter of fact, out of all the deaths annually from major infectious diseases (HIV, malaria, tuberculosis and acute respitory illnesses), approximately 95% of the deaths occur in low-resource settings with Africa shouldering the largest burden (17). Given that rapid POC diagnostic tests are inexpensive to produce, simple to perform, produce prompt visual readouts and often do not necessitate equipment, the potential value for infectious disease POC tests in low-resource settings are greater than in developed regions. This conjecture is widely agreed upon and because of this, diagnostic technology advancement has not garnered the same financial backing and devoted resources as other sectors of bioengineering such as drug delivery and vaccine development (18). It is not until recently that POC diagnostics have looked to be applied for detecting the onset of carcinogenesis.

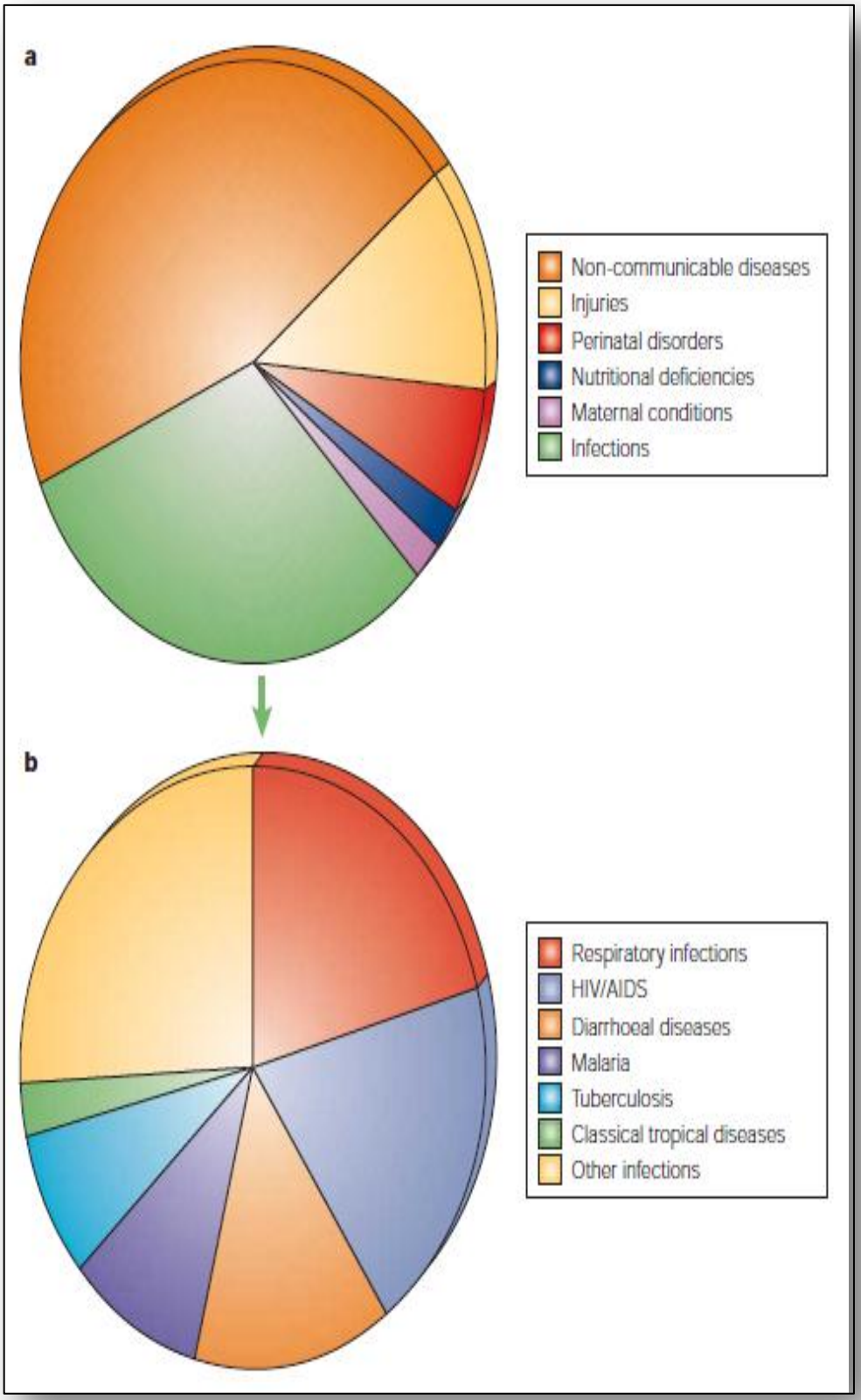

Figure 2.1 Burden of Diseases Worldwide. (18)
(DALYs lost to a. genre of disease b. specific disease)

Table 2.1 Staffing Constraints in Low-Resource Settings.

Country	Physician density (per 1000 population)	Nurse density (per 1000 population)	Year
Malawi	0.02	0.59	2004
Mozambique	0.03	0.21	2004
Uganda	0.08	0.61	2004
Kenya	0.14	1.14	2004
India	0.60	0.80	2005/2004
South Africa	0.77	4.08	2004
Brazil	1.15	3.84	2000
Bolivia	1.22	3.19	2001
United States	2.56	9.37	2000
Denmark	2.93	10.36	2002
Spain	3.30	7.68	2003
Cuba	5.91	7.44	2002

There are a great deal of reasons that infectious disease plague low resource settings. Staffing constraints is one (as seen in table 2.1), as a lack of quality training leads to deficient health care providers. Partial immunity also becomes a problem, as in high-disease endemic regions where locals may have elevated levels of a 'latent' biomarker, otherwise effective diagnostic tests can become inconclusive. Language is also an enormous confounder in deciphering information. In addition there are often local, unique terminologies to describe specific disease conditions which may not be understood by outsiders. Patients implementation of alternative medicine for certain symptoms, can locally impact the timeliness of patient presentation to a health care facility. Overall neglect is alarming as well, in one study conducted in Malawi that evaluated the delivery of tuberculosis (TB) samples to a central reference laboratory

found that only 40% of the test samples collected were successfully transported (19,20). The internal infrastructure of the health care is much to be desired in under-privaledged localities as well. Not only are government obtained supplies of sub-par quality, but routinely the procurement of supplies is unstructured and inconsistant. Coupled with the fact that there are no external quality assessments put into practice, diagnostic tests consistantly underperform. Besides these concerns, specimen collection and processing as well as instrumentation also contribute to the diminished efficacy of diagnostic tests in low resource settings (21).

The characteristics of an appropriate diagnostic tests that could effectively be implemented worldwide according the WHO as mentioned in section 1.2, can be explained by the mnemonic ASSURED. A test passing the criteria of ASSURED can be considered a POC diagnostic, which would allow for circumvention of a great deal of regional limitations described before. Since current diagnostic methods do not reliably and accurately detect ovarian cancer, there is a tremendous need to develop a safe, cost effective, reliable, preferably non-invasive method to detect early stages of the disease. Ultimately goal of this thesis is to develop and medically evaluate a testing platform for a simple, disposable, tiny (sensor component is smaller than human hair or sand particle), low cost (<$1), battery operated (>1 million hours operation with an off-the-shelf AAA battery), ultra-sensitive (~0.15 $^{pg}/_{ml}$) ovarian cancer diagnostic device for early detection at the point of care.

2.1.2 Impact of POC Testing in High-Resource Settings

Perhaps the most overlooked benefit that POC diagnostic tests offer developed regions of the world is that of elimination of the threat of drug resistance. Drug resistance has become a major concern in the management of malaria, TB, HIV and other bacterial infections, comprimising well-being of future generations. Physicians all around the

world may be guilty for the overuse of antibiotics before the definitive identification of the disease. Passive resistance that is acquired over time, threatens the solution of cheap drugs for treatment of various diseases in the developing and developed countries of the world. POC testing might assist in limiting the overuse of antibiotics as well as prevention of therapeutic drug resistance (22). Therefore further execution of POC testing worldwide would encourage prescription of drugs only once the disease state has been identified. Consequently POC diagnostics can be considered a preventative health measure, helping to eradicate drug resistance around the world. If a specific biomarkers can be corresponded to carcinogenesis or any other disease pathology, then novel techniques of specific and sensitive capture of the targeted cancer-marker can be made by means of a POC diagnostic.

2.2 Carcinogenic Pathways for Biomarker Discovery

Cancer research has long been one of the most funded clinical endeavors and as a result, a great deal of strides have been made in understanding carcinogenesis. That being said the sequential, parallel, and interconnected overrides on cellular function that cancer imposes, is still something that engineers, scientist and doctors cannot fully explain (10). Recently, there have been many initiatives in the biomedical world to detect cancer at an incipient stage so as to terminate its' advancement; thereby reducing the mortality rate associated with the disease. There are more than 100 distinctive types of cancers and further range of tumors that can be found in specific organs. Affected cancer succeeds in manifesting physiologic changes due to superseding the defensive mechanisms in the body. Though cancer is present in many names and resides in a multitide of bodily locations, it has been proposed that all cancer cell genotypes exhibit

six distinct modifications (as seen in Figure 2.2), that collectively induce malignant tumor growth. Understanding the alterations that carcinogenic genes perform on our 'molecular signature' will help in exposing discrete analyte biomolecules that can be markers for the onset of specific carcinogenesis (23). Figure 2.2 (described in detail below), defines the essential traits that are present, to some extent, in all carcinogenic behavior.

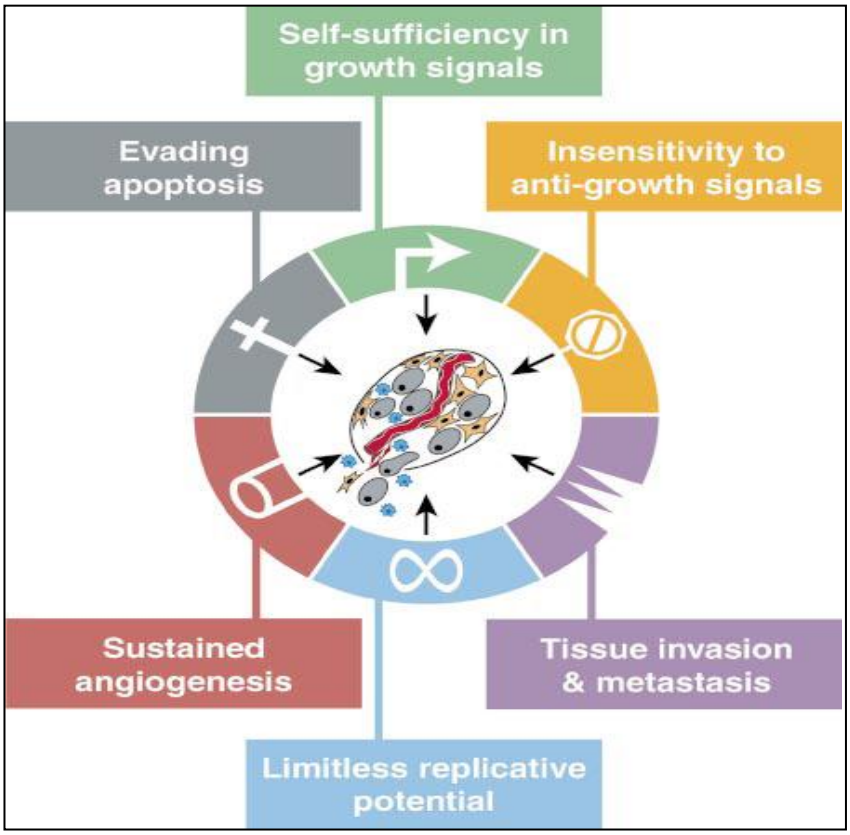

Figure 2.2 Essential Traits of Carcinogenisis. (23)

2.2.1 Sustained Angiogenesis

Angiogenesis is the development of new blood vessels in nascent tissue. Proliferating cells do not have the tendency to encourage blood vessels growth and as a result, angiogenesis is a trait that must be developed. As a result, carefully coordinated

positive and negative signals from the body which induce specific growth, called growth factors or GF, engender angiogenesis. Angiogenic-promoting GF include vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF1 and FGF2) whereas angiogenic-inhibitors include thrombospondin-1 and β-interferon. It is believed that specialized oncogenes (cancer genes), serve as a catalysts to promote and sustain angiogenic activity from otherwise vascular acquiescence. Tumors appear to accomplish this by gene transcription. The combination of increased expression of VEGF and FGF1 and down regulation of thrombospondin-1 and β-interferon is a technique employed by oncogenes to sustain angiogenesis in affected cells. Because of the complexities in transcriptional control of GFs, the mechanisms dictating shifts in angiogenic balances remain unknown (24, 25).

2.2.2 Tissue Invasion and Metastasis

During the progression of most types of cancer, primary malignant cells spawn clones which then invade adjacent tissues. This process eventually disrupts the indispensable homeostasis within the body, resulting in cell and tissue death. As a matter of fact, the distant site of tumor cells (metastasis) causes 90% of human cancer deaths (23). Several cell-cell adhesion molecules (CAM) and integrins are are responsible for transducing proteins involved in tethering for cellular motility. It is an exceedingly complex process which entails multiple signal transductions at diverse locations. As a result, pin-pointing biological effects of specific CAMs and integrin interactions is difficult because they are convoluted by the large amount of ligand-receptors groupings, resulting in a large array of combinatorial expression potential. E-cadherin, present in epethelial cells, is the most widely observed modification to standard cell-to-environment interactions from carcinogenesis. E-cadherin bridges adjoin neighboring cells and allow transmission of anti-growth and other signals.

Successful supression of E-cadherin also muffles the anti-growth signals allowing metastasis to eventually transpire. This fact is corroborated from cell culture evidence: E-cadherin serves as a supressor of invasion and metastasis and its operative elimination represents a key step in carcinogenesis (26, 27).

2.2.3 Insensitivity to Anti-Growth Signals

Anti-growth signals are tranduced to block proliferation in two techniques. Cells may be forced to their quiescent state (indicated as G_0), excluding them from proliferative activity or they can be induced to abandon the proliferative potential and placed in a post-mitotic state: acquiring differentiaton-specific traits. In the early stages of cellular growth (G_1 phase), cells monitor their environment through sensed growth signals and decide whether to proliferate, be dormant, or enter a postmitotic stage. At the molecular level the anti-proliferative triggering signals are initiated by the retinoblastoma protein (pRb). In cervical carcinoma the pRb function is eradicated by viral oncogenes, such as the E7 oncoprotein of the human papillomavirus. The integrin TGFβ also becomes disfunctional because of specific cancer cells. Due to either downregulation of TGFβ receptors or mutation of the adhesion site, the phosphorylation that impedes the pRb function is prevented. Thus, the cell is suspended and is unable to contiue to the following growth stage where DNA replication and differentiaion occurs. Disruption of the vital anti-growth pRb circuit contributes towards cancer's notorius uninhibited spread (23, 28).

2.2.4 Self-Sufficiency in Growth Signals

Coupled with the ability to disregard anti-growth signals, the cability of tumor cells to generate their own growth signals (GS) allows cancer cells complete immunity from the growth signaling defense-mechanisms that are a part of our immune system.

Sufficient laboratory evidence has proven that oncogenes attack by mimiking normal growth signaling in one way or the other. Normal cells require mitogenic GS to serve as the catalyst modifying them to a proliferative state. These GS are transduced by complex transmembrane receptor-ligand interactions from GFs, extracellular matrix and cell-cell adhesions. In culture, oncogenes generate many of their own GS and in doing so reduce the dependency on stimulation from the normal micro-environment. The cell surface receptors that are responsible for growth-stimulation are also deregulated by carcinogenesis. GF receptors tend to possess tyrosine kinase activities in their cytoplasmic region and are overexpressed by many cancer cells. As a result, customary populations of GFs can lead to hyper-activity of the cells proliferative capabilities as a result of the amplified GF receptors on the oncogenes. Moreover, the epidermal GF receptor EGF-R is overexpressed in stomach, brain and breast tumors while HER2 recepetor is upregulated in stomach and mammary cancers (23,29).

2.2.5 Avoiding Apoptosis

The capability of tumor cell populations to increase is influenced by not only cell proliferation, but by the circumvention of cell-programmed death. Cell apoptosis is how cellular removal occurs and is advantagous to the life cycle of an organism. Perhaps the most well known signal transduction cascade is the programmed cell death pathway of apoptosis. Two classes of components govern cellualar apoptosis activity, the sensors and the effectors. The sensors monitor the extracellular and intracellular envionment, processing whether the conditions are abnormal and thus initiating the signal for apoptosis. These lookout mechanisms are cell surface integrins whose signals converge on the mitochondria. Here the bcl-2 family of proteins, whose members include anti-apoptotic and pro-apoptotic constituents, supervise release of of cytochrome C and p53, potent catalysts of cell death. In fact the tumor suppressor protein p53 is

described by some as a ‘master watchman’ implying its role in maintaining stability by preventing genome mutation (30). The most common technique employed by a majority of cancer cells to oppose anti-apoptotic signals involves the p53 supressor gene. Inactivation of the p53 gene is seen in over 50% of human cancers and impinges on the signal transductions of apoptosis (31). A great deal more about the characterization of the bcl-2 family and the overexpression of the bcl-2 protein in ovarian carcinogenesis will be discussed in Chapter 2 .4.

2.2.6 Limitless Replicative Potential

The previous three attained capabilites of cancer cells: GS independence, insensitivity to anti-growth signaling, and opposition of apoptosis leads to the dire ability of the disease to replicate without restrictions. All mamallian organisms possess an intrinsic, autonomous cellular-program which restricts multiplication after a finite number of replications. Therefore for clones of cancer cells to inflict a life-threatening tumor, not only do they need to develop insensitivity towards anti-growth/pro-apoptotic signals but they must also possess the capability to override inherent encoding of the target cell (23). Once cells have progressed through their finite number of proliferation, they cease propagating: a term called senescence. Rarely, a variant (1 in ~10^7 cells) mutant emerges that has limitless replicative potential. Unfortunately, tumor cells grown in culture possess immortality that is imperative for malignant cancerous states. Telomeres, the ends of chromosomes which consist of several thousand short repeat units of RNA, are progressively shortened after each proliferation. Eventually, the telomeres are so short that they cannot protect the valuable chromosomal DNA, leading to the ceasing of replication and further cell-maturation. 85-90% of cancer cells upregulate expression of the telomerase enzyme, while others employ a technique called alternative lengthening of telomeres (ALT), which preserves telomerase-activity

through chromosomal information exchanges. By either upregulation of telomeric enzymes or ALT numerous tumor cells achieve infinite replicative ability (32, 33).

2.2.7 Cancer Biomarkers: Pathway Modifications

The six hallmarks explored explicate specific techniques employed by the progression of cancers and the affected cellular mechanisms. Since genetic and molecular changes are the incipient events in development of cancer, the abundance or lack of signal proteins, GF, specific DNA sequences, integrins, cadherins, cytokines or any cellular indicator whose amplification or depletion indicates the carcinogenic progression can be considered a cancer biomarker.

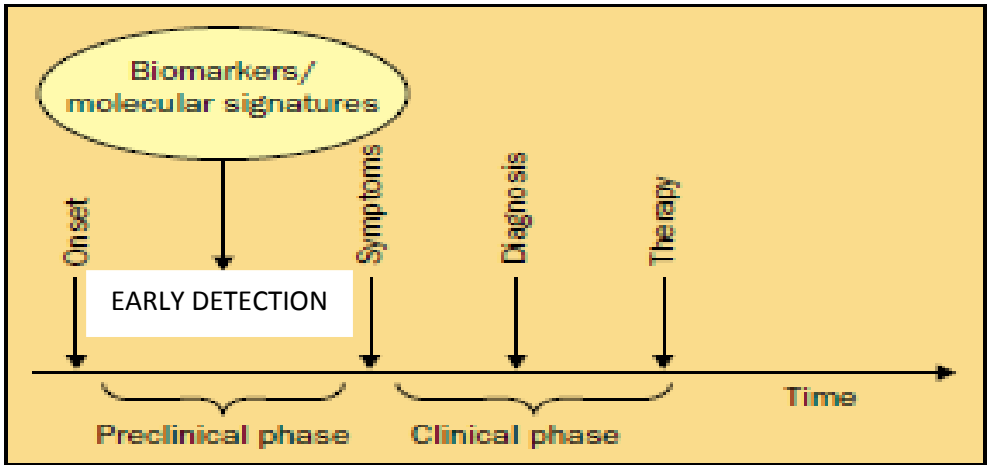

Figure 2.3 Biomarkers Indicate Onset of Disease. (11)

This research is predicated upon the cellular marker (bcl-2 protein) used by ovarian cancer to avoid apoptosis. Biomarkers should be easy to detect, measurable across populations and should provide evidence of early stage development of high-risk individuals as seen in figure 2.3 above. Quantifying specific signaling manipulations that tumor cells are responsible for (such as the silencing of pRb function in insensitivity

towards GS and upregulating VEGF and FGF1 in chronic angiogenesis), is a first step in elucidating biomarkers for early cancer detection. Hence, a variety of distinctive biomarkers can indicate particular cancerous characteristics discussed above, depending on the location of the tumor and defense mechanism manifested at the site. That being said, the complexities of carcinogenesis and the multitudes of defensive signal transductions occuring, often obscure the breakthrough.

The quantification of Dr. Patricia Kruk and her colleague's findings on the ability of ovarian cancer to avoid apoptosis by manipulation of the bcl-2 protein has provided the impetus to undertake this research and complete this master's thesis in hopes of application towards an ovarian cancer recognition device in the near future.

2.3 Proteomics for Biomarker Discovery

The Human Genome Project was an international scientific research endeavor aimed at identifying and mapping the approximately 20,000–25,000 genes of the human genome from both a physical and functional standpoint (34). A working draft of the genome was announced in 2000 and was completed in 2003, however the work on interpretation of genomic data is still in its nascent stages. Knowledge of the human genome provides new avenues for advancements in medicine and biotechnology. Many budding molecular diagnostic companies such as Myriad Genetics and Cepheid have developed easy ways to administer genetic tests that can show predisposition to a variety of illnesses, including breast cancer, cystic fibrosis, sexually-transmitted diseases and many others. Perhaps the greatest asset of the Human Genome Project is the potential to ascertain the etiologies of cancer and other deadly diseases (35).

As the genome is the complete set of genes in an organism, the proteome is the entire set of proteins expressed by a genome, cell, tissue or organism. Simply put, it is the set of expressed proteins in a given type of cells or an organism at a given time under particular conditions. The word "proteome" is a blend of "protein" and "genome", and was coined by Marc Wilkins in 1994 while working on the concept as a PhD student (34). Whereas an organism's genome is unchanging, the entire set of proteins expressed would vary from cell to cell and would be contingent upon the stimuli provided by the micro-environment. The proteome is greatly larger than the genome because there are more proteins than genes and consists of the entire complement of proteins including the modifications made to a particular set of proteins (phosphorylation, methylation, etc.) produced by an organism. The proteome reveals the intrinsic 'genetic code' of the cell as well as the influence of its micro-environment providing valuable cellular signaling information. Since deeper understanding of the disease processes at the level of molecular biology may determine new therapeutic procedures, proteomics allows for the recognition of distinct biological changes caused by pathogens. These changes include altered expression, protein modification, specific activity, inappropriate localization, cell morphology: which can all contribute to damaged cellular function. Fundamentally, proteomics is the systematic study of the correlation of gene activity and protein expression in a cell at defined time (36). Since the proteome and gene expression analysis methods are quantitative, new systematic approaches to characterize protein functions in terms of the interaction within the cell, can lead to discovery of early modifications of proteins (biomarkers) therefore detecting early progression of diseases and potentially disease etiology.

Proteomics dates back to the late 1970s when researchers started to build databases of proteins using the technique of two-dimensional gel electrophoresis (2DE) (37). This resulted in general protein cataloging which resulted in the development of

the first database of expressed proteins. Although the gels could be run reproducibly and consistently, determining the identity of the proteins was problematic because of a lack of sensitive and rapid analytical methods for protein characterization (such the automated sequencer for DNA analysis that we possess today). In the 1990s, biological mass spectrometry (MS) emerged as a powerful analytical method that removed most of the limitations restricting extensive protein analysis. This progress, coupled with the availability of the entire human coding sequence in public databases (via polymerase chain reaction) marks the beginning of a new era in cancer biomarker exposition (38). Today, the term proteomics covers much of the functional analysis of genomic effects or 'functional genomics', involving holistic proteome categorization and identification, as well as specific protein-protein interaction analysis. Figure 2.4 provides a comprehensive synopsis on the various techniques for proteome investigation done today. The four major areas for proteomic research into the analysis of gene function and protein expression and regulation are:

1) Molecular Anatomy: Analysis of protein composition of infectious cells and tissues.

2) Molecular Pathology: Analysis of disease's protein expression and modification.

3) Molecular Pharmacology: Effects of drugs on protein expression and modification

4) Molecular Physiology: Change in protein expression in response to the environment.

Defining proteomics

Proteomics – the classical definition

- Two-dimensional gels of cell lysates and annotation
- Two-dimensional gels to visualize differential protein expression

Proteomics – in the post-genomics era

- Protein identification:
  - One-dimensional gels (for example, analysis after affinity purification)
  - Two-dimensional gels (for example, analysis after affinity purification, body fluids, etc.)
  - Protein chips (chips coated with, for example, proteins or antibodies)
  - Proteins/protein complexes in solution (identification without electrophoresis)
- Post-translational modifications
  - Phosphorylation
  - Glycosylation
- Determining Function
  - Assays for enzymatic activity or determining substrates[75]
  - Bioassays for cytokines, receptor/ligand-binding assays
  - Localization within the cells (GFP fusions)
  - Proteomic analysis using large-scale mouse knockouts[76] or RNA interference[77].
  - Phenotypic analysis using deletion strains[78]
- Molecular Medicine (no longer just pharmaceuticals)
  - Finding molecular (protein) drug targets
  - Disrupting protein–protein interactions using drugs
  - Large-scale animal assays for recombinant proteins, antibodies and inhibitors
- Differential display by two-dimensional gels (superseded by DNA-based array in many situations)
  - Limited applications in:
    - Body fluids (for example, serum and urine)
    - Variants resulting from post-translational modifications
- Protein–protein interaction
  - Direct DNA readout
    - Yeast two hybrid
    - Phage display
    - Ribosome display[79]
    - RNA–peptide fusions[80]
  - Protein identification
    - Affinity purification and mass spectrometry

Figure 2.4 Defining Proteomics. (36)

As mentioned in chapter 1.3, genetic and molecular changes are the incipient events in development of cancer: The abundance or lack of signal proteins, growth factors, specific DNA sequences, integrins, cadherins, cytokines or any cellular indicator whose amplification or depletion indicates the disease progression can be considered a biomarker (10). Proteomic techniques are the most useful approach to biomarker discovery. Proteomics can be divided into two categories, described by their medium of data evaluation: qualitative and quantitave. A review of selected techniques commonly employed by researchers looking to investigate proteomics can be found in appendix A.

2.3.1 Challenges in Biomarker Validation

Many challenges still plague both proteomics and biomarker validation, namely concomitant evaluation of oncogene pathogenesis via proteomics, tissue microarrays and body fluid markers. Another major issue that arises is reproducible sensitivity and specificity of biomarker levels in cancerous solutions. Currently, the major challenge is analytical variability which creates a problem in identifying diagnostic patterns specific to cancer states from the huge dynamic range of biomarker concentration and biological inconsistencies among patient samples. For example, variation in sample collection, handling, storage and profiling techniques may bias the protein profile obtained from a given sample. It is progressively becoming more recognized that repetitive proteomic assays should be applied in the clinical setting to increase reproducibility and validation of cancer biomarkers. Presently effectively integrating proteomic, genomic and metabolic data and their functional consequences in clinical results, will be of major interest. Development of multivariate strategies for concurrent testing platforms is a potential step towards validating carcinogenic biomarkers (39).

Before initiation of clinical testing it should be fully recognized that the specific biomarker is truly in the pathway of the particular carcinogenesis and not simply the

result of an adaptive response. Given the complexities of cancer progression, a multitude of biomarkers spanning a variety or signal transduction networks should be examined for more accurate cellular evaluation. Since a major concern for screening is the eradication of false-positives, a wide variety of facets must display high specificity including similarities between gender and races. Briefly, for a biomarker to be value towards public health worldwide, the assay should be compliant to novel high-thoroughput formats with minimal 'false-positive' misclassifications.

2.4 The Role of the Bcl-2 Family in Tumorigenesis

Bcl-2 or B-cell lymphoma 2 is a protein which is named for being the second member of a range of proteins initially described with chromosomal alterations in follicular lymphoma patients (cancer of the immune system). It was first connected to tumorogenesis in 1988 by the team of Cory and Adams, as they noticed chromosomal translocations, or rearrangements, between positions 14 and 18 resulting in overexpression of the anti-apoptotic bcl-2 gene ultimately contributing to follicular lymphoma. This first introduced the thought that carcinogenic behavior can over-express the bcl-2 protein to impair apoptosis (30). Over the years, Cory and Adams have been pioneers in the study of the bcl-2 family and their role in apoptosis as well as cancer progression. Because of their findings, the link between tumorogenesis and apoptotic-regulation proteins was forever established. As a result, the prevalent misnomer that proliferation and cell-death were under similar genetic control pathways was eradicated and it was discerned that disturbances in both were likely to contribute to neoplasia; signifying a major paradigm shift in the way cancer was subsequently researched in a clinical setting (40).

Apoptosis, the most common form of programmed cell-death, is crucial for formation of the embryo, maintenance of tissue, cellular homeostasis, terminating immune responses, restricting the spread of infections and many other upkeep responsibilities (cellular autophagocytosis, anoikis and necrosis are other examples of programmed cell-death types). There are major signal transduction pathways, known as 'death circuits' that lead to programmed cell death (40). The apoptotic 'death circuit' is predicated on the recruitment of various caspases or cyteine-aspartic proteases, which are enzymes that degrade polypeptides. To date, 12 caspases have been identified and can be categorized into initiator or executioner caspases, indicating their contribution towards programmed cell death. As cells perform apoptosis various caspases are cooperatively responsible for: irregular bulging of the plasma membrane (blubbing), nuclear fragmentation, chromatin condensation and internucleosomal DNA cleavaging and finally removal from the cell through vesicle. The elucidation of the 'death circuits' pathways over the past two decades has raised the possibility of developing therapies that aim at ensuring the inclusion of pro-apoptotic proteins that cancers aim to exclude (30,41).

2.4.1 Bcl-2-Like Proteins: Systematic Control over Apoptosis

Bcl-2-like proteins consists of fully homologous and BH3-only proteins that are the central reason for activation (pro-apoptotic) and inactivation (anti-apoptotic) of an inner mitochondrial permeability transition pore. Activation of this pore induces biochemical stimulus to initiate caspase activity. Therefore, the specific bcl-2 protein is not a strict cell-death regulator, rather it is a range of interactions between a pro-apoptotic and anti-apoptotic constituents of the "bcl-2 family" that govern the cell's fate (figure 2.5). There are 25 genes that display one or more of the four structural domains of homology, entitled the bcl-2 homology (BH1, BH2, BH3, BH4). All anti-apoptotic

proteins contain the BH1 and BH2 domains, and a select few contain an additional BH4 domain. The extent of knowledge about the functional responsibilities of the BH1, BH2

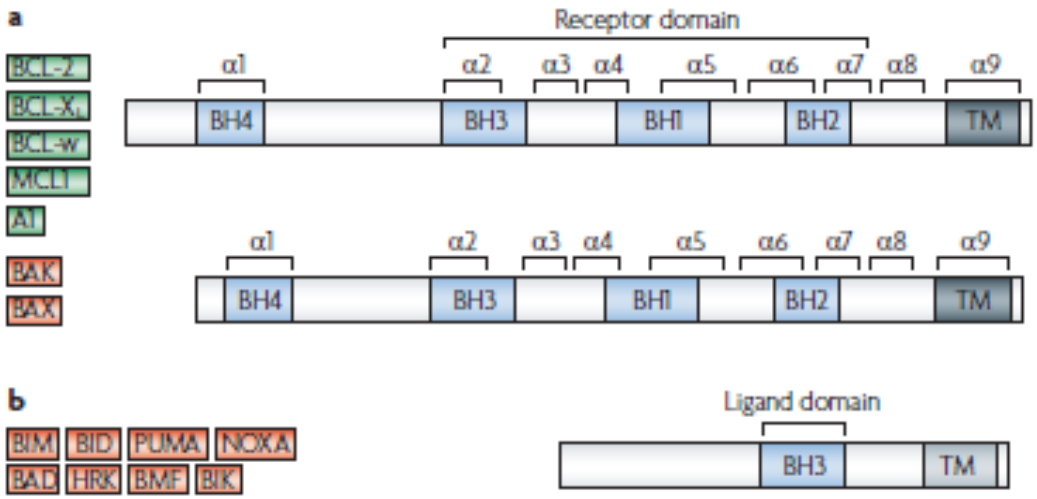

Figure 2.5 Bcl-2 Family Structure. (30)
a) Pro cell-death constituents b) Anti cell-death constituents

and BH4 domains are very limited, however there is a great deal known about the BH3 domain. Sole possession of this domain (BH3-only proteins) indicates pro-apoptotic behavior. These members bind to specific pro-apoptotic proteins of bcl-2 family which leads to their killing in continuation of the anti-cell death defense mechanism component of innate immunity (42, 43). The BH3 domain is also present in some anti-apoptotic proteins as well. It has become increasingly evident that since its' initial inception every cell requires protection by at least one of the bcl-2 homologues. It is no surprise therefore, that abundance of the wide range of bcl-2 homologues indicates tissue homeostasis in organisms. Prominent apoptotic contributors or bcl-2-like proteins are introduced below.

- The *fully homologous constituents* consist of five anti-apoptotic proteins, (Figure 2.5a): Bcl-2: B-cell lymphoma, Bcl-xL (BCL2L1): B-cell lymphoma-extra large, Bcl-w (BCL2L2): Bcl-2-like protein 2, MCL1 (BCL2L3): Myeloid cell leukemia

sequence 1, A1 (BCL2A1): Bcl2-related protein A1 and two pro-apoptotic proteins: BAK (BCL2L7): Bcl-2 homologous antagonist/killer, BAX (BCL2L4): Bcl-2-associated X protein (30,41,43).

- Eight known *BH3-only proteins* induce pro-apoptotic signals, (Figure 2.5b): BIM (BCL2L11): Bcl-2-like protein 11, BAD (BCL2L8): Bcl-2-associated death promoter, NOXA (PMAIP1): PMA-induced protein 1, PUMA (BBC3): Bcl-2-binding component 3, HRK (BID3): BH3-interacting domain-containing protein 3, BIK: Bcl-2-interacting killer and BMF: Bcl-2-modifying factor (30, 41).

Although knowledge is limited regarding the functions of the fully homologous members, it is known that bcl-2 is required for survival of mature T and B lymphocytes. As a matter of fact, bcl-2 is linked to many tumor types overexpression is often connected to poor survival (30, 44). Similarly, Bcl-xL is essential for regulating platelet survival, thus deficiency of the protein might be expected to cause thrombocytopenia or the lack of platelets in blood cells. Both bcl-2 and Bcl-xL bind to the BH3 domains of the tumor-suppressor beclin 1 (BECN1) which has shown to inhibit autophagy, thus indicating the bcl-2 families contributions to programmed cell death extend further than apoptosis. MCL-1 is required for the survival of hematopoietic stem cells and BCL-w is vital for the appropriate development of sperm cells. A1 acts as an apoptosis retardant for blood cells.

To initiate cell death in a healthy cell, BAK or BAX must be relieved of its suppression by the relevant pro-survival proteins. BAK is widely expressed in the mitochondria while quiescent BAX is located in the cytosol and is recruited to the mitochondria once cell apoptosis is commenced. For BAX mediated cell death, binding to bcl-2 is essential whereas MCL1 and Bcl-xL are vital in BAK mediated cell death. BAX and BAK oligomers are credited to provoke the permeabilization of the outer mitochondrial membrane which engenders signaling which is conducive for the initiation

of caspase-mediated cell death. Cytochrome c, an intermediate mechanism in apoptosis, is a large transmembrane protein that is released by the mitochondria in response to pro-apoptotic stimuli and is preceded by elevation in calcium levels. The release of cytochrome c, coupled with presence of apoptosis-activating factor 1 (Apaf1) in turn binds and cleaves caspase 9, the initiator caspase causing mitochondrial death. The apoptotic 'death circuit' pathway is completed with activation of executioner caspases 3 and 7 (51).

On the other hand, selective interactions of BH3-only proteins and their pro-survival family are well documented. Tissue homeostasis seems to be impinged by the disruption of the balance between pro-survival and BH3-only proteins. The majority of the BH3-only proteins act by binding to and neutralizing of their anti-apoptotic relatives, (table 2.2). Perhaps, the small allosteric change generated in the pro-survival proteins by binding with a BH3-only domain affects the association with other proteins in the anti-apoptotic pathway. Thus the BH3-only proteins allow for a greater degree of control over cell death. BID has a vital role in initiation of apoptosis, as it leads to the activation of BAX and BAK and when both of these proteins are excessive, it rapidly (less than a minute) triggers cytochrome c release (43,45).

Table 2.2 Binding Affinities: Bcl-2 Family of Proteins.
Dissociation constants (nM) for interactions between pro-survival BCL-2 family proteins (left column) and BH3-only proteins (top row) are shown. Standard deviations in parentheses. Yellow blocks signify high-affinity binding; blue blocks signify no observed binding. Activators in purple, sensitizers green. (30,41,43)

	BID	BIM	BIDmut		BAD	BIK	NOXA A	NOXA B	HRK	BNIP	PUMA	BMF
BCL-2	66 (6)	<10	▪		11 (3)	151 (2)	▪	▪	▪	▪	18 (1)	24 (1)
BCL-XL	12 (9)	<10	▪		<10	10 (2)	▪	▪	92 (11)	▪	<10	<10
BCL-w	<10	38 (7)	▪		60 (19)	17 (12)	▪	▪	▪	▪	25 (12)	11 (3)
MCL-1	<10	<10	▪		▪	109 (33)	19 (2)	28 (3)	▪	▪	<10	23 (2)
BFL-1	53 (3)	73 (3)	▪		▪	▪	▪	▪	▪	▪	59 (11)	▪

2.4.2 Validation of Bcl-2 as an Ovarian Cancer Biomarker

The uncovering of cell-death pathways over the past two decades has made it possible to target apoptotic proteins to potentially develop therapeutics for cancer and inflammatory disease, as well as diagnostic devices for recognition of tumorigenic stages. While apoptosis is an essential biological process for the development and upkeep of tissue homeostasis it is also centrally involved in a number of degenerative diseases, immunological diseases and cancers (46). Since the bcl-2 family strictly regulates apoptotic initiation and execution, both pro-apoptotic and anti-apoptotic can be targeted for apoptotic-antagonists and biomarker discovery.

Validation of biomarkers is an elaborate process because of problems that arise with the reproducible sensitivity/specificity of biomarker levels in samples and the analytical variability connected with identifying diagnostic patterns specific to cancer states. Variations in sample collection, handling, storage and profiling techniques may bias the protein profile obtained from a given sample. According to the National Institute

of Standards and Technology (NIST), as newly discovered biomarker assays progress from a research setting to the clinical diagnostic laboratory, it should proceed through defined stages of assay confirmation. The preliminary check for biomarker validation is the evaluation of research assay technology, performance, and specifications (*analytical validation*). Ultimately, the biomarker must detect early stage cancer in a clinical setting (*clinical validation*). Upon analytical and clinical confirmation, assays are moved systematically toward a standardized, reproducible, high-throughput format for clinical diagnostic implementation (47).

Examination of the analytical validation of bcl-2 as a cancer biomarker began with a pilot study done from Brazil in 2002. A research team evaluated the importance of p53 and bcl-2 levels in epithelial ovarian cancer, (p53 is a tumor suppressor protein). Tumor tissues from 90 patients were analyzed immunohistochemically for p53 and bcl-2 expression resulting in forty-two (47%) positive for p53 expression and 18 (20%) positive for bcl-2 expression. Positive expression for p53 was less frequent in stage 1 patients (22%) and no direct correlations could be demonstrated between p53 and bcl-2 expression and age or histologic grade. Thus, p53 and bcl-2 expression were not significantly correlated with overall survival, disease-free survival, or progression time (46). By 2004, clinical papers had been accepted for correlation of bcl-2 levels in a variety of mature peripheral B-cell tumors: B-cell chronic lymphocytic leukemia (B-CLL), diffuse large B-cell lymphoma (DLCL), multiple myeloma (MM) and B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (14). The work on elevated bcl-2 levels found in the urine of ovarian cancer patients at the University of South Florida provided the proper clinical validation providing essential impetus for the research in this thesis. In the study two cohorts provided blood and urine samples from women, the first consisting of 92: 19 healthy controls, 38 assorted benign gynecological disorders, 4 with early stage (EOC) and 31 with late stage ovarian cancer (LOC). The second consisted of

296: 58 healthy controls, 123 assorted benign gynecological disorders, 9 with EOC and 106 with LOC. Though it was not seen in the serum, perhaps due to the presence of confounding proteins, the elevated urinary Bcl-2 levels showed a direct correlation with presence of ovarian cancer (6). The average urinary levels of the Bcl-2 : Healthy:0.59 $^{ng}/_{ml}$ Benign:1.12 $^{ng}/_{m}$ EOC: 2.60 $^{ng}/_{ml}$ and LOC: 3.58 $^{ng}/_{ml}$ as seen in figure 2.6.

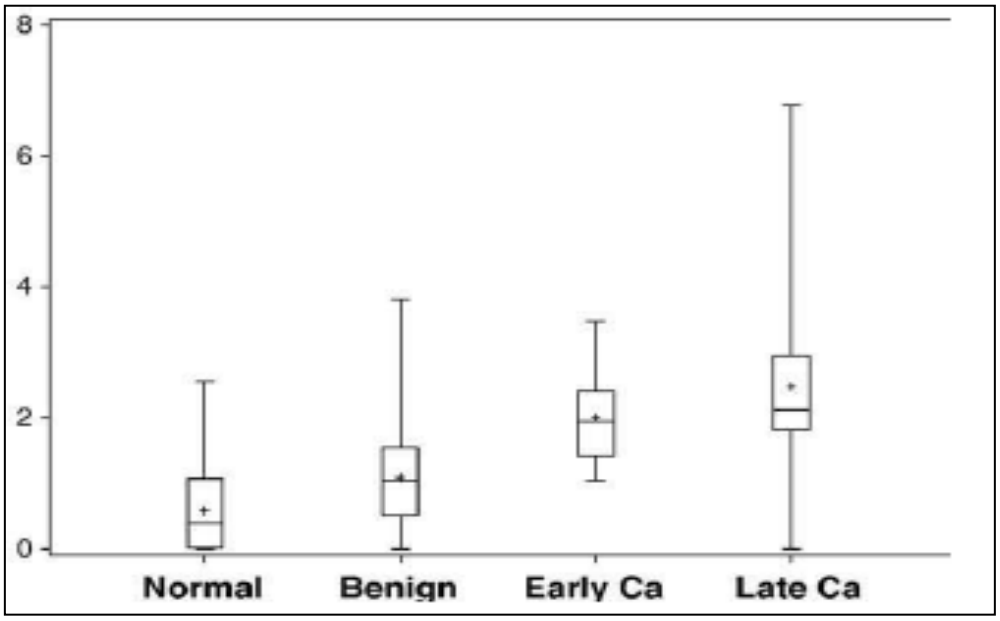

Figure 2.6 Urinary Bcl-2 Levels Elevated in Ovarian Cancer Patients. (6)

In addition, when compared to the CA125 levels which have been shown to be elevated in ovarian cancer (along with benign gynecological disease and hepatic disease), bcl-2 levels showed a favorable correlation. This not only provided further corroboration for Bcl-2 as a biomarker, but also allowed for dual proteomic screening for flawless ovarian cancer diagnosis. It was noted that bcl-2 did not suffer from the false-positives that plague CA125 screening and it was concluded that bcl-2 over-expression in bodily fluids that could result in a novel biomarker for ovarian cancer (6). This is the validation that we need to use the standard bcl-2 protein in this research as an analyte on functionalized surfaces to potentially be implemented in a POC diagnostic.

CHAPTER 3: REAGENTS USED TO FUNCTIONALIZE SURFACE

3.1 Bioconjugation

The technology of bioconjugation over the past decade, has influenced nearly every research branch of the life sciences. Bioconjugation involves novel molecular linking of two or more particles to construct a compound that has properties of its individual constituents. Simply, bioconjugation allows the molecular self-assembly of several reagents with varying properties for a desired purpose. Natural occurring biological systems are often the inspiration that loosely provide 'templates' for novel self-assembly. Biologically assembled compounds can be chemically amalgamated resulting in unique structures with carefully engineered properties. Today, bioconjugation has become a billion dollar industry spanning research in diagnostics, biomarkers and biosensors to name a few (48).

Bioconjugation techniques can be applied to modify the native conformations and overall functions of polysaccharides, nucleic acids, lipids, oligonucleotides, etc. By distinctively conjugating molecules to interact with target analytes in solution, bioconjugation has quickly become a staple of all biological assays in a clinical setting. As a matter of fact, anything that can be chemically derivatized, or isolated, can be conjugated onto inorganic silica or metallic substrates. Proteomic molecules are generally the most common targets for conjugation methods. Through deliberate modifications which allow for specific proteomic binding to a particular target molecule, structure and function of proteins can be elucidated. In this way, without the development of conjugated, labeled and modified molecules the majority of life science

research done today in a laboratorial setting would be impossible to achieve. Thus, molecular self-assembly provides this research with methods to isolate the bcl-2 protein in solution via specific binding to a polyclonal capture antibody which is immobilized due to linkaging of various reagents on glass cover slips.

The methods of molecular self-assembly employed in this research deals with supramolecular chemistry. The forces that directly order of the spatial organization of the self-assembly, are varied from weaker intermolecular (hydrogen bonds or van der Waals forces) to stronger covalent linkages. The study of non-covalent interactions is crucial to understanding many biological processes from cell structure to protein conformation that rely on these forces for organization and function (48).

A self-assembled monolayer (SAM) is the organization of head groups on an inorganic substrate by chemisorption, followed by dense organization of the tail or reactive groups (as seen in figure 3.1). The ability to customize both head and tail

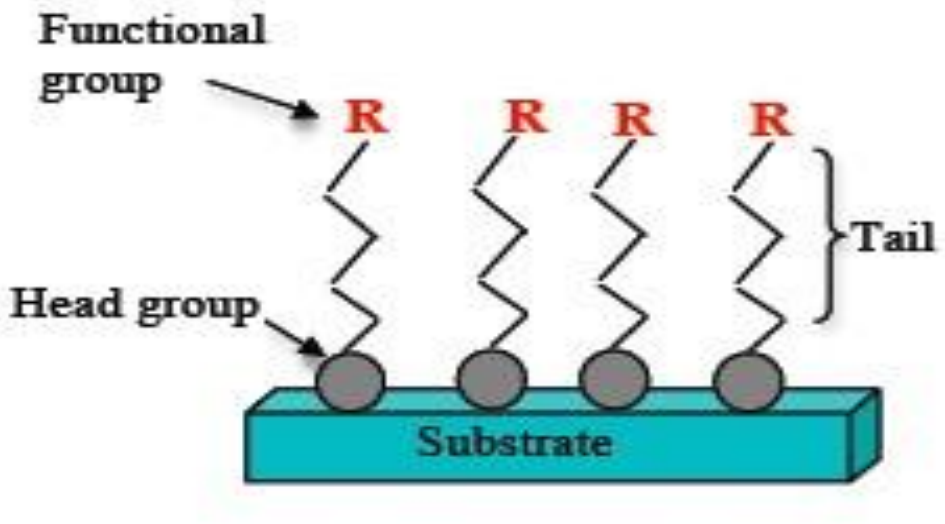

Figure 3.1 Self-Assembled Monolayer.

groups of the constituent molecules makes SAMs excellent systems for a more fundamental understanding of a wide variety of biological phenomena including disease etiology and pathology. SAMs are the precursor to a great deal of bioconjugation techniques as it allows the functional groups to be 'built upon' once the head groups are conjugated on the substrate. In 1946, Zisman published the preparation of a single monolayer layer of a surfactant by adsorption onto a clean metal surface (49). Though at the time the relevance of the research was not fully comprehended, this was the first formation of a monolayer by self-assembly and thereby laid the foundation for the fields of bioconjugation and supramolecular chemistry. Just as research in bioconjugation has made great strides as of late, the field of SAMs has witnessed tremendous growth in synthetic sophistication and quality of characterization. SAMs offer inimitable opportunities to expand fundamental understanding of self-organization, interfacial phenomena and receptor-ligand interactions. Independent control of surface structure and the respective chemical properties are not only scientifically stimulating, but technologically applicable. Since functionalization can occur on many different materials with a large number of molecular structures, the flexibility that SAMs allow for them to be a vehicle for investigation of specific interactions at potentially every biological-interface imaginable (50).

3.1.1 Molecular Self-Assembly Reagents Used

The novelty of this research lies in the fact that it is the first of its kind to attempt to isolate the bcl-2 protein using novel functionalized surface assemblies. Through manipulation of derivatized reagents to target the functional bcl-2 protein, we can compare the overall capture from the bioconjugated substrates with known urinary levels in ovarian cancer patients (6). In this chapter, the various bioconjugation reagents that

are used are introduced and their functional properties and desired functions are elucidated. Briefly, the process of oxygen plasma cleaning results in the dispersion of hydroxyl groups onto the surface, which serves as the proper initiation technique to attach a monolayer of an organofunctional silane on the substrate. This silanization process creates SAMs on the substrate and acts as a link to connect an otherwise inorganic surface with organic molecules, allowing for subsequent molecular assembly on the functional tail group. By linking the silane and a known immobilizer of antibodies, protein A/G, correct orientation of the bcl-2 antibody can potentially be achieved. Two methods are used for protein A/G recruitment: mimicking of hydrophobic *in vivo* binding sites of proteomic targets in our bodies by directly adsorb on the ODMS and secondly, use of amine-reactive homobifunctional reagents to crosslink onto the 3-APTMS (51). Lastly, a non-fouling surface must be maintained by introducing chains of (poly)ethylene glycol (PEG) and if adsorbed extensively, will allow the substrate to be resistant to non-specific binding. Through extensive research into assembly techniques using various reagents, five target surfaces are functionalized and subjected to novel ELISA assay quantification. All of these bioconjugations reagents will be explored in this chapter.

For the research to be diagnostically applicable, certain factors reveal themselves as more important than others. First of all the substrate must display highly specific capture of the bcl-2 analyte, via high affinity of a bcl-2 recognizing capture antibody. Secondly, since a diagnostic device can be rendered inaccurate if it provides false-positive results, the quantification of only bcl-2 must be achieved. By successfully introducing PEG layers onto the surface, background has been shown to be eliminated and a non-fouling surface, void of any non-specific binding, can be upheld (52). Three different PEGylation reagents are experimentally varied, to assess the efficacy of attachment on the surface. Moreover, the substrate with the highest capture of bcl-2 with the lowest background non-specific binding serves as the ideal bioconjugated

platform. Other relevant factors which potentially can affect the assembly, such as surface energy and wettability parameters are also varied in the experimental setup as an amine reactive hydrophilic silane (3-APTMS) is tested against a hydrophobic silane (ODMS). Once the head groups of each silane could be covalently linked, the functional tail allowed for bioconjugation on the substrate to orient the antibody (anti-bcl2) needed to target the analyte bcl-2 protein. To arrive at the most effective assembly method, a rigorous literature review revealed target reagents.

Antibodies are expressed as IgA, IgD, IgE, IgG and IgM and they each correspond to different sequence homologies and therefore different ligand binding which results in initiations of a multitude of signal transductions. In its four forms, IgG, provides the majority of antibody-based immunity against invading pathogens. It is well established that the "Y" shape of IgG has F_{ab} (antigen binding) and F_c (constant) regions, as seen in figure 3.2. Upright orientation ensures that maximum binding can occur with the desired analyte and confirms that the F_{ab} receptors are sterically unrestricted, increasing the desired proteomic binding. There are a number of immunoglobulin binding proteins which are used in biological research to purify, immobilize or detect immunoglobulins by binding their F_c domains which include: Staphylococcal Protein A, G and L (protein-A, protein-G and protein-L) (53, 54).

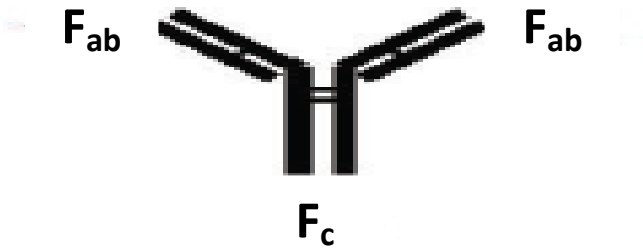

Figure 3.2 Antibody Structure.

Protein-A/G is a recombinant protein that combines IgG binding domains of both protein-A and protein-G ensuring maximum antibody recruitment. Protein-A/G contains four Fc binding domains from Protein A and two from Protein G. The binding of protein-A/G is less limited than Protein A which is of the dependent upon pH and has the additive properties of protein-A and protein-G. A great deal more about protein A/G is mentioned in chapter 3.3.1.

3.1.2 Test Substrates Chosen

To ensure that the surfaces displaying the highest efficacy of capture were arrived at, a variety of reagents were used (table 3.1). The commonly used amine crosslinking technique was employed in substrates 1-3 to covalently link the protein-A/G on the 3-APTMS SAM by targeting amine groups (51). Hence the proven amine-crosslinking method was experimentally tested against direct physical absorption of protein-A/G on hydrophobic surfaces. Subsequently, all of the substrates received a polyclonal anti-bcl2 IgG that would recruit the analyte protein. The final step in the functionalization was to ensure widespread integration of PEG chains on to the surface to maintain a non-fouling surface, thereby only allowing specific bcl-2 capture.

Table 3.1 Experimental Surfaces Chosen.

Substrate #	Silane Used	Orientation By	Capture Antibody	Surface Block
1	3-APTMS	Gluteraldehyde (GA)-A/G	anti-bcl2	$MS(PEG)_4$
2	3-APTMS	$BS(PEG)_5$-A/G	anti-bcl2	$BS(PEG)_5$
3	3-APTMS	$BS(PEG)_5$-A/G	anti-bcl2	$BS(PEG)_5$ $MS(PEG)_4$
4	ODMS	protein A/G	anti-bcl2	$MS(PEG)_4$
5	ODMS	protein A/G	anti-bcl2	Pluronic

Three PEGylation reagents were tested, two being amine reactive $BS(PEG)_5$ and $MS(PEG)_4$ and the third a triblock copolymer named Pluronic F127. Table 3.1 explores the five functionalized surfaces whose silanes, crosslinkers and PEGylation reagents displayed the most effective self-assembly. Commonalities between the surfaces include initial plasma cleaning and introduction of hydroxyl followed by a silane treatment, protein-A/G recruitment to orient the primary antibody and adsorption of a surface block with PEG layers to ensure prevention of non-specific binding. All of these reagents and their reactive chemistries will be discussed at greater lengths in later sections of this chapter. A schematic displaying reagent assembly on the 3-APTMS based glass substrates can be seen in figure 3.3 below.

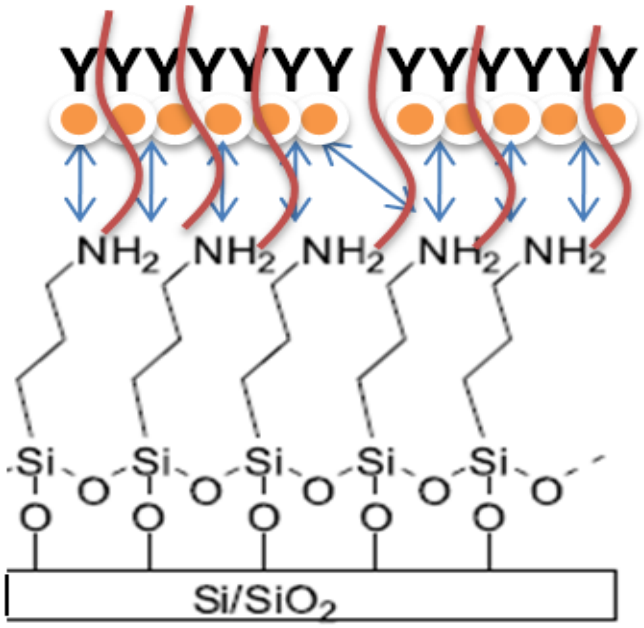

Figure 3.3 Schematic of 3-APTMS Surface Functionalization.

Substrate 1, 2 and 3 all employ the amine-crosslinking chemistry seen above with the blue arrows, with GA being used in substrate 1 and $BS(PEG)_5$ in substrate 2

and 3 to recruit protein A/G to orient the antibody. To avoid non-specific binding, substrate 1 and 3 link $MS(PEG)_4$ after the capture antibody and substrate 2 uses only $BS(PEG)_5$. The PEG chains can be seen with the red lines. Below, in figure 3.4, a schematic displaying reagent assembly on the ODMS based substrate can be seen.

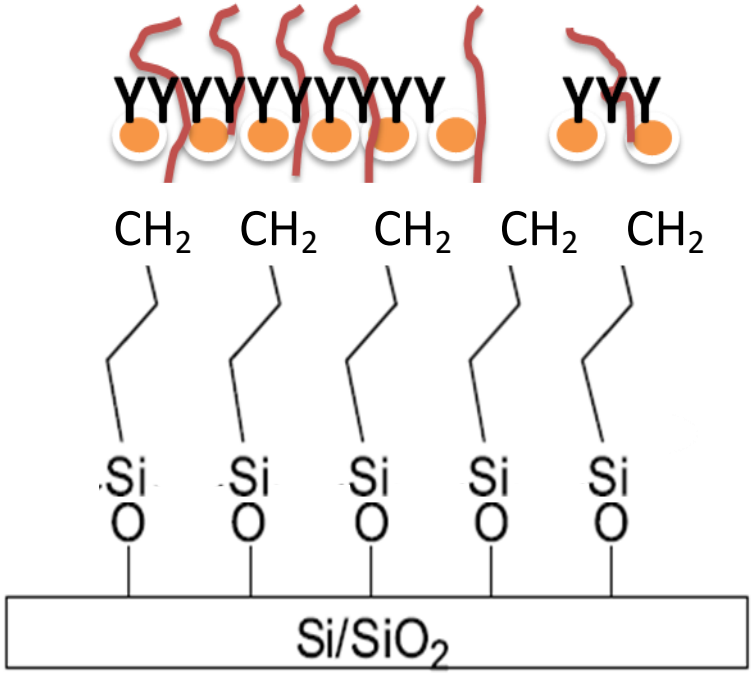

Figure 3.4 Schematic of ODMS Surface Functionalization.

Substrate 4 and 5 employ the direct physical adsorption of protein A/G (in orange) on the hydrophobic silane to recruit the capture antibody. Substrate 4 adsorbs $MS(PEG)_4$ on the surface and substrate 5 uses Pluronic to maintain a non-fouling surface after the antibody step (red chains).

3.1.3 Validation of Substrate: Contact Angle Measurements

Selection of the test surfaces was not as linear as simply choosing the reagents and randomizing the order for assembly on the substrate. Rather, once the most suitable reagents were arrived upon, an analysis on the surface properties was conducted through sessile contact angle measurements. This revealed the critical surface tension and adhesion properties, which allowed for investigation into the extent of effective binding occurring on the test surfaces. Gathering measurements at each step of the bioconjugation process and determining surface contact angles between the applicable silanes, homobifunctional crosslinkers and PEGylation reagents revealed if proper reagent coverage was occurring on the substrate. Since each reagent is expected to engender distinct fluctuations in the overall substrate surface energy, statistically relevant variations in contact angle measurements can perhaps indicate suitable reagent coverage. Thus, by identifying relevant changes in contact angle measurements, the validation of the techniques to recruitment protein A/G and PEGyation reagents on the 3-APTMS and ODMS substrates could be arrived at.

According to Gelest's handbook on hydrophobicity, hydrophilicity on silane surface modification, any surface with less than 30^{o} contact angle with water can be considered hydrophilic and greater than 90^{o} to be hydrophobic (51). This is will serve as a good parameter to discuss the findings, as the contact angle measurements were tested with deionized water (diH_20). A KSV CAM 101 contact angle measurement device was used to gather sessile contact angles of the glass substrates. Since the two major factors that need to be addressed in this research are efficient tethering of antibodies via protein A/G immobilization and elimination of non-specific binding via recruitment of PEGylation reagents, these two issues will be addressed by both the contact angle measurements as well as analysis of the chemistries of the various chemistries. Figure 3.5 display an analysis of the contact angles of an amine-crosslinker, GA, on both

silanes. It should be noted that all data has been statistically verified with a significance level (p-value) of 0.05 unless otherwise mentioned. Also, all of the contact angle measurements found in this research can be found in appendix C.

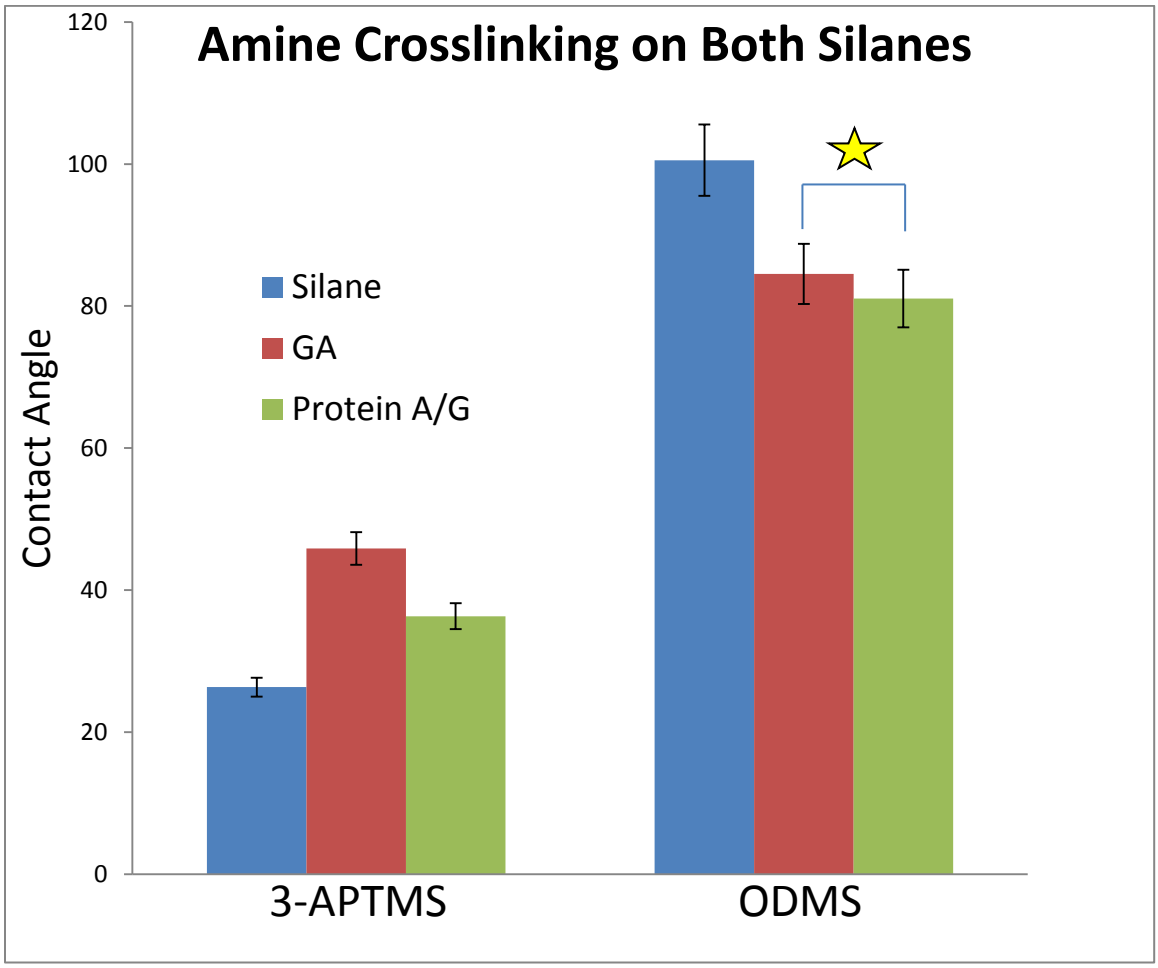

Figure 3.5 Efficacy of Amine-Crosslinkers to Recruit Protein A/G.

Contact angles obtained from both organofunctional silanes were as expected with the ODMS displaying hydrophobic properties and 3-APTMS being very close to published results (51). When adsorbing protein A/G on both silanes with the prior adsorbtion of GA: we see a statistically significant increase in hydrophilic behavior

(45.9 ^{o} vs. 36.3^{o}) for the 3-APTMS and statistically insignificant increase in hydrophilic behavior for the ODMS (84.525^{o} vs. 81.044^{o}). It can therefore be established that ODMS is an inefficient silane to adsorb an amine-crosslinkers to immobilize protein A/G upon. This conjecture is achievable because of the published research into GA being able to covalently link with amine groups (48, 53), present on protein A/G and 3-APTMS. As a result, the biological evidence of proteomic binding in hydrophobic settings was mimicked in the research to physically adsorbing the protein A/G on the ODMS (51). Consequently, direct physical adsorption of protein A/G on the hydrophobic ODMS will be tested against the introduction of amine-reactive homobifunctional crosslinkers, GA and BS(PEG)$_5$, on the 3-APTMS test assemblies to crosslink protein A/G. Following protein A/G all test substrates will be introduced to the polyclonal capture antibody.

The resilient linkaging of PEG layers onto the test substrates was also tested with the sessile drop technique. Consequently, the 3-APTMS with GA and A/G and the ODMS with A/G had all three non-fouling surfactants adsorbed and were examined for contact angle measurements. Since the PEGylation reagents increase surface wettability, (because of PEG repeat 'mers'), we can gauge the effective adsorption or covalent linkaging of the PEG chains on the surface by observing the smallest contact angle found on each substrate (55). Since an increase in surface wettability represents an increase in hydrophilic behavior, the reagent that can decrease the samples contact angle are of interest, especially for the ODMS because of its' innate hydrophobic properties. Figure 3.6 illustrates the effective recruitment of the three PEGylation reagents onto the 3-APTMS and the ODMS substrates.

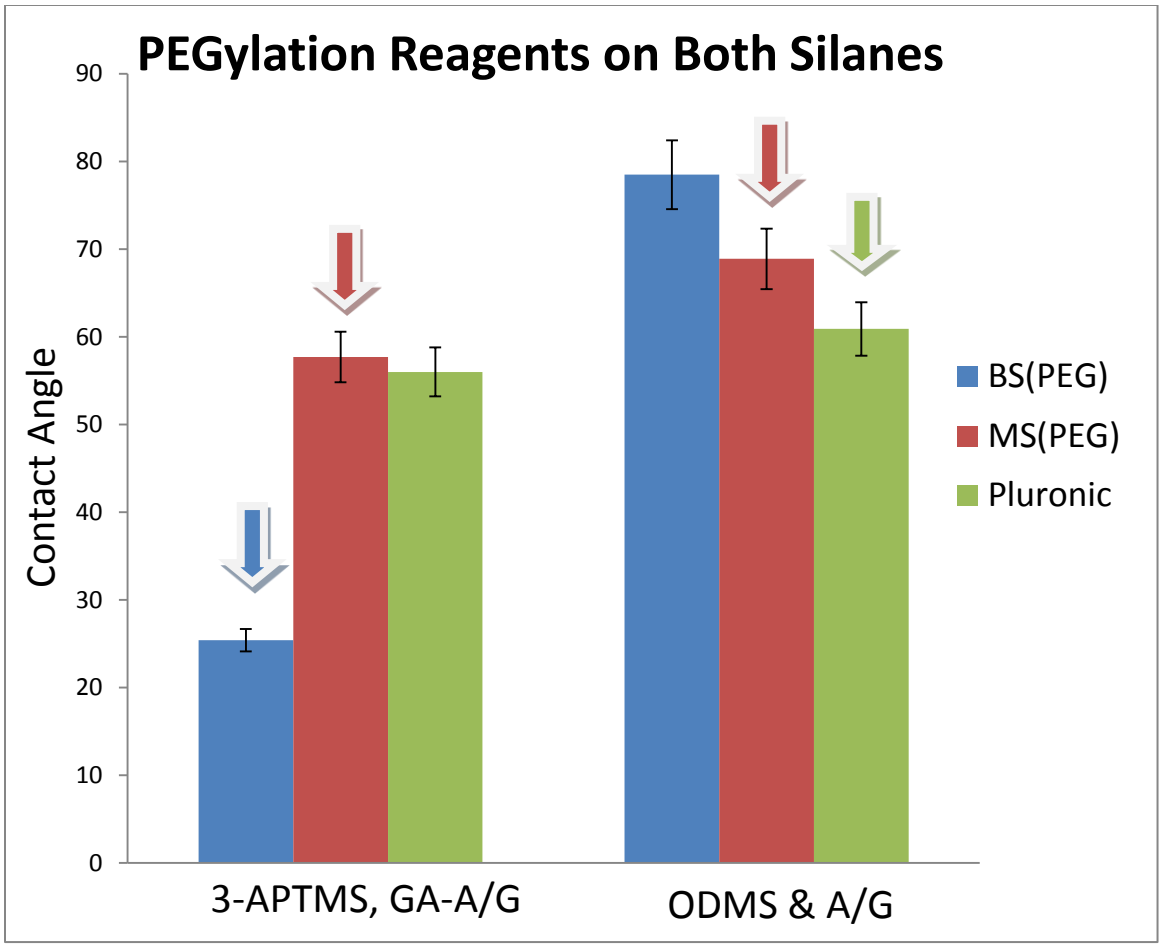

Figure 3.6 Efficacy of PEGylation Coverage on Both Silanes.

Covalent linking of the $BS(PEG)_5$ was efficiently done on 3-APTMS, which can be seen by the extremely hydrophilic behavior. This results were expected and this demonstrated that the $BS(PEG)_5$ is a versatile complex for amine-crosslinking as well as PEGylation on the silane. For this reason, it is used in two of the 3-APTMS assemblies, substrates 2 and 3. However, it did not increase the hydrophilicity of the ODMS to the extent that might be expected. Since ODMS does not introduce amine groups on the surface, the amine reactive *N*-Hydroxysuccinimide (NHS) ends of $BS(PEG)_5$ were perhaps not be linked to the surface. Furthermore, due to its short length and hydrophilic nature, it is unlikely to adsorb this reagent to the hydrophobic ODMS.

The $MS(PEG)_4$ has four repeat PEG 'mers' with an amine reactive NHS ester on one end and an unreactive hydrophobic methyl group on the other. When attached to the surface a 'brush' of PEG chain is formed. For the 3-APTMS, the hydrophobic methyl group present at the free end of $MS(PEG)_4$ influenced the contact angle and may be the reason for the increase in hydrophobicity compared to the $BS(PEG)_5$ (57.7^o vs. 25.4^o). There is also a statistically relevant difference in contact angle for the ODMS substrates with $MS(PEG)_4$ compared with $BS(PEG)_5$ representing enhanced coverage perhaps because of inherent affinity of the silane to the methyl-terminated tail of the $MS(PEG)_4$. For this reason it is employed in substrate 4 to test the value of the PEGylation reagent.

Finally contact angle measurments were taken for the Pluronic F127, which is a tri-block copolymer composed of a central hydrophobic chain of poly-propylene oxide (PPO) enclosed by two hydrophilic PEG chains. Pluronic resulted in the highest surface energy for the ODMS representing the reagent's worth for PEGylation coverage on hydrophobic substrates (55). Conversely, for 3-APTMS we see an increase in hydrophobic behavior with adsorption on the surface or a decrease in surface energy. Interestingly, there is not a statistical difference between the measurements found from the 3-APTMS assembly with Pluronic and $MS(PEG)_4$ (56.0^o vs. 57.7^o). A potential explanation for this is that the PEG chains are highly attracted to the hydrophilic 3-APTMS surfaces, causing inverse binding of the Pluronic with the hydrophobic PPO section to be oppositely oriented. Further insight into this as well as a figure of Pluronic is found in section 3.4. The variability of the validated reagents' bioconjugation upon the two distinct organofunctional silanes can be seen on the next page in figure 3.7

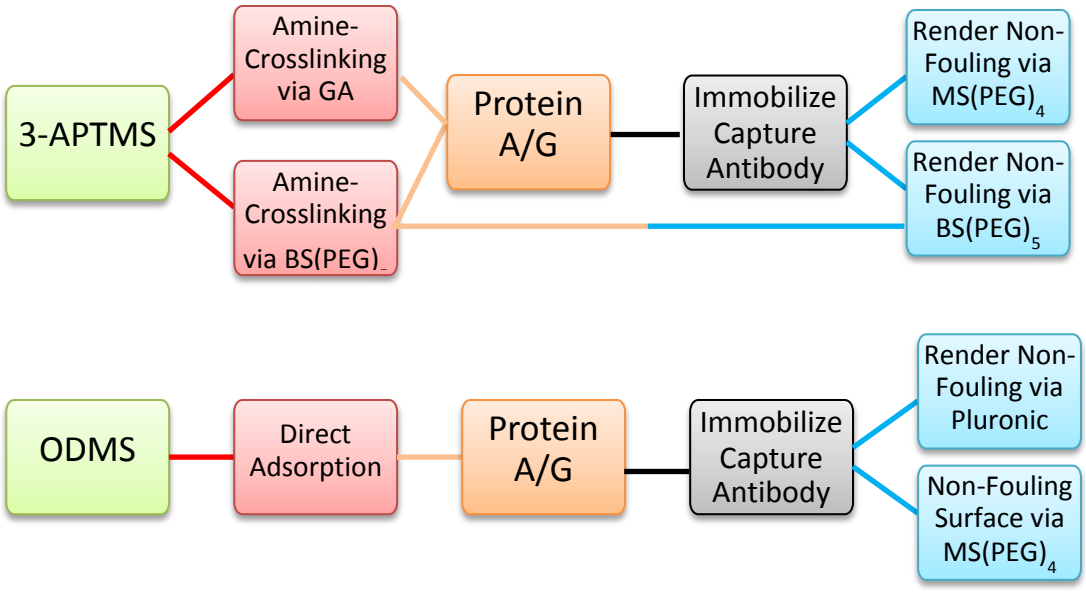

Figure 3.7 Variability of Reagents on the Test Substrates.

3.2 Organofunctional Silanes

The linking between inorganic substrates and organic building blocks for bioconjugation is a key aspect of surface functionalization. It has been shown over the years that organosilanes are the most effective at creating widespread, tough covalent linkages or SAMs on a variety of metallic and silica surfaces. Organosilanes contain at least one silicon-carbon bond. The carbon-silicon bond is very stable and possesses a unique tendency in nature to form four covalent linkaging, thus being ideal for molecular self-assembly techniques. Organofunctional silanes are molecules carrying two different reactive groups on their silicon atom so that they can react and couple with inorganic surfaces (56). The value of organofunctional silanes as coupling agents was discovered in the 1940s, during the development of fiberglass-reinforced composite beams (50). Some common applications for silanes today are the windshield coatings by creation of

strong hydrophobic layer, as additives in inks, coatings and sealants to improve adhesion and in plastics/rubbers to allow for cross-linking. Many combinations are possible between the molecular constituents of silanes, however the only prerequisites is that the head group is hydrolyzable (such as alkoxy or chlorine), so it can build strong, tight bonds with solids. The large number of possibilities of hydrolyzable head groups (1-3) and organofunctional ends make silanes extremely adaptable to many applications in life science as well as nanotechnological research (57).

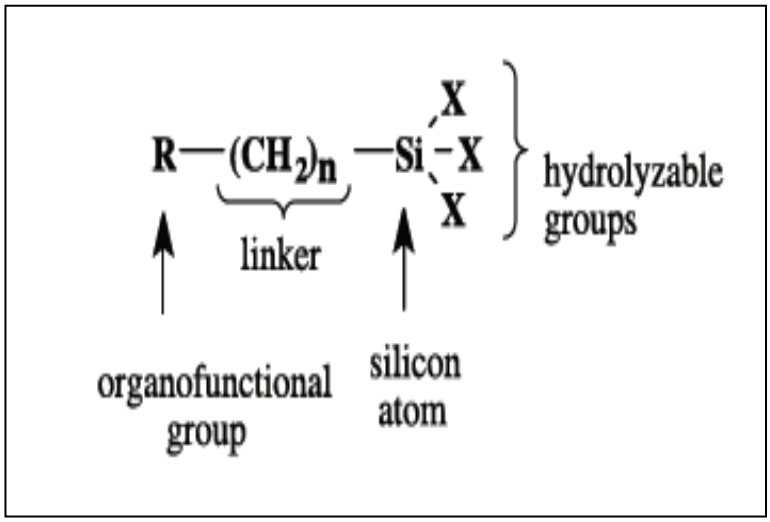

Figure 3.8 Chemistry of Organofunctional Silanes.

3.2.1 Chemistry of Silanization

Today, silanization is a commonly practice which uses biochemistry properties in novel methods for a wide variety of applications. Because of this, silanization is employed in a range of engineering fields for microfabrication, crosslinking and self-assembly. Most of the widely used organosilanes possess an organic substituent (tail groups) and one to three hydrolyzable substituents (head group) (56). This can be seen in figure 3.8, where the X's are the hydrolyzable groups and R is the organofunctional

group. It should be noted that silicon reacted with three alkyl groups are usually referred to as a silyl group. These hydrolyzable groups merge the linkage with the inorganic substrate in well-documented steps. The first step is the physical adsorbtion of the silane onto the silica substrate, where hydrolysis occurs of the hydrolyzable head groups. The majority of silanes have linking groups containing a silane binded to three actively saturated hydrogen-carbon molecules (trialkoxyl-silane). Formation of silanol (Si-OH) groups from the initial silyl groups occurs through hydrogen bonding. These silanol groups can then condense with each other to form the final monolayers with covalent siloxane bonds (Si-O-Si). The self-organization that ensues is driven by the van der Waals interactions amongst adjacent alkyl chains (51, 56).

The formation of covalent bonds to the surface ensures a certain amount of reversibility. Because of this silanes with three alkoxyl groups are widely used to crosslink inorganic substrates. Levels of hydroxylation on the substrate are also a factor when attempting to silanize. An important issue in controlling the effectiveness and overall surface properties is the linker length $(CH_2)_n$. Typical linker length is three carbon atoms which is suitable for inorganic-based molecular self-assembly (56). Because of the steric constraints imposed by inorganic surfaces, the linker length allows greater mobility of the organofunctional groups. Simply, longer linker lengths have shown to create dense SAMs which correspond to a larger surface area for bioconjugation. However, it has been found that excess in linker length (greater than 18 carbon atoms) in silanized SAMs causes mingling of chains and thus a high level of disorder (57). Depending on the application of the silane, a monolayer or multilayer adsorption may be desired. The thickness of a polysiloxane layer is regulated by the concentration of the siloxane solution and is also a factor which governs surface properties. It has been calculated that deposition from a 0.25% silane solution onto glass could result in three to eight molecular layers, which is suitable for creating a platform for bioconjugation (51).

3.2.2 3-Aminopropyltrimethoxysilane

3-APTMS is a common organosilane used with a wide variety for crosslinking molecular reagents especially in proteomic analysis because of the presence of the amine group in its functional tail (57). A figure of 3-APTMS (figure 3.9) shows that it has a molecular formula of $H_2N(CH_2)_3Si(OCH_3)_3$, density of 1.027 $^{g}/_{ml}$ and a molecular weight of 179.29 $^{g}/_{mol}$ (58). A schematic of 3-APTMS was retrieved from the Sigma-Alrich website. It can be seen that the hydrolyzable head consists of three methoxy groups, which create a highly polymerized SAMs on the substrate. 3-APTMS has a short organic 3-Amino(Propyl) group which terminates in a primary amine. This allows 3-APTMS to be react with GA-mediated linkaging, or other amine-related tethering. Essentially, any crosslinking reagent containing an amine reactive group can be used to modify the hydrophilic silane surface. The silane can be diluted in any anhydrous solvent such as toluene, in preparation to be covalently linked to inorganic substrates. 3-APTMS will be linked to amine-reactive homobifunctional crosslinkers (chapter 3.3), in hopes of efficiently tethering of protein A/G. It should be noted that the 3-APTMS surface may contain some thick hydrophobic patches due to the random polymerization and may contain unreacted methoxy groups, which can lead to increased background via nonspecific interactions with untargeted protein molecules (59).

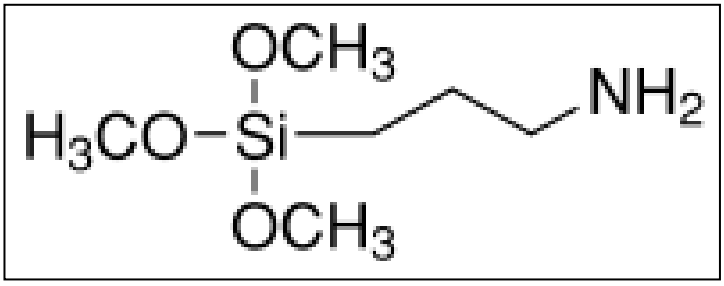

Figure 3.9 3-APTMS Structure. (58)

3.2.3 Chlorodimethyloctylsilane

ODMS has a molecular formula of $CH_3(CH_2)_7Si(CH_3)_2Cl$, density of 0.836 $^{g}/_{ml}$ and a molecular weight of 179.29 $^{g}/_{mol}$ (60). A schematic of ODMS was retrieved from the Sigma-Alrich website (figure 3.10). It can be seen that the ODMS methyl groups (CH_3), both to crosslink to the substrate and as organofunctional tail to allow for molecular self-assembly to occur. However ODMS only has one hydrolyzable groups, two less than the highly reactive 3-APTMS. Intuitively, this may seem to be detrimental to the successful functionalization on the inorganic surface. However, it was noted that the lack of hydrolyzable groups may have favorable effects on the SAM organization. This was determined by the overall hydrophobic characteristics of the substrate (roughly 101^{o}) as well as clear uniform deposition on the surface, because of the presence of a single hydrolyzable Si-Cl bond. Whereas the 3-APTMS substrates appeared semi-transparent, due to unregulated silanol formation, ODMS substrates remained clear indicating well-defined, tight monolayer dispersion. Thus, the sole hydrolysable group on the ODMS provided a consistent, homogenous base for subsequent bioconjugation to occur. Because of this the ODMS could be concluded as the more reproducible silane of the two.

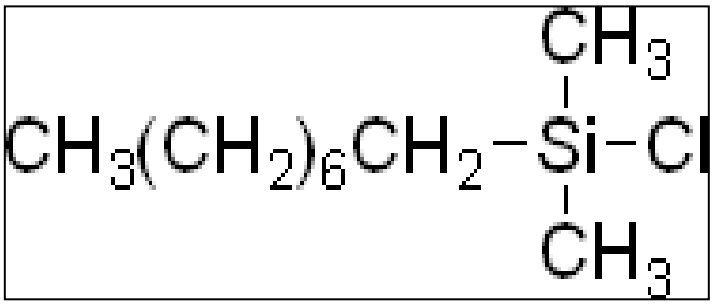

Figure 3.10 ODMS Structure. (60)

3.2.4 Methods and Materials for Plasma Cleaning and Silanization

Deposition of silanes from aqueous alcohol based solutions is the most facile method for preparing silylated surfaces on inorganic substrates and this method will be employed in this research. Before silanization and bioconjugation on the square glass slips, they must be thoroughly cleaned and have hydroxyl dispersion on the surfaces. To accomplish this they were subjected to five minutes of plasma treatment in the PE-50 benchtop plasma cleaner,. The square glass substrates were purchased from Bellco (catalog number 463109, model number 1916-09009) and had a side length of 9 mm. Before entering the oxygen plasma cleaner, the glass cover slips were also thoroughly cleaned with nitrogen (N_2). The silanes were diluted in anhydrous toluene, which was purchased from Sigma Aldrich (product number 244511). The plasma cleaner successfully removes any organic materials and oxidizes the surface, providing high density of hydroxyl (-OH) groups for silane reactions to occur.

3-APTMS was purchased from Sigma Aldrich (product number 281778). Anhydrous liquid phase deposition was done by dissolving the silane in toluene. Toluene was purchased from Fisher Scientific (product number T324). A working molar concentration of 0.1 $^{Moles}/_{Liter}$ is desired in toluene, to covalently link the silane to the inorganic substrate. Since the molecular weight for 3 -APTMS is 179.29 $^{grams}/_{mole}$ and the density is 1.027 $^{grams}/_{mole}$; the volume of silane needed can be calculated. Sample calculation can be seen below for diluting a total of 20mL of 3-APTMS to 0.1M.

$$\text{mass of 3APTMS} = (\text{total volume})(\text{molarity needed})(\text{molecular weight})$$

mass of 3APTMS = (.02L) (0.1 $^{M}/_{L}$) (179.29 $^{grams}/_{M}$)

mass of 3APTMS = 0.3586 grams

$$\text{3-APTMS needed} = (\text{mass of 3APTMS})/\ (\text{density of 3APTMS})$$

3-APTMS needed = (0.3586 grams)/ (1.027 $^{grams}/_{ml}$)

3-APTMS needed = 0.349 mL ≅ *349 µL*

After the samples have been oxygen plasma cleaned, they were careful deposited in the solution for a fixed duration of 1 hr at room temperature. After the hour, the samples were thoroughly rinsed with generous amounts of toluene then ethanol and subsequently blow-dried with N_2.

ODMS was purchased from Sigma Aldrich (product number 246859). Anhydrous liquid phase deposition was done by dissolving the silane in toluene. A working molar concentration of 0.1 $^{Moles}/_{Liter}$ is desired in toluene to covalently link the silane to the inorganic substrate. Since the molecular weight of the ODMS is 206.83 $^{grams}/_{Mole}$ and the density is 0.836 $^{grams}/_{Mole}$; the volume of silane can be calculated. Sample calculation can be seen below for diluting a total of 20mL of ODMS to 0.1M.

$$\text{mass of ODMS} = (\text{total volume})(\text{molarity needed})(\text{molecular weight})$$

$$\text{mass of ODMS} = (.02\text{L})\ (0.1\ ^{M}/_{L})\ (206.83\ ^{grams}/_{M})$$

$$\text{mass of ODMS} = 0.41366 \text{ grams}$$

$$\text{ODMS needed} = (\text{mass of ODMS})/\ (\text{density of ODMS})$$

$$\text{ODMS needed} = 0.474 \text{ mL} \cong 474\ \mu L$$

Post oxygen plasma cleaning, the samples must be carefully deposited in the solution for a fixed duration of 1 hour at room temperature. After the hour, the samples were thoroughly rinsed with toluene and ethanol and subsequently blow-dried with N_2.

3.3 Immobilization of Antibodies

Antibodies are an ideal tool that are used in variety of diagnostics, enzyme-linked immunosorbent assays (ELISA) and biosensors (59). The high specificity that antibodies display towards discrete proteomic targets essentially guarantees recruitment of target proteins, allowing for novel assays and protocols to be established in a variety of engineering, chemistry and biological fields. The term antibody and immunoglobulin are often interchangeably used as antibodies are glyco-proteins that belong to the immunoglobulin family. Of them, IgG is the most prevalent type of surface immunoglobulins. IgGs are thus the basis of affinity techniques to biologically activate specific complex formations. Because of this, not only does this allow for isolation and

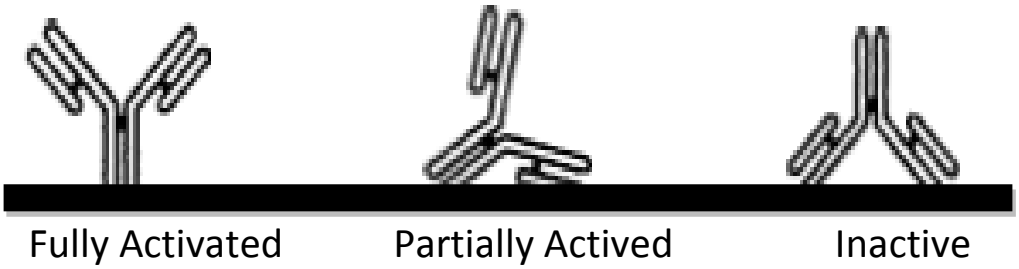

Figure 3.11 Upright Orientation of Antibody is Desired.

quantification of a multitude of biological markers such antigens, signal proteins, nucleotides, etc. but also allows for the study of signal transductions in relationship with the cell's microenvironment. Thus, with the rapid development of bioconjugation techniques, immobilization of antibodies has quickly become integrated with immunoaffinity chromatography, immunosensors, optical fibers and diagnostic immunoassays to name a few current applications (61). There are two primary techniques to immobilize antibodies on a desired surface: direct chemical immobilization methods which covalently links the IgG on the surface and physical methods of

immobilization such as adsorption (62). In chemical immobilization, functional groups on the surface of the desired protein, such as amino and carboxyl groups are targeted. However, when antibodies are directly adsorbed to solid supports, the specific binding capacity is usually less than that of a soluble antibody. The primary reason for this reduced activity is that the random orientation of the antibodies on surfaces, masking some of the antigen binding sites (figure 3.11). In fact, conventional direct adsorption of antibodies can potentially denature and reduce proteomic functional sites by more than 90%, as seen in figure 3.11. A common strategy to covalently immobilize antibodies on surfaces utilizes the free amines of lysine residues. Unfortunately, this approach also results in random antibody orientation because numerous lysines are present in antibodies (63). This can inevitably lead to low detection sensitivity and signal-to-noise ratio, which are two factors that must be avoided in this research. Antibodies are considered properly oriented and completely active when immobilized by the F_c fragment since this allows the F_{ab} fragments, or biologically active regions, to be sterically unrestricted and hence able to maximally bind antigens. Thus the key in immobilization of antibodies to recruit target proteins is the proper upward orientation of the active F_{ab} segments (61-64). With the advances in molecular self-assembly techniques and the knowledge of reagents with strong affinity to F_c recruitment, a methodology shift in orienting antibodies has occurred in the past ten years from direct to chemical adsorption. Today, protein A and protein G are commonly employed as tools to bind the F_c domain of antibodies, with affinities that vary with species and immunoglobulin type. However, they provide a tool that can ensure sterically unrestricted binding to antigens via upright orientation (54,62). In the following sections, the affinities of protein A and protein G binding to F_c regions in antibodies are explored. There will also be a discussion about the homobifunctional crosslinking chemistry that are employed in

tethering protein A/G. Two separate methods to immobilize protein A/G and the capture antibody are used on the 3-APTMS and ODMS test substrates.

3.3.1 Protein A/G

Protein A was first used 1978 to bind to an antibody whereas almost a decade later in 1989, protein G was similarly used to link to a target antibody. Since that time, both proteins have both been demonstrated to be powerful affinity ligands to recruit, or purify specific antibodies because of their high specificity to bind F_c regions of IgGs (65, 66). Today, these proteins are included when oriented immobilization of an IgG is needed in bioengineering, biochemistry, medical studies and organic chemistry. The protein A molecule contains four high-affinity (Ka = 10^8/ mole) binding sites capable of interacting with the F_c regions from IgGs of several species including human and rabbit. Optimal binding occurs at pH 8.2, although binding is also good at neutral, physiological conditions (pH 7.0-7.6). The disadvantage of protein A lies in the fact that only certain subclasses of immunoglobulins are able to be recruited (67). In comparison, protein G binds with significantly greater capacity than protein A to several IgG subclasses such as human IgG_3, mouse IgG and rat IgG. Differences in binding characteristics between protein A and protein G may be elucidated by the dissimilar compositions in the F_c binding sites of each protein. The tertiary structures of these proteins are very similar, however their amino acid compositions significantly differ which contributes to their different affinities (62,68).

Recombinant fusion of the F_c-binding domains of protein A and protein G has resulted in production of a functionally chimeric protein A/G with broader binding than either of its primary constituents alone. Binding is less pH-dependent than both protein A and protein G occurring efficiently at pH 5-8. The enhanced F_c-binding properties of

protein A/G make it a popular tool in the investigation and purification of antibodies. Since protein A/G binds to all human IgG subclasses, IgA, IgE, IgM and to a lesser extent IgD it is an excellent tool for detection of mouse monoclonal antibodies from IgG subclasses without interference from these other serum proteins. Individual subclasses of mouse and rabbit monoclonal antibodies are most likely to have stronger affinity to this chimeric protein than to either protein A or protein G (69).

It should be noted that interactions of enzymes and amino acid *in vivo* occur at hydrophobic interfaces (51,59). A combination of this and contact angle data which indicated that adsorbtion of protein A/G on the hydrophobic silane occurs, allows for the conclusion that recruitment of protein A/G can be directly linked to hydrophobic SAMs. This facile assembly will be discussed at greater lengths in the next chapter.

3.3.2 Amine-Reactive Homobifunctional Crosslinkers

A homobifunctional crosslinker can be described as any reagent which has the capability of covalent linkage between amine-containing molecules (48). When a complex has two, identical amine-reactive groups it is called homobifunctional and the two specific crosslinkers used in this research are GA and $BS(PEG)_5$. GA can be present in at least 13 different homologous forms and all demonstrate the ability to network proteins, leading to an extensive range of possible bioconjugates. The crosslinking of proteins, either to an inorganic substrate or between protein molecules, involves lysine residues of the ε-amino group. All of these forms of GA might be reactive toward lysine residues of proteins and this unique characteristic is why GA is considered one of the most effective amine-reactive crosslinking reagents (70). In the first test-assembly, GA is covalently linked to the surface since it is amine-reactive and successively recruits protein A/G's lysine residue (48). $BS(PEG)_5$ is a PEGylation reagent with reactive ends of *N*-hydroxysuccinimide, or simply NHS esters. These NHS esters react specifically

with lysine and N-terminal amino groups at pH 7-9 to form stable amide bonds (71). Because $BS(PEG)_5$ can double as both an amine-reactive crosslinker as well as a PEGylation reagent, this versatile reagent is employed in the second and third assemblies to immobilize protein A/G as well maintain non-fouling surfaces. A great deal more about the characteristics of non-fouling surfaces will be discussed in section 3.4. Because of the lack of a target amine groups in the ODMS structure, it should be noted that the amine-reactive crosslinking reagents can only be applied on the 3-APTMS based substrates.

3.3.3 Methods and Materials: Protein A/G, GA and Capture Antibody

Since GA is a reactive crosslinker in the presence of an amine group, it will be directly adsorbed onto one of the three experimental samples which possess the hydrophilic silane (substrate 1). The other two will employ the $BS(PEG)_5$ as a crosslinker while using it as a primary surface-blocking polymer brush (substrate 2) and a secondary surface block (substrate 3). The assemblies of these two surfaces will begin with the adsorption of $BS(PEG)_5$, subsequent to the silanization, followed by the attachment of protein A/G to immobilize the primary polyclonal antibody. The ODMS, with an active methyl-monolayer, will be subject to direct adsorption of protein A/G mimicking native hydrophobic interactions amongst proteins, as mentioned before.

Gluteraldehyde was purchased from Fisher Scientific (product number G151). The known amine, cross linking reagent can be diluted in ethanol to a working percentage of 5% $^{weight}/_{volume}$. (If the stock solution of GA is at 25% concentration: to prepare 25 ml, 5 ml of GA must be combined with 20 ml of solvent). However to ensure maximum amine linkage of the amino-silane, a physiological pH of 7.4 is desired (48). To raise the pH levels of the GA solution a few drops of 1 N sodium hydroxide should be adequate. 1 normal (N) solution of sodium hydroxide can be understood as the

molecular weight, 40 grams, in 1 liter of distilled water. The sodium hydroxide was obtained from Fisher Scientific. Samples were submerged in the solution for a fixed time of two hours at room temperature. Subsequently, the samples were generously rinsed with ethanol and blow dried with N_2.

Pierce Recombinant Protein A/G (product number 21186) was first made into a stock solution with a concentration of 1M ($1\ ^{mg}/_{ml}$). The solution was made by combining the vial of protein A/G, 5mg, with 2.5 ml of glycine and 2.5 ml of Delbecco's Phosphate Buffer Solution (DPBS). Both the glycine and DPBS were purchased from Invitrogen. Since the total volume of stock 1 M Protein A/G is 5 ml, ten aliquots of 500 µl were portioned and stored at -20 C to maximize longevity of the mixture. A working solution of 1 mM ($1\ ^{\mu g}/_{ml}$) was then prepared by diluting the stock solution in DPBS. For example, 10 µl of stock Protein A/G was diluted in 10 ml of DPBS. Substrates were incubated in the solution for a fixed time of 15 minutes at room temperature. Afterwards the substrates were thoroughly rinsed with and stored, if needed, in DPBS at 4° C.

The polyclonal anti-bcl-2 IgG derived from rabbit was purchased from Sigma Aldrich (product number PRS3337) at a concentration of $1\ ^{mg}/_{ml}$. The initial antibody was aliquot into ten equal portions of 10 µl and stored at -20° C to ensure longevity. Upon use, the antibody needed to reach a working concentration of $5\ ^{ug}/_{ml}$ in DPBS (as suggested by Sigma-Aldrich). For antibody immobilization on each substrate, 50 µl of working solution, for example if 30 samples are desired: 1500 µl of DPBS and 7.5 µl of stock. To apply the antibody on one side of the glass substrate 50 µl of the working solution is placed on parafilm (purchased from Cole Parmer). Because of the hydrophilic properties of the film the solution agglomerates. Slides should be carefully deposited on top of the drop of solution for a fixed time of an hour at room temperature. Afterwards, the slides should be thoroughly rinsed in DPBS.

3.4 PEGylation Reagents for Protein Resistant Surfaces

A primary concern for this research is functionalizing non-fouling properties onto bioconjugated surfaces. A non-fouling, or protein-resisting surface is a key characteristic of many biomaterial and bio-nanotechnological surfaces (72). In assays looking to recruit analyte proteomic markers from urine or serum samples it must be certain that there is minimal non-specific binding with other proteomic molecules which would bias the data. In a diagnostic setting, the threat of false-positive results has provided a great deal of motivation to study a multitude of non-fouling surface techniques in a wide variety of fields. The most common approach to reduce protein adsorption on a surface is via a coating of poly(ethylene glycol) (PEG) chains. Studies over the last few decades have shown that surface modification with long-chain PEG chains (about 2,000 Daltons and above) can drastically reduce protein adsorption (52,55,72). As a result, a number of methods to immobilize PEG-layers onto surfaces have been developed that include physisorbtion and covalent linkaging onto the surface of interest. The large majority involve the deposition of the PEG layers onto a surface from solution (73). Amine-reactive PEGylation reagents have become very popular to for a variety of protein modifications including tethering of proteins and in this case recruitment of protein A/G. As mentioned in section 3.3.2, $BS(PEG)_5$ is a PEGylation reagent terminated on both sides with amine-reactive NHS esters allowing for protein crosslinking. On the otherhand, $MS(PEG)_4$ can be considered a unifunctional PEGylation reagent with reactive NHS esters one end of a PEG chain (figure 3.12). The third PEGylation reagent that was used was a tri-block copolymer named Pluronic F127. Pluronic, as seen in figure 3.12 below, contains a central hydrophobic poly(propylene-oxide) (PPO) chain surrounded by two PEG chains (note: for over 50 repeat units of ethylene oxide termed PEG, otherwise PEO). Intuitively, the protein resistance is directly dependent on the

adsorbed amount of Pluronic, and since the PPO is responsible to immobilization onto the surface anything greater than 30 chains have been shown to be easily linked onto hydrophobic surfaces (52). A large number of Pluronic species exist with the initial letters corresponding to the physical state and subsequent numbers defining the weights of the PPO and PEG chains. Pluronic F127 contains 56 repeat units of PPO flanked by two 101 repeat units of PEG. It has been clinically demonstrated that surfactants with longer PEG chains will be better at resisting proteins, if they can be successfully adsorbed on the surface (74). The Pluronic reagent is important in this study because it indirectly measures the efficacy of adsorbed PPO chains versus covalent amine-reactive NHS esters, to immobilize PEG chains. The purpose of including a variety of PEGylation reagents is to arrive at the reagent which not only powerfully attaches to the surface but can also disperses the thickest PEG brush on the test substrates.

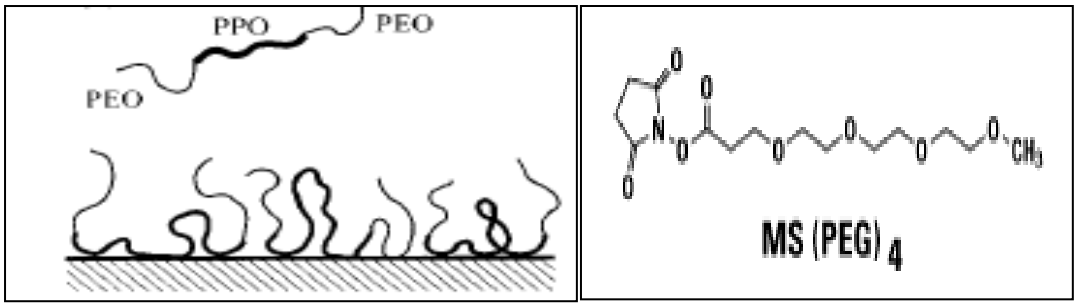

Figure 3.12 Pluronic and MS(PEG)$_4$ Structure. (75)

3.4.1 Methods and Materials: $BS(PEG)_5$, $MS(PEG)_4$ and Pluronic

For all of the experimental substrates, the PEGylation reagents were adsorbed after the introduction of the primary antibody, at the end of the surface assembly protocol. The only discrepancy is for substrate 3 which contains both the $BS(PEG)_5$, after the silanization, and similarly ended with $MS(PEG)_4$. The functionalized hydrophobic surfaces employed Pluronic for substrate 5 and $MS(PEG)_4$ in substrate 4. Although greater capture would inevitably have been seen with adsorption of the analyte bcl-2 standard directly after the primary antibody, the research looked to emulate the technique the functionalized surface would implement within a diagnostic device, which would need to introduce the analyte subsequent to full assembly.

$MS(PEG)_4$ was purchased from Thermo Scientific (product number 22341) and must first be made into a stock solution at a concentration of 250 mM. This was accomplished by mixing 1100 µl of dimethyl sulfoxide (DMSO), or any other polar aprotic solvent, with the 100 mg vial of $MS(PEG)_4$ to create a stock solution. The solution was then aliquot into 10 equal vials of 120 µl and stored at -20° C to ensure longevity. Upon use, a solution of 1mM in DPBS was used (as prescribed by the manufacturer). For example in 20ml of DPBS 80 µl is needed. Substrates were deposited in the solution for a fixed time of 15 minutes at room temperature. Afterward, the samples were liberally rinsed with DPBS. Samples were stored in DPBS at 4° C if necessary.

$BS(PEG)_5$ was purchased from Thermo Scientific (product number 21581). A stock solution must first be prepared combining the 100mg mass of the $BS(PEG)_5$ with 650 µL of DMSO. The 250 mM stock solution was then aliquot into seven vials of 100 µL and stored at -20° C. Upon use, a solution of 1mM in DPBS was required (as prescribed by the manufacturer). For example in 20ml of DPBS 80 µl of $BS(PEG)_5$ is needed. Substrates were deposited in the solution for a fixed time of 15 minutes at room

temperature. Afterward, the samples were liberally rinsed with DPBs. Glass slips were stored in DPBS at 4° C if necessary.

Pluronic F-127 was purchased from Sigma Aldrich (product number P2443). F127 implies $3600^{g}/_{mol}$ molecular weight of the central poly(propylene glycol) and 70% polyethylene glycol chains content. The letter 'F' infers its solid nature and to create a working solution it was diluted in deionized water to 1% $^{weight}/_{volume}$ (55). The Pluronic solution was given 12 hours to settle and the slides were incubated for a fixed time of an hour at room temperature for both procedures.

CHAPTER 4: NOVEL ELISA TO ASSESS EFFICACY OF BCL-2 CAPTURE

4.1 Sandwich ELISA Technique

After an understanding regarding the various bcl-2 test surfaces has been established and the bioconjugation reagents have been chosen, the method for testing the analyte capture will be fully revealed. In this chapter, the intricacies of bcl-2 standard recruitment, quantification and selection of the best test substrates are all clarified. Once the most suitable surface was identified a serial dilution of bcl-2 concentrations not only confirmed the overall sensitivity of the bioconjugated surface, but also allowed for comparison against published concentration of early stage and late stage ovarian cancer. Therefore, an ovarian diagnostic testing platform is revealed in this chapter.

The overall capture cannot simply be measured in a one-step quantification process. Rather novel assay protocols must be established to test the efficacies of these substrates. ELISA, or enzyme-linked immunosorbent assays have long been employed in clinical settings in a wide variety of fields to be a diagnostic tool in measuring the presence or concentration of an analyte in a complex solution (76). Though today some ELISA protocols might not necessarily be ‘enzyme-linked’ the general principles and techniques are similar and thus can be grouped into the same broad category as ELISAs. Before the development of ELISA techniques the only real option for conducting an immunoassay was through radioactively-labeled target antibodies. Quantifications for radioimmunoassays are somewhat accurate; however they pose a potential health risk. In 1971, a safer alternative was published by researchers at Stockholm University in Sweden which employed the knowledge of

discrete antibodies to recruit analytes which could be enzymatically traced through signals. A common signal is an enzymatically responsible change in color in the presence of clinically tested receptor-ligand bindings (76). Because of the multitude of receptor-ligand interactions *in vivo*, the ELISA method was quickly employed for *in vitro* analysis of concentrations of analytes in all life science and engineering fields. Performing an ELISA involves at least one capture antibody which has affinity for a particular proteomic target. Briefly, a complex solution with an unknown concentration of an analyte is exposed to a detection antibody. A secondary antibody, which has affinity for the detection antibody as well as an enzyme, then converts the substrate into a visible signal thus indicating a quantifiable reading of the concentration of the analyte (77). A more comprehensive 'sandwich ELISA' method is employed in this research to assess the bcl-2 capture on the substrate and can be seen in figure 4.1.

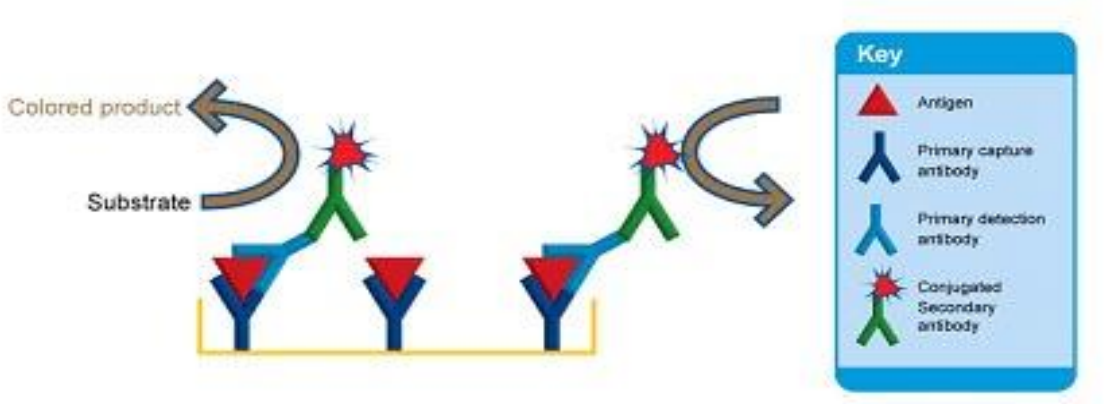

Figure 4.1: Sandwich ELISA Quantification Process.

In the novel sandwich ELISA method employed in the research, the idea was to quantify the bcl-2 concentrations immobilized by the capture polyclonal antibody (dark blue) by analyzing the levels of targeted mouse monoclonal anti-bcl2 (light blue) and alkaline phosphatase IgG (green) levels. Finally, an enzymatic reaction, mediated by

the secondary detection antibody (in green) converts the substrates to a product with unique optical properties which can then be measured at a specific wavelength by any plate reader with spectrometer capabilities.

The analyte in this assay is a recombinant bcl-2 protein standard which was purchased from R & D systems. Though natural bcl-2 contains a carboxyl-terminal, mitochondria targeting sequence not present in the recombinant bcl-2: both are able to maintain the ability to neutralize their fellow pro-apoptotic bcl-2 family members (78). Therefore for the research at hand, the recombinant bcl-2 is functionally identical to the anti-apoptotic protein that is elevated in ovarian cancer. The primary detection antibody is a mouse antibody which targets one site on the bcl-2 protein and was purchased from Santa Cruz Biotechnologies to initiate the novel ELISA protocol (79). The enzymatic antibody that was used was an alkaline-phosphatase IgG which was obtained from Jackson Immunology. The para-Nitrophenylphoshate (pNPP) liquid which turns yellow in the presence of the alkaline phosphatase enzyme was purchased from Sigma-Aldrich. When in the presence of alkaline phosphatase IgG, phosphoric acid (H_3PO_4) and the important para-Nitrophenol ($C_6H_5NO_3$) a soluble end-product is produced. This para-Nitrophenol is reason for the yellow color in the reaction, allowing measurements at 405 nm which corresponds to the yellow wavelength. This then revealed the concentrations of analyte on the substrate. Ideally, only the detection of bcl-2 should be measured

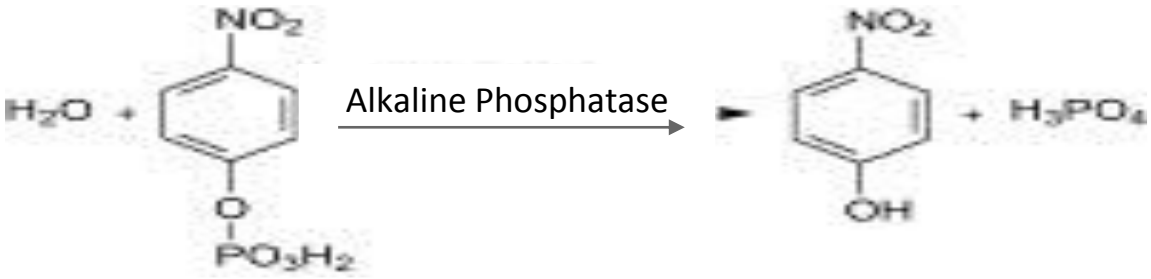

Figure 4.2 Enzymatic Alkaline Phosphatase Reaction.

by the specific, direct binding to the antibody on the surface. However, often indirect interactions or non-specific binding occurs on the surface by competitive receptor-ligand binding. It is a challenge in any ELISA protocol to avoid inflation of the data that occurs because of non-specific binding. This non-specific binding that potentially occurs is often referred to as background and in a diagnostic setting it is vital to minimize background. The ELISAs were able to be executed with and without the presence of the analyte of the non-specific interactions could be quantified on each surface.

The BioTek Synergy HT plate reader was used to read the output yellow color at 405nm through endpoint analysis. When absorbance measurements are made, the instrument switches to a Xenon Flash Lamp and a monochromator for wavelength selection. The use of a xenon flash lamp allows for both UV and visible light absorbance measurements. The monochromator provides wavelength selection from 200 to 999 nm in 1 nm increments and offers repeatability of 0.03% and reads a 96-well plate in 14 seconds (80). The output units of the reading are expressed in optical density of relative absorbance units (AU). The governing equation that is employed by the device to provide a reading follows.

$$\text{Absorbance Units}_{405nm} = \log_{10}(I_o/I) \quad (1)$$

When the intensity of the light before it enters the sample is divided by the intensity of the light at a specific wavelength, in this case 405 nm, the transmittance of a sample can be found. Taking the $\log_{10}$ of the transmittance the relative AU can then be found. As far as the logarithmic AU scale is concerned: a reading of 1 can be understood as the ten-fold reduction in optical light density and a reading of 3 is a thousand-fold reduction in optical light density (81). This method of analysis is commonly used in ELISA because of the ease of operation of a monochromator and the. In this way, specific absorbance readings are statistically relevant when comparing and

contrasting them to one another. This is to say that each plate that is read, allows for comparison of measurements with each other. Each plate that is read, therefore, cannot be compared to one another as there could potentially be discrepancies in the readings. For example a higher sensitivity can be expected from readings of a 96-well plate than that of a 24-well plate by potential gradients that can form in a 24-well plate. Accordingly, each reading by the plate reader was analyzed and expressed in later absorbance plate readings by subsequent manipulations of the test substrates. Therefore the novel sandwich ELISA method was used to test a multitude of surface assemblies until the substrate with highest absorbance and lowest background was conclusively found. This novel surface-assembly which displayed capture with the highest efficacy can be considered a facile bioconjugation technique for developing a testing platform for an ovarian cancer diagnostic device.

4.2 Methods and Materials of ELISA

The novel sandwich ELISA was performed in three primary steps: capture of the bcl-2 standard protein, binding of the primary and secondary detection antibodies and the enzymatic reaction following the introduction of the liquid pNpp substrate to produce a soluble yellow end product.

Recombinant Human Bcl-2 Standard: This protein was obtained was from R & D Systems (product number 827-BC) at a concentration of 306.6 $^{ug}/_{ml}$. The protein was aliquot into twelve equal portions of 15 µl and stored at -20° C to ensure longevity. Upon use, a working concentration of 0.1 $^{ug}/_{ml}$ in DPBS was used (6). To apply the protein on one side of the glass substrate, 50 µl of the working solution is placed on parafilm. Slides should be carefully deposited on top of the drop of solution for a fixed time of an

hour at room temperature. Afterwards, the slides should be thoroughly rinsed and stored if needed at 4° C in DPBS if necessary.

Bcl-2 (8c8) Monoclonal Antibody: This antibody derived from mouse was purchased from Santa Cruz (product number SC65392) at a concentration of 0.2 $^{mg}/_{ml}$. The initial antibody was aliquot into ten equal portions of 10 µl and stored at -20° C to ensure longevity. Upon use, the antibody needed to reach a working concentration of 1 $^{ug}/_{ml}$ in DPBS. For antibody adsorption on each substrate, 50 µl of working solution is needed for example if 30 samples are desired: 1500 µl of DPBS and 7.5 µl of stock. To apply the antibody on one side of the glass substrate, 50 µl of the working solution is placed on parafilm for a fixed time of an hour at room temperature. Afterwards, the slides should be thoroughly rinsed and stored if needed at 4° C in DPBS.

X-mouse Alkaline Phosphatase IgG and pNPP substrate: This antibody was obtained from Jackson Immunology (product code 200-052-037) and aliquot at -20^{o}C at a concentration of 0.3 $mg/_{ml}$. Upon use, the antibody needed to reach a working concentration of 0.3 $^{ug}/_{ml}$ in DPBS. For antibody adsorption on each substrate, 50 µl of working solution is placed on parafilm for a fixed time of an hour at room temperature. Afterwards, the slides should be thoroughly rinsed with DPBS and deposited carefully into a standard 24 well plate. Para-Nitrophenylphoshate (pNPP) substrate was purchased from Sigma-Aldrich (product number P7988) and 250 µl was added in each well. Slowly over the next thirty minutes a soluble yellow end product appears in each well, as seen below. Adding 62.5 µl of 3M NaOH quenches the reaction and either 200 µl of each sample can be transferred to a 96-well plate or plates can be directly read in the 24-well plate. The yellow end product can be seen in figure 4.3 on the following page in both 96 and 24-well plates.

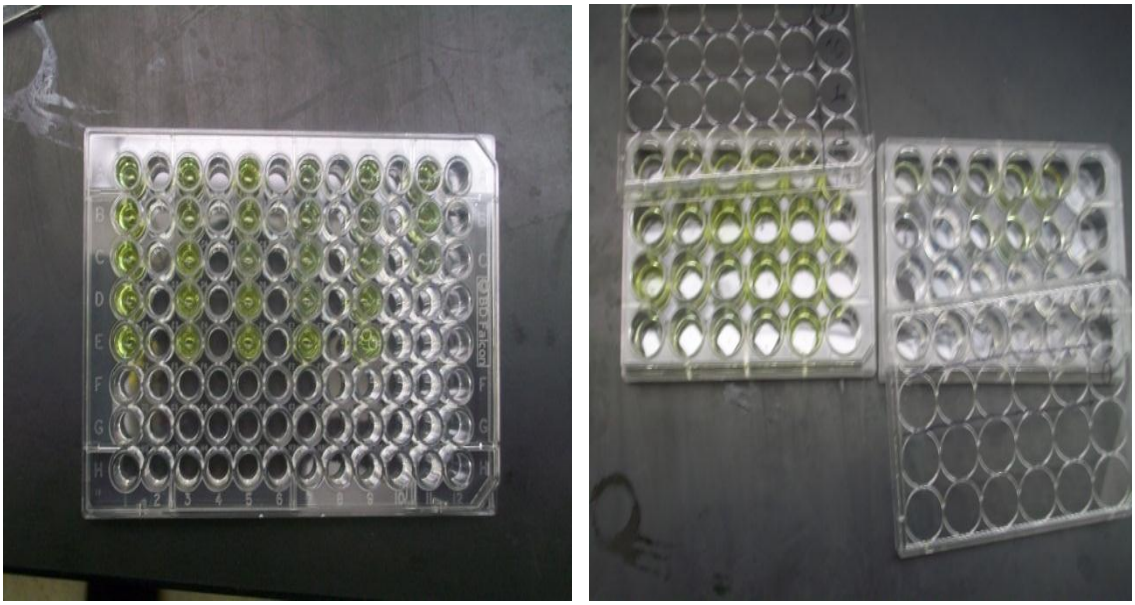

Figure 4.3 Novel ELISA Read in 96-Well and 24-Well Plates.

4.3 Bcl-2 Quantification

Each of the assemblies shown in table 3.1 on page 41 were tested for the specific recruitment of the bcl-2 standard analyte. The five substrates were compared with and without analyte and the results can be seen below in figure 4.4, with the signal-to-noise ratio being shown. Two trends were desired to be seen in data. Firstly, high overall signal with the presence of analyte (corresponding to sensitivity) and high signal-to-noise ratio (corresponding to specificity). Note the units are in relative AU for this chapter.

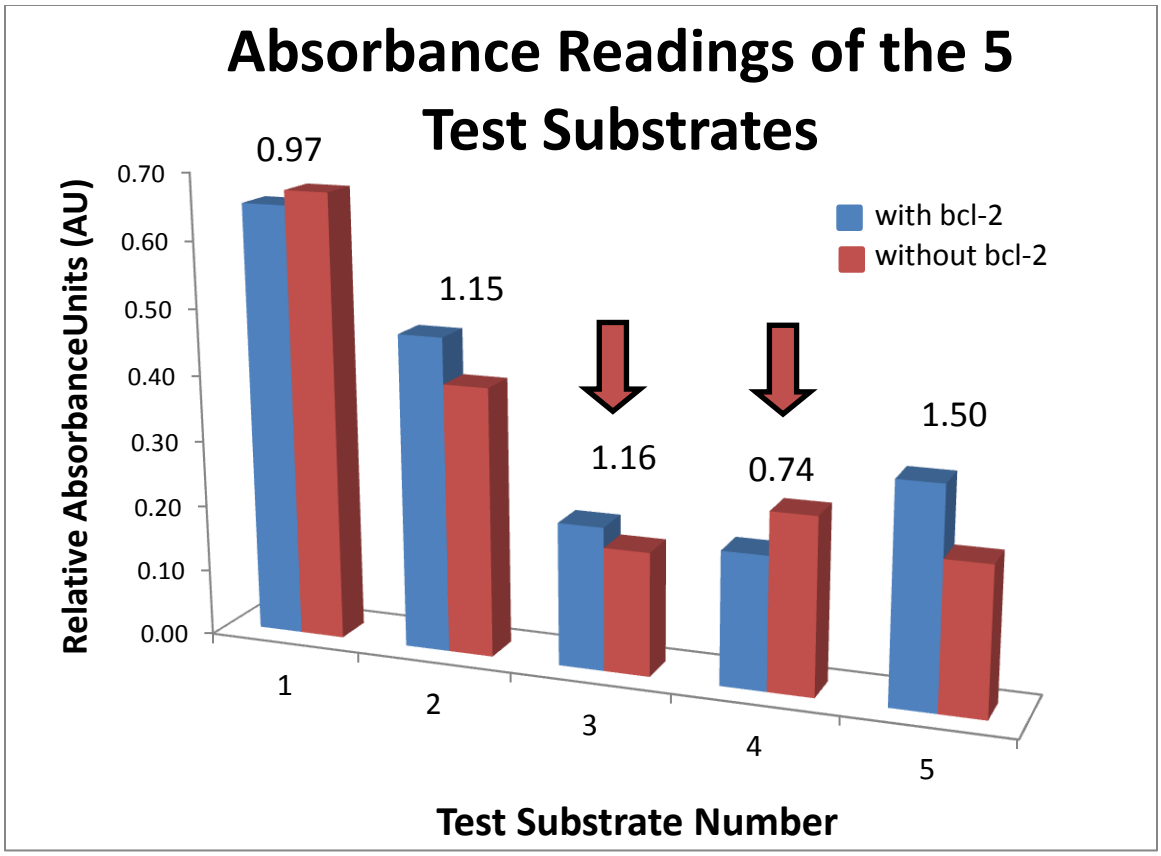

Figure 4.4 Absorbance Readings of the 5 Test Substrates.

It can be seen that the substrate 1 showed the highest absorbance with or without the presence of the analyte on the substrate, roughly 75% more than substrate 2, almost four times that of substrate 3 and 4 and two and a half times more than substrate 5. However, substrate 5 was the only assembly where a favorable signal-to-noise ratio could be seen (approximately 1.5). After the initial trial it could be surmised that this surface had the highest capture efficacy. Although substrate 1 displayed the highest relative absorbance, the signal-to-background ratio was low and therefore indicated poor specificity for bcl-2. As a result substrate 5 showed the highest capture efficacy. Surface 3 and 4 resulted in the overall lowest readings from the initial ELISA

and because of statistically significantly lower capture (almost half of the other surfaces), they were eliminated as test substrates for bcl-2 recruitment in the following trials.

Some preliminary conjectures can be made about the bioconjugation reagent effectiveness after the initial reading. Substrates 2 and 3 are very similar with the additional covalent linkaging of the $MS(PEG)_4$ in substrate 3 being the only difference. Attachment of both amine-reactive PEGylation reagents greatly suppressed the overall signal with and without the analyte, thus not offering the sensitivity that is desired in this research. It can therefore be concluded that an excess amount of PEGylation reagents on surface 3 obstructs the recruitment of the analyte bcl-2 as well as the capture and detection antibodies. Substrates 4 and 5 are similar with the only difference being the PEGylation reagent being used, $MS(PEG)_4$ and Pluronic respectively. Substrate 5 displays twice as much signal-to-noise than substrate 4, (0.74 vs. 1.50), confirming that Pluronic is a highly suitable at maintaining a non-fouling surface on a hydrophobic self-assembled monolayer (SAM,) such as the ODMS based surfaces. It can therefore be concluded that substrate 3 and 4 are incapable of diagnostically relevant bcl-2 capture, as seen with the black arrows in figure 4.4.

4.3.1 F_c Region Antibody to Lower Background

Since the majority of the surfaces displaying a large amount of non-specific binding, a strategy was to be used to counter this problem. Because of the high affinity of protein A/G for F_c regions of antibodies, conceivably the primary detection and alkaline phosphatase antibodies could be recruited by vacant A/G on the test-assemblies, biasing the overall signal. Thus, a recombinant Human IgG_1 F_c portion was obtained from R & D systems (catalog number 110-HG) to be adsorbed onto the substrates before attachment of the secondary ELISA antibodies to occupy any unoccupied protein A/G sites. These F_c regions were diluted in DPBS to an active

concentration of 0.5 $^{\mu g}/_{ml}$ and applied on the surface using the parafilm technique that was employed for the other antibodies. It should be noted that this is the first research which employed the novel use of F_c portions to address high non-specific binding contributing to background. In the following data, the presence of bcl-2 as well as the presence of F_c regions were varied and tested for the three remaining test surfaces: substrates 1,2 and 5. The results can be seen below in table 4.1.

Table 4.1 F_c Antibodies: Do Not Lower Non-Specific Binding.

substrate 1	With BCL2		substrate 2	With BCL2		substrate 5	With BCL2	
	with Fc	*no Fc*		*with Fc*	*no Fc*		*with Fc*	*no Fc*
	0.56	0.36		0.48	0.37		0.78	0.54
	0.44	0.67		0.58	0.46		0.43	0.36
	0.22	0.96		0.44	0.28		0.40	0.44
	0.39	0.60		0.39	0.46		0.38	0.22
avg	0.40	0.65	avg	0.47	0.39	avg	0.50	0.39
std	0.14	0.24	std	0.08	0.08	std	0.19	0.14
substrate 1	No BCL2		substrate 2	No BCL2		substrate 5	No BCL2	
	with Fc	no Fc		with Fc	no Fc		with Fc	no Fc
	0.41	0.67		0.66	0.44		0.17	0.23
	0.54	0.35		0.51	0.31		0.24	0.25
	1.10	0.59		0.56	0.48		0.35	0.33
	0.39	0.77		0.52	0.52		0.40	0.11
avg	0.61	0.59	avg	0.56	0.44	avg	0.29	0.23
std	0.33	0.18	std	0.07	0.09	std	0.10	0.09

In the test substrates with the presence of bcl-2 standard the introduction of the F_c portions caused a decrease in relative AU of 37.73% for substrate1 and a surprising increase of 20.5% and 28.2% for substrate 2 and 5, respectively. Without the presence of the analyte, however an opposite trend was seen for substrate 1, indicating statistically irrelevant changes in signal with or without the F_c regions on the surface. Substrate 2 displays no real correlation with or without the analyte and thus the 3-

APTMS based assemblies failed to improve the signal-to-noise ratios. Substrate 5 displayed a 70% increase in signal in the presence of the analyte, with or without the F_c portions, confirming that the reagent was unable to decrease background. In fact, none of the test surfaces displayed significantly higher signal-to-noise ratios with the application of the F_c portions. Substrate 1 actually decreased in signal-to-noise with F_c regions compared to without (1.09 vs. 0.65) with a similar trend being seen in substrate 2 as well (0.90 vs. 0.84). Substrate 5 displayed a small increase in signal-to-noise ratio with the introduction of F_c portions, 1.72 vs. 1.70, however because the increase was so small (about 1%), it is deemed statistically irrelevant. It is worth mentioning, that with or without the F_c portions the most efficient bcl-2 capture continued to occur on assembly 5 above all other substrates. This was assessed by the surfaces ability to display adequate sensitivity and superior specificity towards the bcl-2 protein. That being said, it can be concluded that the F_c regions did not contribute to improving of the capture efficacy of the surfaces.

4.3.2 Analysis on Orientation of Capture Antibody

Molecular self-assemblies' variability leads to the presence of 'defect' that occur on bioconjugated surfaces. Each ELISA trial revealed valuable information commenting on the ability of the reagent to link on the substrates. A key point of interest was low variability in the assemblies to ensure reproducibility of the technique. Both substrate 1 and 2 having low signal-to-noise ratio and marked inconsistencies in overall signal (high standard deviations), failed to display reproducible, specific capture Because of the fact that the substrate 1 consistently displayed highest overall signal, substrate 2 was eliminated from subsequent ELISA trials. The high degree of 3-APTMS polymerization onto hydroxylated substrates led to high variability in the initial monolayers that formed (57), undoubtedly affecting the immobilization of subsequent reagents.

To arrive at a surface that would be best for bcl-2 capture with the highest efficiency, substrates 1 and 5 were further assessed. A 'scaled down' approach was used to assess the efficiency of both surfaces to successfully bind protein A/G therefore immobilizing the capture antibody. Thus, the GA-protein A/G step in substrate 1 and the direct adsorption of A/G in substrate 5 were tested. By modifying these crosslinking reagents inclusion in the functionalization process, a better understanding on the individual contributions of GA and protein A/G on the recruitment of the analyte and could be arrived at. This ELISA was conducted looking to maximize the sensitivity of the substrates towards the bcl-2 protein. The test assemblies are listed in figure 4.5 and the results for substrates 1 and 5 can be seen in figures 4.6 and 4.7 respectively.

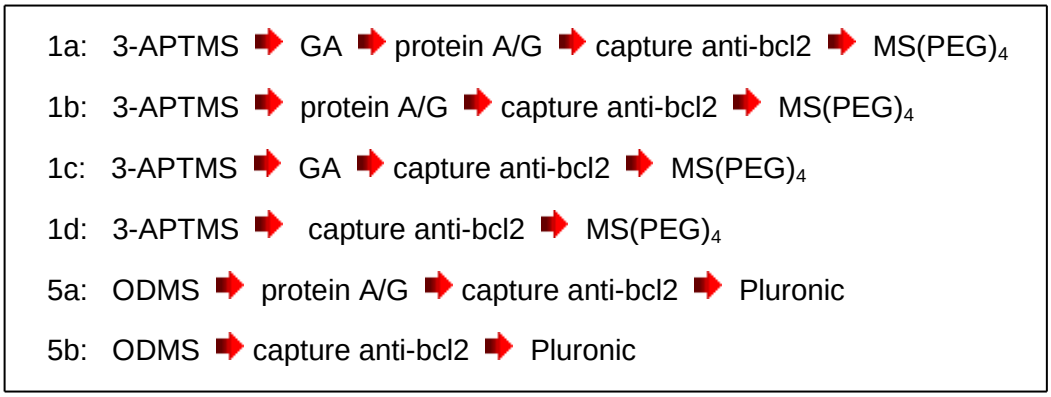

Figure 4.5 Assessing Maximum Antibody Immobilization on Substrate 1 and 5.

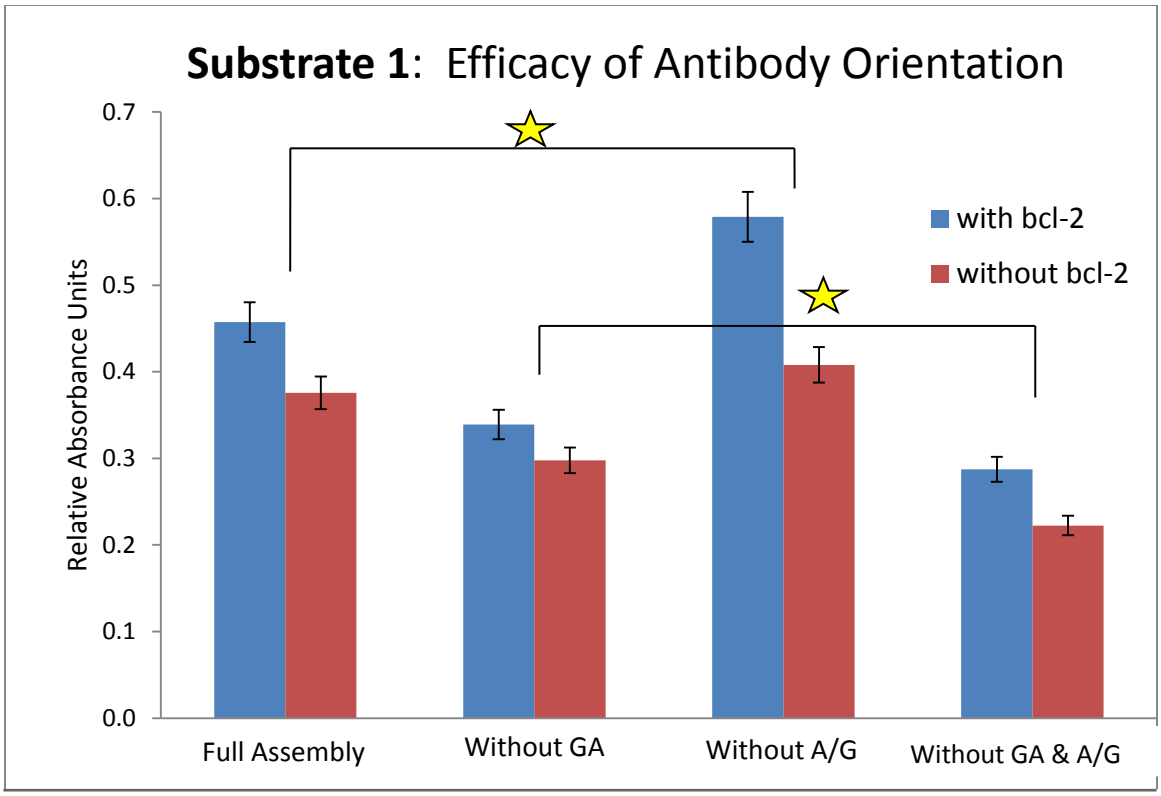

Figure 4.6 Amine-Crosslinking for Antibody Recruitment on Substrate 1.

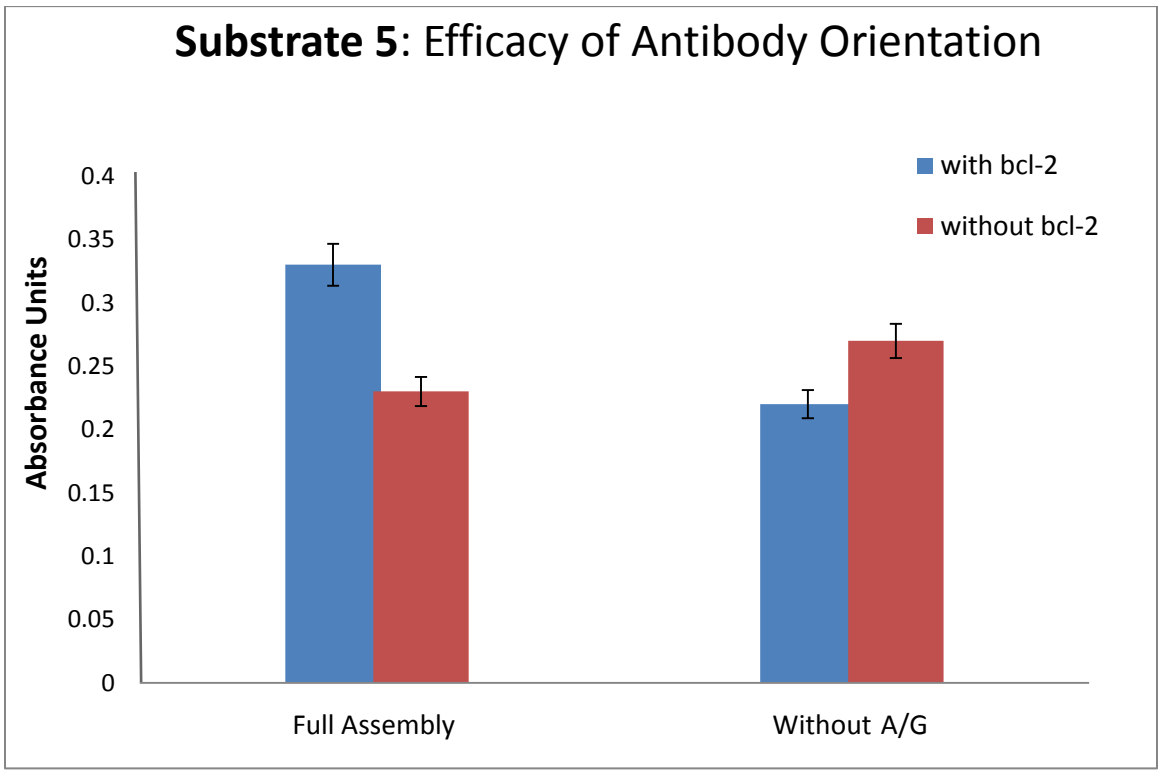

Figure 4.7 Amine-Crosslinking for Antibody Recruitment on Substrate 5.

When analyzing if improvements in sensitivity for bcl-2 were made, it was seen that for substrate 1, the full assembly and without protein A/G displayed highest overall absorbance. However, a statistically relevant increase in signal is not achieved with only GA and therefore one surface cannot be deemed more suitable for sensitive bcl-2 capture than the other. When addressing direct adsorption of protein A/G on the 3-APTMS (substrates 1b and 1d) it was seen that the maximum capture was similar with or without the presence of protein A/G (0.33 vs. 0.28, respectively). This perhaps indicated that the need for an amine-crosslinker, such as GA, on a 3-APTMS SAM for antibody tethering was crucial. Thus, the full assembly of substrate 1 was demonstrated a highly sensitive but insufficiently specific capture of bcl-2, and could not be improved upon. However, for substrate 5, it can be seen that the highest signal and best overall signal-to-noise ratio (1.412) was seen with the full assembly. Without adsorbing protein A/G onto the surface, a statistically significant decrease in the overall signal and capture efficacy was seen (signal to noise ratio of 0.824). Thus, protein A/G attachment onto the hydrophobic silane is an efficient method to immobilize target antibodies.

4.3.3 Selection of 'Best' Bioconjugated Surface

Substrate 1 lacks in the two major parameters which are important in this research. First, the surface continually fails to specifically quantify the bcl-2 capture, being biased by the presence of other non-specific interactions. Second, the surface fails to show the reliability and consistency needed to be diagnostically relevant. In fact, the standard deviation is 250% percent lower in the ODMS based substrate 5 (0.06 vs. 0.138) demonstrating that although 3-APTMS SAMs were built-up and widespread, they are not as reproducible as the hydrophobic, ODMS surfaces. Substrate 5 consistently displayed favorable overall signal and signal-to-noise ratio. This facile assembly can therefore be selected as the best substrate for efficient capture of the bcl-2 analyte.

4.4 Diagnostically Relevant Bcl-2 Capture on Substrate 5

To ensure that the proper surface was selected further novel ELISA testing must be done to verify the decision. Again, two paramount features of an ideal substrate are efficient immobilization of the primary antibody and prevention of non-specific binding. For substrate 5 the two reagents which directly regulated these interactions are protein A/G and Pluronic. By varying these two reagents, with and without the presence of the analyte (as seen in the tree diagram below), a comprehensive analysis can be conducted quantifying the impacts of these important reagents on recruiting antibodies and preventing unwanted binding, respectively. Likewise, if a better functionalization assembly is found, that can be assessed as well.

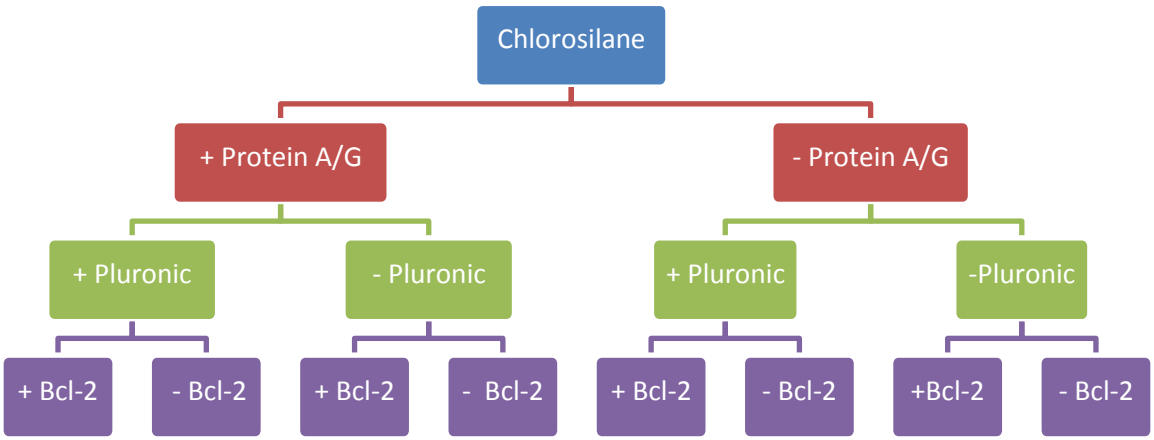

Figure 4.8 Analysis on Protein A/G and Pluronic Immobilization on Substrate 5.

Before presenting the results it should be noted of a couple of modifications in the ELISA method that were made at this juncture in the research. All of the initial readings discussed were read in a 24-well plate. As mentioned, the output of a plate reader can be compared to readings across one individual plate. It was noted that there was an increase in signal from a 96-well plate compared to the of a 24-well plate,

however the relative differences were similar. Additionally, a known surfactant called Tween-20 (Sigma-Aldrich, product number P2287) was employed as a washing agent in the ELISA steps of the detection antibody and enzymatic antibody. Adding this reagent, at a concentration of 0.05% in a common phosphate buffer solution, helps to prevent non-specific antibody binding in immunosorbent assays (82). The results of the assay follow. Note, the signal-to-noise ratios are displayed above the graphs.

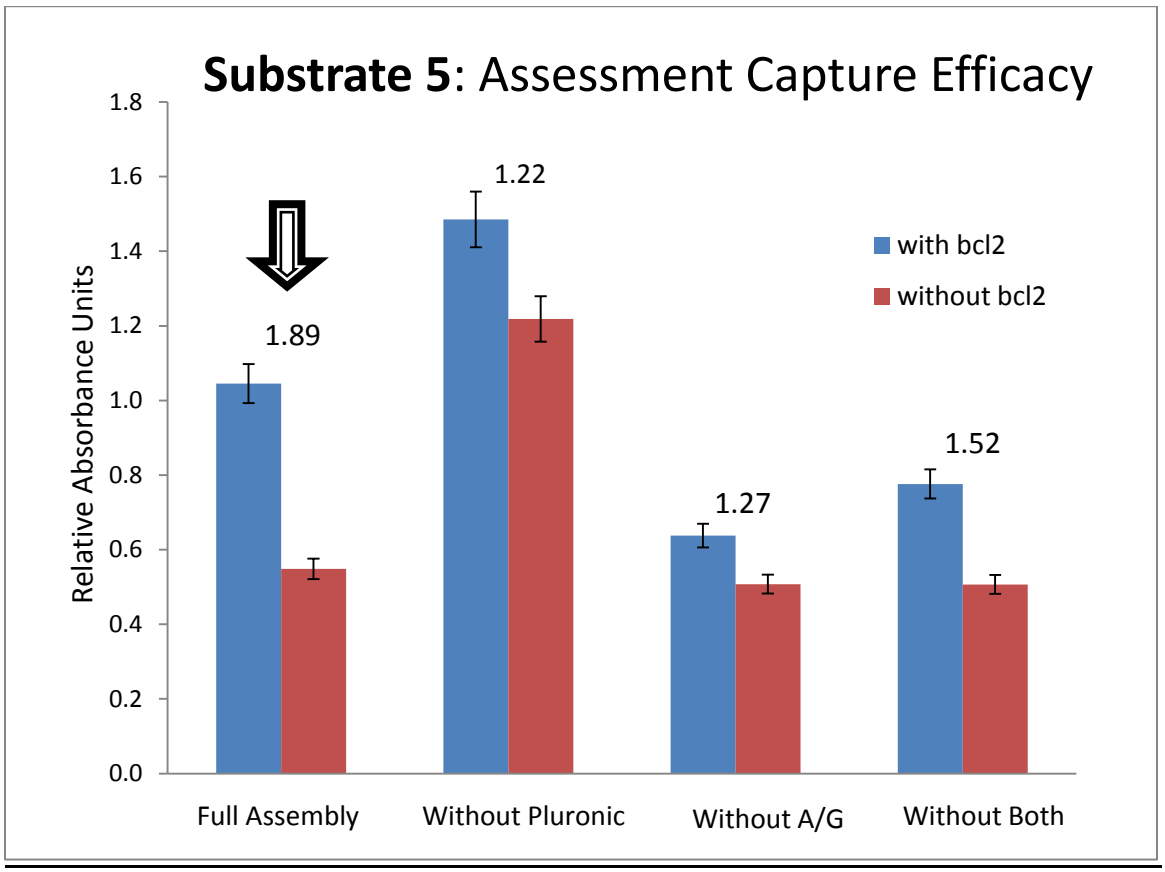

Figure 4.9 Substrate 5: Displays Superior Selectivity and Specificity of Bcl-2.

Analysis of the results reveals some valuable information. The noticeable increase in signal-to-noise will be credited to the tween 20 reagent used in the dilution and rinse step, which aims at reducing non-specific binding. To assess the crosslinking efficiency of substrates with protein A/G to the ones without, (substrates 5a and 5b vs.

substrates 5c and 5d), a 76% increase in signal was seen (1.27 vs. 0.76, respectively). Substrate 5b (with no Pluronic) resulted in a marked increase in signal, both with and without the presence of the analyte when compared to substrate 5d (with neither Pluronic nor A/G). Therefore because of the direct correlation between protein A/G and increased signal, it was concluded that the direct adsorption of protein A/G on a hydrophobic surface is a viable technique to orient desired antibodies. To assess Pluronic's effectiveness the comparison of the full assembly (5a) to the assembly only missing the PEGylation reagent (5b) reveals a 43% increase with and a 122% increase without the presence of the bcl-2 analyte, respectively. Coupling this with the overall lowest signal-to-noise ratio of the full assembly of substrate 5 (1.89), we can safely conclude that the Pluronic reagent maintains a non-fouling surface on the substrate. Certainly it can be concluded from this data that protein A/G is effective at orienting the capture antibody and that Pluronic limits non-specific interactions on the hydrophobic, ODMS based assembly. Because of this and the facile functionalization method, substrate 5 is further validated as the best overall choice for the capture of the bcl-2 biomarker protein, which corresponds to early stage ovarian cancer.

Although the 'best' substrate of those tested has been elucidated, to ensure future nanosensor integration endeavors of this research the surface must display extremely sensitive capture of bcl-2 level. Up until this point, the concentrations of the bcl-2 concentrations were held constant at 100 $^{ng}/_{ml}$. Referring back to Dr. Kruk's findings, levels of bcl-2 in early and late stage ovarian cancer were found to be 2.60 and 3.58 $^{ng}/_{ml}$ respectively. In addition, it was seen that healthy levels of bcl-2 are 0.59 $^{ng}/_{ml}$ (6). Thus, application of the analyte at the concentrations that were previously executed (100 $^{ng}/_{ml}$) would not guarantee the bioconjugate surface suitable for recognition of urinary bcl-2 levels. Since it is experimentally verified that mass-loaded nanosensors possess sensitivity in the order of 0.15 $^{pg}/_{ml}$ (9), early ovarian cancer detection is possible

if the functionalized surface can, at the very least, recognize bcl-2 levels of 0.1 $^{ng}/_{ml}$. Six different concentrations were therefore tested: 100 $^{ng}/_{ml}$, 10 $^{ng}/_{ml}$, 1 $^{ng}/_{ml}$, 0.1 $^{ng}/_{ml}$, 0.01 $^{ng}/_{ml}$ and 0 $^{ng}/_{ml}$. Thus by a ten-fold reduction in concentrations, a logarithmic serial dilution of bcl-2 levels was done. The results can be seen below in figure 4.10 with the arrow signifying the bcl-2 concentrations corresponding to early stage ovarian cancer (red) and the healthy levels (light blue) (6) .

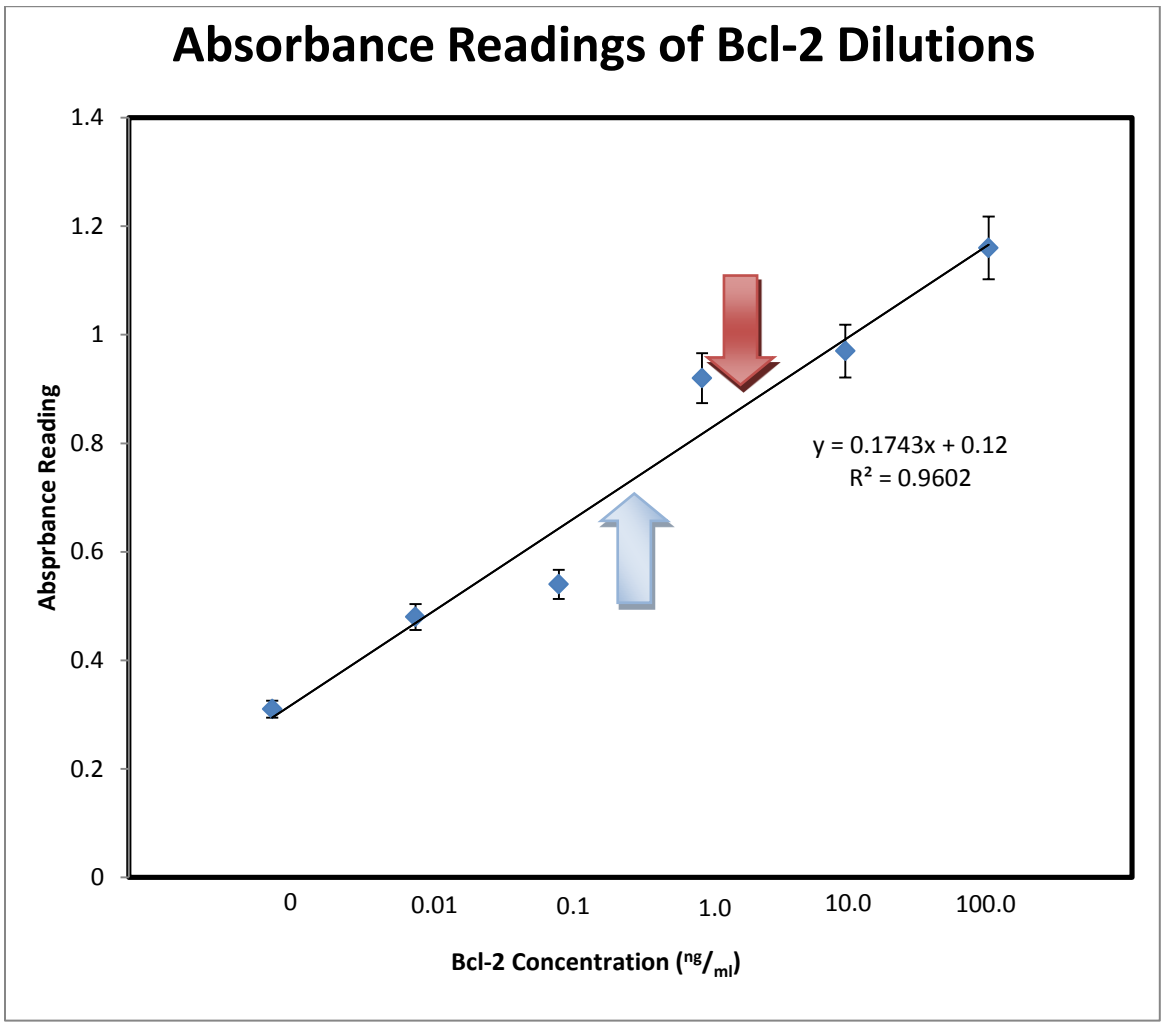

Figure 4.10 Serial Dilution of Bcl-2.

The serial dilutions of the bcl-2 analyte on the substrate revealed absorbance data that displayed a favorable, linear relationship when assessed. This data was fit to a first degree regression trendline, which can be seen in the graph above. The coefficient of determination for the line is 0.9602 signifying a very close linear relationship seen between the data, which is an indication of the applicability of the ODMS based surface assembly towards ovarian cancer diagnostics. At the concentrations of interest of 0.1 $^{ng}/_{ml\ and}$ and 0.01 $^{ng}/_{ml}$ the capture efficacy can be calculated at 1.72 and 1.52 respectively, ensuring that the majority of the signal that is being measured at those thresholds were because of the analyte not because of non-specific interactions. The serial dilution demonstrated that the sensitivity of the substrate to recognize bcl-2 is done at a level ten times than that needed (0.01 $^{ng}/_{ml}$). This is extremely important because recognition at such small concentrations would ensure that elevated and healthy levels of urinary bcl-2 can both be effectively detected by the bioconjugated surface. Thus, an assembly of hydrophobic ODMS with direct adsorption of protein A/G to orient the capture antibody and Pluronic F127 to maintain non-fouling properties: produces a surface that displayed sufficient sensitivity and specificity to be deemed diagnostically relevant to recognize levels of the bcl-2 protein. Therefore, this research has demonstrated that integration of the testing platform with a novel nanosensor can successfully create an ovarian cancer POC diagnostic device for worldwide implementation.

CHAPTER 5: PROJECT SYNOPSIS AND FUTURE CONSIDERATIONS

The research presented in this thesis is the first of its kind to quantify a confirmed, ovarian cancer biomarker, the bcl-2 protein, on novel bioconjugated substrates. After identification of applicable target reagents, a series of contact angle measurements along with published results and expected chemistry outcomes, allowed for validation of the test assemblies that were chosen. A novel sandwich ELISA method was then employed to quantify the bcl-2 capture on the substrate, to assess the overall sensitivity and specificity the test assemblies displayed towards the target analyte. Progressively, substrates displaying substandard relative absorbance units and signal-to-noise ratio were eliminated and the remaining substrates were thoroughly assessed for the efficacies of the crosslinking of protein A/G to recruit antibodies and for the extent of PEGylation coverage on the surface. After a series of ELISA trials, the assembly of ODMS with directly adsorbed protein A/G and Pluronic was identified as the 'best test substrate' due to continually superior capture efficacy, reproducibility and ease of assembly. To examine the diagnostic relevance, a logarithmic serial dilution of bcl-2 analyte concentration was done to test the overall sensitivity of this test surfaces. It was demonstrated that the sensitivity of the substrate to recognize bcl-2 is done at a level ten times than that needed ($0.01\ ^{ng}/_{ml}$), ensuring that elevated and healthy levels of bcl-2 can be detected by the bioconjugated surface. Thus, the functionalized surface of hydrophobic ODMS, protein A/G and Pluronic displays sufficient accuracy and sensitivity in recognizing levels of the bcl-2 protein, and can therefore be successfully integrated into an ovarian cancer POC diagnostic device.

This research opens the door to fabricate an ovarian cancer diagnostic device which can be employed at the POC worldwide. Since the field of ovarian cancer diagnostics is nascent in its development, future endeavors should be done to integrate the surface with a novel transducer or other appropriate nanosensor. Current nanosensors employ piezoelectric currents which are not only expensive, but are not biocompatible, because of the presence of lead. Because of integration capability, large bandwidth and by effectively eliminating the need for a matching layer, the capacitive machined ultrasonic transducer (CMUT) is often employed for microfabricated biosensor development. Dr. Rasim Guldiken from University of South Florida has designed a dual-electrode CMUT that has been fabricated and has been experimentally operated to display mass recognition level up to 0.15 $^{pg}/_{ml}$: easily sufficient diagnostic implementation. Since the nanosensors typically use very small amounts of energy in mass quantification applications, one AAA battery will operate a dual-electrode CMUT for over 1 million hours, truly providing a cost-effective method to quantify the capture on the aforementioned testing platform. Consequently, calibrating a suitable nanosensor such as the dual electrode CMUT with the ODMS based testing platform elucidated in this thesis will allow for development of a threshold that can be corresponded with healthy and elevated bcl-2 levels. Achievement of both integration of the substrate with a novel nanosensor and calibration of the nanosensor to threshold concentrations will result in the creation of a POC ovarian cancer diagnostic device in the near future.

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APPENDICES

Appendix A Review of POC Tests in Low-Resource Settings

Group A Streptococci, also known as a sore throat, is most commonly of viral origin. Microbiological assays are needed, as clinical presentation alone does not allow for dependable distinction between bacterial and viral stimulation. Throat swab culture on a blood agar plate has long served as the reference criterion. Ever since the 1980s, several rapid tests have been developed targeting the streptococcus gene, which is a known sore throat biomarker. Initial assays using agglutination techniques were improved by enzyme immunoassays and more recently immunochromatographic (lateral flow) tests. Lateral flow tests can be considered a competitive sandwich ELISA procedure, as once the surface is exposed to a fluorescent antibody, the analyte protein can then be measured at a discrete wavelength. Lateral flow tests are upwards of 95% and cost-effective they enable treatment to be started in the case of a positive test result. The wide availability of sore throat tests has led to a steady decline in antibiotic prescriptions and in doing so contributing to avoidance of problem of antibiotic resistance (1-2).

Malaria continues to be a leading cause of death caused by infections. The poor prognosis of malaria is largely because the disease displays a clinical presentation that is markedly erratic, and key symptoms such as fever and chills might be missing in a substantial percentage of patients. Thus, about 60% of cases are initially misdiagnosed in North America (3). A diagnostic assay is therefore needed in the setting of the broad differential diagnosis and the possibility of co-infections that are perhaps overt in their clinical presentation. Giemsa thick blood smears are still considered to be the reference standard for malaria diagnosis. Although it is moderately inexpensive in the developed worlds, accurate diagnosis of malaria necessitates microscopy and consequently related expertise. Unfortunately, there is a scarcity of adequate microscopes and trained clinicians in emergency rooms of low resource settings all around the world. In recent

Appendix A (Continued)

years rapid diagnostic techniques have been developed in to amerliorate the restrictions. First introduced in the 90's, these lateral flow tests, supply results in less 15 minutes and have showed encouraging results when validated with microscopic evaluation. Sensitivity, which varies with the level of parasitaemia (quanitative measure of parasites in the blood), is usually higher than 90%, and as a matter of fact recent findings indicate that rapid tests might perform better than microscopy in low resource settings. Even so, rapid tests cannot produce reliable quantification of antigen amount and so cannot replace microscopic evaluation for this infectious disease (4).

C. trachomatis is the most common bacterial sexually transmitted disease worldwide. Molecular amplification tests serve as the diagnostic reference standard because of their higher sensitivity and better acceptability when performed on urine samples. That being said, molecular diagnosis remains expensive, and the delay before results are available implies a second visit for the initiation of treatment. This second appointment may often be missed in low resource settings, leaving numerous infected patients untreated. On the other side, the high frequency of erratic infections makes microbiological diagnosis necessary before empirical therapy. In this setting, a POC test would have a major impact, allowing patients to receive diagnosis and treatment during the same consultation (5).

Appendix A (Continued)

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Appendix B Review of Selected Proteomic Techniques

Qualitative proteomic assays aim to study changes in protein expression by monitoring changes in protein mixture composition under various physiologically-analogous conditions. Similar to genomics experiments, a standard qualitative proteomics analysis compares the relative levels of thousands of protein species in biological samples by prevailing successful protein-profiling technologies: namely 2-Dimensional Gel Electrophoresis (2-DE) (1). Two-dimensional gel electrophoresis (2DE) has been the most widely used means for protein purification or the isolation of a specific protein from a complex mixture. Proteins are separated along two axes, the first dimension based on their isoelectric point (magnitude of charge) and the second dimension based on the respective molecular masses, (via mass spectrometry and the mass-to-charge ratio). Because of the controlled pH gradient, detection of lower abundance proteins is possible via high resolution microscopy. Discrepancies between samples can be compared relatively by quantifying the ratio of spot intensities. Mass spectrometry (MS) is the top technique for determining masses of particles. Utilizing the power of MS, advancements in identification of isolated proteins have made 2-DE an ever more comprehensive option for protein mixture analysis. Some of these MS advancements, namely time-of-flight (TOF) have brought these two chosen assays to the forefront of qualitative proteomic analysis:

1) Matrix-Assisted Laser Desorption/Ionization, (MALDI) is an ionization technique allowing the analysis of biomolecules (like proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers, other macromolecules). A nitrogen laser beam triggers the ionization and MALDI enables the conversion of biomolecules into a charged gaseous state to be investigated by TOF MS. MALDI-TOF instruments are typically equipped with an "ion mirror", deflecting ions with an electric field, thereby increasing the resolution (2)

Appendix B (Continued)

2) Surface-enhanced laser desorption/ionization, (SELDI) is a variation of MALDI, which immobilizes specific antibodies allows for high-throughput screening because of its versatility, ease of use, low cost and reproducibility. Through the use of specific biomolecular binding sites, the SELDI platform allows for highly specific proteomic analysis (in the femtomolar range). Much like MALDI, time-of-flight mass spectrometry (TOF-MS), is the most appropriate mass separation technique used with SELDI. Because of these qualities, SELDI has advantages over other technologies such as 2DE and enzyme-linked immunosorbent assays (ELISA) (3).

Quantitative proteomics aims at exposing numerical disparities in protein concentration within a sample. Quantitative proteomics assays offer all of the biomolecule identification capabilities as qualitative assays, while including quantification as an additional advantage. Qualitative proteomic techniques have provided indispensable knowledge about cancer progression and early biomarker discovery. While monitoring qualitative changes is imperative, further development of quantitative tools can provide insight into disease mechanisms (in particular characterization of cell function, disease mechanism and biomarker discovery), that qualitative proteomic assays cannot. Recently, enabled by the influx of novel quantitative proteomics technologies, rapid advancements in global detection and quantitation of proteins have provided both opportunities and challenges to discover molecular mechanisms of cancer and other diseases (4). Two prevalent quantitative proteomics methods are illuminated below.

Appendix B (Continued)

1) Stable isotope labeling with amino acids in cell culture, (SILAC) has become a popular labeling strategy for peptide quantification. It not only provides quantitative analysis of relative changes in protein abundance from specific cell treatments, but also of proteins for which there are no antibodies available. SILAC assays consist of growing cell populations with 'light' or 'heavy' nonradioactive isotopes, expressed through standard cellular protein synthesis. They are grown identical, except that one of them contains a 'light' and the other a 'heavy' form of a particular amino acid. Once equal amounts of proteins from both cell populations are combined, they are separated using protein purification techniques, such as 2DE. The proteins are enzymatically digested into smaller peptides using a protease, such as trypsin. Because of the chemical similarities of 'light' and 'heavy' labeled proteins, they both detected simultaneously and the relative peak intensities of multiple distinct peptides which are gathered from the MS analysis can then be manipulated to determine the average change in protein abundance in the treated sample. SILAC is economical and reproducible and labeling is virtually 100%, thus marking is as a promising technique that is currently being extensively applied for biomarker discovery, cell signaling dynamics, identification of posttranslational modification sites, protein-protein interaction and cellular proteomics all over the world(5).
2) Isobaric tags for relative and absolute quantification, (iTRAQ) is a robust, multiplexed quantitative proteomic analysis technique. Labeling of peptides, like SILAC assays, are employed with a compound that produces isobaric reagents, or reporter ions, within the sample. Once the proteins are enzymatically digested through an appropriate protease, current identification and quantification of multiple samples can be achieved via MS analysis. The key advantage that

Appendix B (Continued)

iTRAQ provides is the amine specificity of these reagents, which possess high affinity to most peptides in the complex, limiting the loss of information from samples involving post-translational modifications. Applying this technology to the tandem MS fragmentation data, a low molecular mass reporter ion is generated that can be used to relatively quantify specific proteins (6-7).

Appendix B (Continued)

Approach type	Approach	Advantages	Disadvantages
Qualititative Analysis	Protein microarray	> good for unknown protein functional assay > high throughput	> limited information > relative expensive
	2-DE	> simultaneously monitor thousands of proteins > compatible with various stain methods > high throughput	> require relatively large amounts of starting material > only identify the most abundant proteins > not good reproducibility
	2-D LC	> greater throughput potential > good reproducibility > easy configure to MS analysis	> difficulty data analysis > nonquantitative > relative expensive
	MS-based proteomics	> highly sensitive > relative simple protocol > posttranslational modification analysis	> nonquantitative > too many redundant sequence
Quantitative Analysis	Radioactive labeling	> highly sensitive > very good quantitative > posttranslational modification analysis	> safety
	Fluorescence labeling	> highly sensitive > reduced 2-DE variation > compatible with MS analysis	> expensive > marginal reproducibility > only good for high abundance proteins
	ICAT	> highly sensitive > good quantitative	> limited application > difficulty data analysis
	iTRAQ	> good proteome coverage > simultaneously comparison of multiple samples > good statistic relevance > good quantitative and good for biomarker validation	> possible false positive > reduced sensitivity because of chemical labeling
	SILAC	> known expected mass difference prior to identification, simple quantition > highly labeling yield, easily labeling in mammalian cells > protocol simple and straightforward > highly sensitive > potential application *in vivo* study	> difficulty data analysis for low or partially labeled species
	mSILAC	> known expected mass difference prior to identification, simple quantition > highly labeling yield, easily labeling in mammalian cells > protocol simple and straightforward > highly sensitive > application for *in vivo and* cell culture studies	> to be validated

Figure 1.A Summary of Proteomic Strategies.

Appendix B (Continued)

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Appendix C Contact Angle Measurements

Table 1.A Contact Angle: Amine-Crosslinking to Immobilize Protein A/G.

3-APTMS	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	16.8	29.1	23.0	9.0	1.9	48.8	69.5
	26.0	25.7	25.9	8.2	2.0	43.6	61.5
	36.1	24.2	30.1	7.0	1.5	26.7	43.9
avg	26.3	26.4	**26.3**	8.1	1.8	39.7	58.3
ODMS	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	100.6	100.5	100.5	2.3	1.3	4.0	9.6
	100.0	99.9	100.0	1.9	1.1	2.1	6.4
	101.2	101.1	101.1	2.4	1.4	4.5	10.5
avg	100.6	100.5	**100.5**	2.2	1.3	3.5	8.8

3-APTMS GA	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	44.2	44.2	44.2	3.6	0.7	3.8	11.6
	46.7	47.4	47.1	3.4	0.7	4.2	11.8
	46.4	46.0	46.2	3.5	0.7	3.7	11.2
	45.7	46.3	46.0	3.6	0.8	4.2	12.1
avg	45.8	46.0	**45.9**	3.5	0.7	4.0	11.7
Chlorosilane, GA	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	87.3	88.0	87.6	2.8	1.3	5.1	11.4
	82.7	81.9	82.3	2.8	1.2	4.6	10.6
	85.3	83.1	84.2	2.8	1.2	4.8	11.0
	84.2	83.8	84.0	2.8	1.2	4.7	10.8
avg	84.9	84.2	**84.5**	2.8	1.2	4.8	11.0

3-APTMS, GA, A/G	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	35.8	37.0	36.4	3.8	0.6	3.7	12.9
	35.7	36.5	36.1	3.4	0.6	3.2	11.2
	32.0	35.9	33.9	3.7	0.6	10.7	22.9
	61.3	36.4	38.9	3.0	0.5	4.5	11.1
avg	41.2	36.5	**36.3**	3.5	0.6	5.5	14.5
Chlorosilane, GA, AG	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	85.0	84.3	84.6	2.6	1.1	3.7	9.3
	87.1	86.5	86.8	2.5	1.2	3.8	9.3
	75.5	73.9	74.7	2.8	1.0	3.9	9.8
	78.9	77.2	78.1	2.7	1.0	3.6	9.2
Avg	81.6	80.5	**81.0**	2.6	1.1	3.8	9.4

Appendix C (Continued)

Table 1.B Contact Angle: PEGylation Coverage on the Substrates.

3-APTMS, GA, A/G BS(peg)	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	30.3	28.7	29.5	4.3	0.6	6.3	18.7
	23.5	23.3	23.4	4.9	0.5	4.7	19.9
	18.8	19.4	19.1	4.9	0.4	4.0	19.9
	25.2	27.2	26.2	4.1	0.4	3.4	14.8
	29.1	28.6	28.9	3.8	0.5	3.4	13.4
avg	25.4	25.4	**25.4**	4.4	0.5	4.4	17.4
Chlorosilane, A/G BS(peg)	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	78.3	77.0	77.7	3.1	1.2	5.3	11.8
	63.8	77.3	70.6	3.2	1.3	5.9	12.7
	84.3	83.4	83.9	2.9	1.3	5.3	11.8
	81.5	82.2	81.8	3.0	1.2	5.4	11.8
avg	77.0	80.0	**78.5**	3.0	1.2	5.5	12.0

3-APTMS, GA, A/G MS(peg)	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	69.4	68.0	68.7	2.8	0.9	3.4	9.0
	56.5	62.9	59.7	3.2	1.0	5.4	12.4
	51.8	53.9	52.8	3.1	0.8	5.3	12.5
	50.3	48.9	49.6	3.3	0.8	4.8	12.2
avg	57.0	58.4	**57.7**	3.1	0.9	4.7	11.5
Chlorosilane, A/G MS(peg)	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	69.9	69.9	69.9	3.1	1.0	4.4	10.8
	69.7	68.3	69.0	3.0	1.0	4.1	10.3
	69.3	69.1	69.2	3.0	1.0	4.1	10.3
	66.5	68.4	67.4	3.0	1.0	4.1	10.4
avg	68.8	68.9	**68.9**	3.0	1.0	4.2	10.4

Appendix C (Continued)

Table 1.B (Continued)

3-APTMS, GA, A/G Pluronic	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	56.6	57.8	57.2	2.6	0.7	2.8	8.1
	54.0	53.3	53.6	2.8	0.7	2.5	8.1
	56.7	57.4	57.0	2.6	0.7	2.5	7.6
	56.0	56.3	56.2	2.6	0.7	2.0	6.8
avg	55.8	56.2	**56.0**	2.7	0.7	2.4	7.7
Chlorosilane, A/G Pluronic	Left Contact	Right Contact	Average	Length	Height	Volume	Area
	63.0	61.0	62.0	2.8	0.8	3.3	9.0
	62.2	63.0	62.6	2.9	0.9	3.5	9.4
	58.5	57.1	57.8	3.1	0.8	4.2	10.8
	61.4	60.6	61.0	2.7	0.8	2.6	7.8
avg	61.3	60.4	**60.9**	2.9	0.8	3.4	9.3

Appendix D Selected ELISA Readings

Table 1.C ELISA Readings for all 5 Substrates. (Note: corresponds to figure 4.4)

with bcl2		1	2	3	4	5	control
		0.87	0.27	0.19	0.16	0.23	0.05
		0.24	0.66	0.18	0.16	0.41	0.05
		0.71	0.23	0.21	0.20	0.28	0.05
	avg	0.61	0.39	0.19	0.18	0.31	0.05
	std.	0.33	0.24	0.02	0.02	0.09	0.00
Without bcl2		**1**	**2**	**3**	**4**	**5**	**control**
		0.97	0.34	0.15	0.21	0.25	0.06
		0.38	0.27	0.13	0.21	0.11	0.06
		0.41	0.55	0.20	0.26	0.15	0.05
	avg	0.59	0.39	0.16	0.23	0.17	0.06
	std.	0.33	0.14	0.04	0.03	0.06	0.00

Table 1.D ELISA Readings for Antibody Orientation on Substrates 1 and 5.

(Note: data corresponds with figures 4.6 and 4.7).

	Substrate 5a		Substrate 5b	
	+bcl2	-bcl2	bcl2	-bcl2
	0.46	0.25	0.26	0.16
	0.29	0.21	0.20	0.33
	0.29	0.24	0.20	0.25
	0.28	0.23	0.22	0.33
avg	**0.33**	**0.23**	**0.22**	**0.27**
std	0.09	0.02	0.03	0.08

	Substrate 1a		Substrate 1b		Substrate 1c		Substrate e1d	
	+bcl2	-bcl2	+bcl2	-bcl2	+bcl2	-bcl2	+bcl2	-bcl2
	0.46	0.46	0.35	0.31	0.36	0.37	0.33	0.34
	0.46	0.48	0.31	0.28	0.47	0.33	0.38	0.28
	0.53	0.39	0.33	0.30	0.95	0.49	0.16	0.13
	0.36	0.16	0.35	0.29	0.47	0.44	0.27	0.18
avg	**0.45**	**0.37**	**0.33**	**0.29**	**0.56**	**0.41**	**0.28**	**0.23**
std	0.07	0.15	0.02	0.02	0.26	0.07	0.10	0.10

Appendix D (Continued)

Table 1.E ELISA Reading Assessing Assembly of Substrate 5.

(Note: data corresponds with figure 4.9).

	5a		5b		5c		5d
With BCL-2	0.78		1.63		0.54		0.80
	1.55		2.04		0.56		0.81
	1.04		1.22		0.64		0.72
	0.78		1.09		0.87		0.80
avg	1.04	avg	1.49	avg	0.65	avg	0.78
std	0.36	std	0.43	std	0.15	std	0.04
Without BCL-2	0.42		0.57		0.44		0.29
	0.43		1.51		0.38		0.70
	0.65		1.28		0.48		0.49
	0.70		1.53		0.74		0.56
avg	0.55	avg	1.22	avg	0.51	avg	0.51
std	0.14	std	0.45	std	0.16	std	0.17