Multianalyte Detection of Breast Cancer by Fabrication of Hybridmicroarrays on Polymer-Based Microanalytical Devices

Suying Wei
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MULTIANALYTE DETECTION OF BREAST CANCER BY
FABRICATION OF HYBRIDMICROARRAYS ON
POLYMER-BASED MICROANALYTICAL DEVICES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Suying Wei
B.S., Shandong University of Science and Technology, China 1996
M.S., Beijing University of Chemical and Technology, China 2000
December, 2006
To my son: Wilson
DEDICATION

This dissertation is dedicated to the very people that have been indispensable in my life.

To my mom and dad, Yanhua Guan and Chenghai Wei, for giving me life, raising me up, and having me educated. I cannot appreciate more than enough your love and care for Wilson. Thank you so much for taking over the burden! You never realized how much it helped my graduate studies.

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Sujuan Wei
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ABSTRACT

Breast cancer is one of the most common and fatal cancer diseases that affect women worldwide. As is true with most other cancer diseases, early detection of breast cancer is very crucial for proper medical treatment because treatment of advanced breast cancer will be much more difficult and inconsistent. Screening and testing of breast cancer biomarkers, either genetic or proteomic, are among techniques used for diagnosis of breast cancers. Nevertheless, none of the biomarkers is by itself sensitive and selective enough for diagnosis of breast cancer, and thus, multi-analyte assays towards detection of multiple breast cancer biomarkers from different classes are desired for accurate diagnosis of this disease.

Described is a methodology with which both genetic and protein biomarkers of breast cancers are detected simultaneously on the same platform. This methodology consists of a novel hybrid biosensor system in a universal Zipcode DNA array format on the platform of polymer-based microfluidic devices. Detection of the genetic mutated material and the protein targeting material is through hybridization events between the arrayed universal Zipcode DNA sequences and the corresponding complementary Zipcode DNA sequences that are incorporated into both biomarkers during materials preparation. Signal generation and detection are through near-IR, laser-induced fluorescence imaging method. The hybrid biosensor system combines the strengths of microfluidic devices—high throughput, low sample consumption, and high kinetics—with that of the universal DNA array format, which uncouples detection from hybridization event, thereby increasing the sensitivity of detection. Near-IR laser-induced fluorescence detection method adds further sensitivity to this system.
In this work, surface properties of the microfluidic device substrate, PMMA have been manipulated in surface functionalities, surface topography, and surface wettabilities. Biomolecules including both antibodies and DNA have been successfully immobilized onto the UV-modified PMMA surfaces. The targeting biomarker materials were prepared using distinct protocols: PCR/LDR combined assays were adopted to prepare the breast cancer gene marker \textit{BRCA1} mutated material, while the protein antigen CEA targeting complex was achieved by a semi-synthetic method. Monitoring and characterization of surface manipulation, bio-functionalization, and targeting materials preparation were accomplished by unique analytical tools.
CHAPTER 1. INTRODUCTION

1.1 RESEARCH MOTIVATION, GOALS, AND SYNOPSIS

Breast cancer still imposes a significant health burden on women worldwide. As is true with most other cancer diseases, early diagnosis and implementation of prompt treatment are crucial in battling this disease. Biological markers from blood, urine, and tissue can signal predisposition, onset, response to drug treatment, and recurrence of breast cancer. Thus discovery and early detection of biomarkers are among the techniques that play significant roles in early diagnosis, effective prognosis, and close monitoring in treatment of breast cancers.

There are several major classes of biomarkers for each specific type of cancer: mutations (changes) in genetic materials, usually deoxyribonucleic acid (DNA, Figure 1.1 and 1.2) including small-scale mutation such as deletion, insertion, point mutation or exchange of a single nucleotide for another and large-scale mutations such as amplifications, deletions, formation of fusion genes, and loss of heterozygosity—DNA mutation. Over/under expression of gene activity reflected by mutations in messenger RNA (DNA $\rightarrow$ mRNA $\rightarrow$ protein), and the presence of functional proteins secreted in serum or on circulating tumor cells (protein biomarkers). Unfortunately, none of the biomarkers monitored to date are present in sufficient abundance to allow them to be sensitively and selectively detected using diagnostic assays such that they can act as sole prognostic of the disease. Thus it is highly desirable to test for the presence of multiple biomarkers in tandem so as to generate and interrogate information on one specific type of cancer. Moreover, different classes of biomarkers need to be evaluated in order to provide significant molecular profiling information for clinical practice in cancer diseases. Therefore, novel and sensitive technologies capable of simultaneous detection
of multiple breast cancer biomarkers would greatly improve the treatment and cure of this cancer disease.

**Top**

Adenine (A)  Guanine (G)  Cytosine (C)  Thymine (T)  Uracil (U)

**Bottom**

*Figure 1.1* Composition and structure of DNA. Top-bases composing DNA: Adenine, Guanine, Cytosine, and Thymine (Uracil is found in RNA). Bottom-schematic of the double-stranded DNA which is held together by hydrogen bonding between the base pairs Adenine and Thymine, and Guanine and Cytosine. Each strand is comprised of four types of nucleotides (A, G, C, and T) linked together by phosphodiester bonds.
Overall, the project described here focuses on development of a methodology for early diagnosis of breast cancer using simultaneous detection of biomarkers via a microfluidic device platform. This methodology is intended to combine the strengths of microfluidics — high throughput, low sample consumption, and low detection limit — with the highly parallel characteristics of the microarray technologies and the high sensitivity/low limit of detection near-IR fluorescence techniques. By achieving this methodology, it is to make multianalyte (multiple biomarkers originating from the same disease) detection will be a reality and, the accuracy of diagnosis will be improved by reductions in the false negative and false positive outcomes.

In brief, the goal is a hybrid biosensor system fabricated on a polymer-based microfluidic device that utilize a unique format — Zipcode universal DNA arrays that will direct each capture probe (genetic and protein capture probes for a given breast cancer biomarker) to a specific locus on the polymer device surface — that decouples the

![Figure 1.2 Chemical structure of a deoxyribonucleotide (nucleotide) composed of a nitrogen-containing base (purine or pyrimidine), a deoxyribose sugar, and a phosphate.](image)
hybridization event from the detection event, and thereby improves the accuracy of the technique. Here, the genetic biomarker (gene mutational sequence) that is named breast cancer type I gene, or BRCA1, will be prepared through a polymerase chain reaction (PCR)/ligase detection reaction (LDR) coupled assay using genomic DNA isolated from a breast cancer cell line as the template; the protein biomarker (cell surface over-expressed protein) will be semi-synthesized by cross-linking the anti-protein biomarker antibody with the cZipcode oligonucleotide (oligo) DNA sequence. Signal generation and transduction are achieved through near-IR fluorescence detection which minimizes background fluorescence interference and therefore increase the sensitivity of detection.

The specifics of the methodology are outlined in Figure 1.3, and have its heart the use of short, single-stranded DNA sequences (24mers, named “Zipcode oligos”) arrayed on PMMA microfluidic device surfaces. The genetic biomarker targeting material cZip11-BRCA1 consists of a mutated base (the red star in the sequence), a 24-mer sequence that is complementary to the Zipcode11 oligo DNA sequence or cZipcode11 sequence (the short piece of DNA sequence in orange color) on one end and a near-IR dye on the other end. The protein biomarker capture probe, for example, is a conjugate of the anti-human carcinoembryonic antigen (CEA) antibody with the cZipcode1 sequence (sequence in blue color). This antibody-oligo DNA conjugate will react with a specific antigen (the protein biomarker-red heart shape) and a detection antibody (labeled with a near-IR fluorescent dye) to form a sandwich immunoassay complex, or a protein biomarker targeting material. Finally, both genetic and protein biomarker targeting materials will be introduced to the arrayed Zipcode oligo DNA surfaces, and the hybridization interaction event will be interrogated by fluorescence generation, detection, and analysis.
1.2 GENERAL INFORMATION ON CANCER DISEASES

Cancers are still one of the most detrimental diseases at present. Cancers can be defined as a group of diseases in which cells grow uncontrollable either in a specific organ or spread to other parts of the body. They may be caused by external factors, such as alcohol, radiation, and chemicals, and internal factors, including inherited mutations, hormone, and immune conditions. General prevention strategies for cancers may include appropriate food and nutrient intake, reducing alcohol and tobacco consumption,
avoiding obesity, and considering chemoprevention. Detection and diagnosis of cancers varies among different types of cancers, but generally it is done by a series of screening and testing techniques for a specific type of cancer and further confirmed by biopsy or tissue examination.

Among the screening and testing methods, tumor markers are one of the targets used for early detection and diagnosis of cancers. Tumor markers are materials produced by tumor and other cells of the body induced by cancer and some select noncancerous conditions. Usually, these materials may be found in the blood and urine, and tumor and other tissues. Genomic and proteomic-based biomarkers are the most widely studied tumor markers for early detection of cancers. Genomic biomarkers are characteristic genetic mutations, or alterations in specific genes, while proteomic biomarkers are changes in protein shape, function, and pattern of expression due to cancerous conditions. Genetic alterations typically manifest themselves as the accumulation of multiple mutations in three types of genes: oncogenes such as K-ras genes, tumor suppressor genes of which p53 is an example, and DNA replication and repair genes. Compared to genomic biomarkers, protein biomarkers are much more complex due to the large total number of proteins in a cell, and the variations in proteins that exist among individuals, different cells, and even different stages of the same cell. Basically, protein biomarkers are very dynamic while genomic biomarkers are relatively static. Protein biomarkers can also be used for monitoring the progress of cancer diseases, the body’s response to drug treatment, and the relapse of cancer diseases.

Cancer researchers have so far achieved some success in promotion of the application of cancer biomarkers. For example, prostate specific antigen (PSA), a very specific protein biomarker for prostate cancer, has become an important clinical
screening target in the diagnosis of prostate cancer. For ovarian cancer which is usually
diagnosed at an advanced stage, the main efforts have been dedicated in searching for
early biomarkers such as those found in protein expression patterns between diseased and
normal people. Combined investigations of three candidate proteins in blood have also
proved to be helpful in differentiating between breast cancer from benign breast diseases
and healthy controls.

In terms of treatment, cancer can be treated in various ways such as surgery,
radiation, chemotherapy, immunotherapy, and hormones. Treatment is mainly determined
by an overall evaluation of given cancer conditions by a physician and preference of the
patient. For general information about cancers and their treatment, I refer you to the
American Cancer Society (ACS) web site (http://www.cancer.gov/).

1.3 THE BREAST CANCER PROJECT AT LSU

Breast cancer, one of the most burdensome cancers affecting women worldwide
(second most detrimental in the US), possesses most of the characteristics of all other
cancers. According to the most recent annual cancer report, approximately 1 in 8 women
living today will be diagnosed of breast cancer in their lifetime. However, the occurrence
rate is a function of geography: in high-risk regions such as North America and Western
Europe it is 1 in 4 females, while it is as low as 1 in 16 females in areas such as China
and Japan.

Regarding prevention of breast cancer, the most updated data show obesity,
exposure to alcohol, ionizing radiation, and hormone treatment all lead to an increased
risk of breast cancer, while exercise is one of the factors that can actually decrease breast
cancer occurrence. Chemoprevention, such as taking a drug named tamoxifen (a
compound with both estrogen-like and anti-estrogen properties, or a selective estrogen
receptor modulator) has proved to be effective in reducing the breast cancer occurrence or recurrence, but chemoprevention is only effective for women in high risk populations, and thus, it is not recommended for lower- and average-risk women because the possible harms may outweigh the benefits. Common screening techniques available for breast cancer include self-examination, mammography and clinical examination. It has been proved that mammography, a technique that has been used for almost 30 years, is able to produce much more sensitive results than self-examination and can locate tumors that are not commonly felt by self-examination; its implementation has been attributed to the decreased rate of breast cancer occurrence according to a recent survey (cancer facts and figures, 2006). Despite the fact that mammography has been the most frequently used modality for screening breast cancers it also produces false negatives, thereby failing to locate tumors in the breast, and also produces false positives, especially for dense-tissue breasts. Recent screening experiments have shown that magnetic resonance imaging (MRI) is more powerful and more sensitive than mammography, ultrasound, and clinical breast examination in detecting breast cancers. Biopsy is an invasive technique because a small portion of tissue is removed while all other methods of detection are non-invasive because no tissue will be removed for these tests. Although there is still some disagreement regarding which technique is more accurate, the most recent research by researchers in the Agency for Healthcare Research and Quality, a division of the U.S. Department of Health and Human Services suggests that noninvasive tests cannot replace the invasive test, i.e., biopsy, in order to get an accurate diagnosis because none of the non-invasive diagnosis techniques (mammogram, ultrasound, MRI, PET, Scintimammography) can eliminate false positives and false negatives. A summary of prevention techniques is listed in Table 1.1. For general information on breast cancers,
one of the most popular informational websites can be referred to at
http://www.breastcancer.org/.

**Table 1.1 Detection and Prevention Recommendations for Breast Cancer.**

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Average-risk women</th>
<th>High-risk women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer screening</td>
<td>Annual mammography every 1-2 year beginning at age of 40 years</td>
<td><em>BRCA</em> mutation carriers: monthly self-examinations beginning by age of 18-21, annual or semiannual clinical examinations beginning at age 25-35 years old, and annual mammography beginning at age of 25-35 years old.</td>
</tr>
<tr>
<td>Ovarian cancer screening</td>
<td>No screening</td>
<td><em>BRCA1</em> mutation carriers: annual screening using transvaginal ultrasonography and CA-125 serum levels beginning an age of 25-35 years old; optional for <em>BRCA2</em> mutation carriers.</td>
</tr>
<tr>
<td>Chemoprevention for breast cancer</td>
<td>None</td>
<td>Women at increased risk for breast cancer as defined by the Gail model and low risk for complications: tamoxifen chemoprevention.</td>
</tr>
<tr>
<td>Prophylactic mastectomy and oophorectomy</td>
<td>None</td>
<td>Women with ≥ 2 first-degree relatives with ovarian cancer: offer prophylactic oophorectomy after completion of childbearing or at age of 35 years old.</td>
</tr>
</tbody>
</table>

Treatment of breast cancer can vary for each and every individual, but nowadays the most commonly used treatment for breast cancer is comprised of radiation, chemotherapy, hormone, biological therapy, and surgery. As is true with most other cancer diseases, it is often futile and disfiguring to treat the cancer when it reaches its advanced stages. Therefore, it is crucial to develop techniques that are capable of detecting breast tumor formation accurately at its earliest stage and subsequently take appropriate measures for treatment. Strategies for early diagnosis of breast cancer or
breast cancer predisposition are usually carried out by screening for genetic mutations in genetic biomarkers, especially for high-risk populations, examining proteomic biomarkers such as Her2/neu, CEA, MUC-1, and mammoglobin. Genes that have been identified to be related to hereditary breast cancer are BRCA1, BRCA2, TP53, CHK2 and ATM, and they are generally involved in the maintenance of genomic integrity and DNA repair. Mutations in these genes usually result in truncated or malfunctional respective proteins in breast or ovarian tissues.\textsuperscript{15} A comprehensive breast cancer mutation database can be found at the Breast Cancer Information Core (BIC) website (http://research.nhgri.nih.gov/projects/bic/). Around 10% of breast cancers are caused by inherited genetic mutations (or abnormal genetic sequences), among which are mutations in two most commonly involved genes, namely Breast Cancer Type I Gene (BRCA1) and Breast Cancer Type II Gene (BRCA2), while a higher risk is found with those having BRCA1 abnormalities. People who inherit mutations in this gene will have a higher risk of developing breast cancer (50-85\% chance of developing breast cancer in their lifetime\textsuperscript{16}) as well as others, such as ovarian cancer. The BRCA1 gene, which expresses a specific protein in breast and ovarian tissue, has been found to be a DNA damage repair and tumor suppressor gene,\textsuperscript{17} meaning that, at its normal stage, it maintains normal cell growth of breast tissue and suppresses tumor formation through the process of protein expression.

Several of the most common and characterized protein biomarkers for breast cancers are listed in the following Table 1.2. The majority of proteins are membrane proteins and as a result have unique features. Displayed in Figure 1.4 is a schematic of different types of membrane proteins. Her2/neu, carcinoembryonic antigen (CEA), MUC-1 or CA15.3, mammaglobin have been found to be associated with breast cancer.
MUC-1 (CA15.3, a carbohydrate antigen marker), one member of the transmembrane mucins family, is found to be over-expressed in various human carcinomas including breast cancer carcinoma.\(^{18, 19}\) Mammaglobin, a member of a protein family recently designated as secretoglobins, has been identified as a serum-born breast cancer biomarker, and studies of the protein have shown that 70-80% of breast tumors are positive for it.\(^{20}\) Her2 is referred to human epidermal growth factor receptor 2 and also known as neu, Her-2/neu and c-erbB-2. This is a transmembrane glycoprotein, of which the intracellular domain has tyrosine kinase activity, while the extracellular domain (97–115kDa) is shed into the circulating body fluid and can be measured to test the expression level using serum samples.\(^{21}\) Her-2 overexpression is associated with a number of cancers although most investigations have been focused on breast cancers. Her-2 positive breast cancer
tends to result in a lower survival rate than Her-2 negative type. The Her-2 positive type of breast cancer is a candidate for treatment by trastuzumab (brand name Herceptin), a very specific monoclonal antibody against Her-2 antigen. Her-2 was initially found to be correlated to breast cancers at advanced stages with protein overexpression identified in 10-34% of invasive breast cancers.\(^{22}\) Her-2/neu amplification was also detected at a very low level and proved to be a valuable biomarker for early stage breast cancer.\(^{23}\) CEA was the first identified human tumor biomarker and is one of the most thoroughly characterized tumor associated antigens.\(^{24}, 25\) CEA has one IgV-like and a variable number of IgI-like domains, a putative transmembrane domain, and a cytoplasmic tail which can be replaced by a glycosyl phosphatidylinositol anchor. CEA's are encoded from the chromosome 19q branch, which form a family gene cluster (at least 20 genes including CEACAM1 to CEACAM8, CEACAMP1 to CEACAMP11 and PSG). The cellular functions of CEA consist of homophilic adhesion, heterophilic adhesion, E-selectin binding, tumor suppression, type I fimbriae binding, and transmembrane signaling.\(^{26}\) The CEA structure\(^{27}\) is illustrated in Figure 1.5.

In terms of clinical relevance, overexpression of CEA has been found in various types of tumors, including those from the colon, breast, and lung.\(^{28}\) CEA is especially valuable for evaluation and monitoring of metastatic breast cancer because protein overexpression was found in the serum of about 40-50% of patients.\(^{29}\) However, the percentages of cases targeting elevated levels of CEA in early breast cancer development vary considerably, primarily depending on the primary tumor site.\(^{30}, 31\) In addition, serial CEA levels are of particular clinical importance and are persistently elevated (\(>10\) ng/mL) in cases of relapse.\(^{32}\) The mostly used serum-born protein biomarkers are listed in Table 1.2.\(^{33}\)
Figure 1.5 Schematic of CEA structure. The molecular weight of CEA is raised up to 180kDa due to glycosylation.

Table 1.2 List of most widely used serum tumor markers in breast cancer.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Protein(s) detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 15.3</td>
<td>MUC-1</td>
</tr>
<tr>
<td>BR 27.29 (CA 27.29)</td>
<td>MUC-1</td>
</tr>
<tr>
<td>CEA</td>
<td>CEA</td>
</tr>
<tr>
<td>TPA</td>
<td>Fragments of cytokeratin 8, 18, and 19</td>
</tr>
<tr>
<td>TPS</td>
<td>Fragments of cytokeratin 18</td>
</tr>
<tr>
<td>Her-2 (shed from)</td>
<td>Extracellular form of Her-2</td>
</tr>
</tbody>
</table>
In summary, each of the biomarkers, either gene or proteins, can provide some assistance in prevention, diagnosis, prognosis, and monitoring the treatment of breast cancer, but none of them is by itself conclusive enough to evaluate the status of the disease. Thus a novel analytical platform is urgently needed where multiple biomarkers from different classes can be assayed with high sensitivity and specificity.

1.4 STRATEGIES FOR DISCOVERY AND EARLY DETECTION OF BREAST CANCER BIOMARKERS

Since it was initially mapped to chromosome arm 17q in the early 1990s, considerable efforts have been devoted to \textit{BRCA1} research including development of a reliable, inexpensive, accurate, and sensitive testing methods, such as a combination of PCR amplification and 2-D electrophoresis and fluorescence-assisted mismatch analysis for screening.

Microarray technology has also been extensively explored for detection of mutations in the breast cancer related gene \textit{BRCA1}. In these reports, all \textit{BRCA1} coding exons from genomic DNA were PCR amplified using intronic forward and reverse primer pairs containing T3 and T7 promotor sequences to allow transcription using RNA polymerases and biotinylated or fluorescently labeled dUTP analogs to permit detection. These transcribed RNA targets were then hybridized to the \textit{BRCA1} array, and two-color imaging was implemented to obtain information in regards to the polymorphic nature of specific sites in the coding regions.

Screening tests on \textit{BRCA1} and \textit{BRCA2} genes, especially among high-risk populations, have been more and more acceptable to the public. Appropriate measures, such as adjustment of lifestyle, undergoing chemoprevention, and mastectomy (surgery to remove the affected breast) would be taken by people if diagnosed of carrying
mutations in these genes. A great amount of effort has been devoted to developing practical, inexpensive, fast, sensitive, and accurate techniques for screening gene variations of particular connection to cancer diseases. The common strategies used for analyzing genetic mutations can broadly be categorized into two different types, direct and indirect. Direct methods, which include DNA sequencing, document the existence of the particular mutation and also reveal its exact nature (A, C, G or T in the case of single-point-mutations). Indirect methods typically screen large sections of genes for mutations but do not provide information relating to the exact composition of the mutation. Techniques commonly performed for screening mutations in these two breast cancer related genes are summarized in Table 1.3. As noted in Table 1.3, there are a number of potentially powerful techniques to analyze various types of mutations in BRCA1 or BRCA2, all possessing particular advantages and disadvantages. For example, the protein-truncation test (PPT), an in vitro translation technique, has been used mainly for searching for small-insertions or deletions, or nonsense mutations that lead to the introduction of a stop codon, and hence a result of truncated proteins. This technique is fast and inexpensive but not very sensitive, so it is limited to screening only for mutations in large exons, such as exon 11 in the BRCA1 gene, and exon 10 and 11 in the BRCA2 gene.

Since it was developed in mid 1990s, denaturing high-performance liquid chromatography (DHPLC) has proved to be a promising, sensitive, and cost-effective technique for detecting genetic alterations predisposed for a particular disease. It has since attracted interest from various fields and has been applied to identifying mutations related to a variety of genetic diseases, such as NPM1 mutations in acute myeloid leukemia, specific variants in breast cancer related genes BRCA1 and BRCA2.
In comparison to the conventional HPLC method, this technique involves a unique stationary phase (DNASep, Transgenomic Inc., San Jose, CA) made of alkylated nonporous poly(styrene-divinylbenzene) particles 2-3 microns in diameter, which enables the separation of nucleic acids by means of ion-pair reverse-phase liquid chromatography.\(^5\) Briefly, the principle governing the separation of nucleic acid with DHPLC is that the positively charged triethylammonium ions are adsorbed at the interface between the nonpolar stationary phase and the hydroorganic mobile phase, and

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-truncation test</td>
<td>Cheap; rapid; allows detection of genomic deletions</td>
<td>Mutations can be missed when gene product is very short; does not missense mutations; RNA required to examine small exons</td>
</tr>
<tr>
<td>Single-strand-conformation analysis of genomic DNA</td>
<td>Simple; well-established techniques</td>
<td>Low sensitivity; labor-intensive; does not detect exon deletions</td>
</tr>
<tr>
<td>Denaturing high-performance liquid chromatography</td>
<td>Detects almost all intra-exonic and splice-site mutations; rapid</td>
<td>Expensive equipment required; does not detect exon deletions</td>
</tr>
<tr>
<td>DNA chips</td>
<td>Can potentially identify all sequence variants; very rapid</td>
<td>Expensive equipment required; high cost per chip</td>
</tr>
<tr>
<td>Direct sequencing</td>
<td>Identifies most intra-exonic and splice-site mutations</td>
<td>Expensive; exon deletions can be missed if detailed single-nucleotide-polymorphism analysis is not carried out</td>
</tr>
<tr>
<td>Multiplex ligation-dependent probe amplification</td>
<td>Detects all exon deletions</td>
<td>Cannot detect intra-exonic mutations</td>
</tr>
</tbody>
</table>
therefore leads to a positive stationary phase surface potential and retention of dsDNA results from electrostatic interactions between the positive surface potential on the stationary phase and the negative charges of the phosphodiester groups of the dsDNA. Despite all the advantages of DHPLC, it needs to be coupled to other techniques to determine base compositions, and further development is necessary to make it more amenable and quantitative in order to reach gene mapping levels.

The most sensitive and reliable gene screening technique is probably direct sequencing because this technique is able to provide detailed base information quantitatively; it is, therefore, especially useful as a screening of genetic mutations corresponding to particular cancer diseases. Unfortunately, it is often technically demanding, costly, and time-consuming. Researchers have invested significant effort in finding fast, sensitive, friendly techniques for detecting alterations in large genes as alternatives of direct sequencing or prior to sequencing. Gross and coworkers compared mutation analysis on \textit{BRCA1} gene using direct sequencing, single-strand conformation polymorphism (SSCP)\textsuperscript{51} and DHPLC, and found out that DHPLC resolved 100\% of the DNA alterations while SSCP detected 94\% variations as were observed in cyclic sequencing.

Single-strand-conformation analysis of genomic DNA (SSCA) is a technique traditionally used in locating gene alterations. It has been used to successfully detect mutation spectrum in Danish young female breast cancer patients by screening regions of \textit{BRCA1} and \textit{BRCA2} genes.\textsuperscript{52}

Multiplex ligation-dependent probe amplification (MLPA) is another quantitative technique that has been proved to be powerful in determining multiple deletions and duplications in \textit{BRCA} genes simultaneously in a single reaction.\textsuperscript{53} In brief, this technique
Involves two probes hybridized to the target sequence (genomic DNA) and joined together by a ligase to make a copy of that sequence. The probes are so specially designed that all the sequence products will be amplified using the same pair of primers. This feature contributes to the simultaneous nature of this amplification technique, but this technique cannot detect intra-exonic mutations.

In general, most of the screening techniques described above involve a PCR step to amplify the genomic DNA materials preceding the sequencing, followed by gel electrophoresis to analyze the fidelity or to identify the compositions of the sequencing target. It turns out that all of these techniques are labor intensive, and some, such as SSCA, cannot provide sufficient levels of sensitivity.

During the past one and a half decades, a new technique named oligonucleotide arrays, or DNA chips has evolved, and it is clear that this is a powerful tool in acquiring genomic information in a parallel, rapid fashion. Since the pioneering work by Fodor and coworkers in the early 1990s on light-directed spatially addressable combinatorial chemical synthesis of oligonucleotides, which makes high density oligo arrays possible and readily available (Affymetrix), DNA chips have found numerous applications from rapid DNA sequence analysis and mutation detection in various types of genes to gene expression monitoring, gene function analysis, and even optimization of antisense oligo design. Generally speaking, this technique is based on the differentiation of fluorescence signals between perfect-matched hybridization reactions and mismatched hybridization reactions; it is so sensitive that it can detect single-base pair mismatch among targeting sequences due to the high density, highly diversified probes arrayed on the chip surfaces. Specifically for breast cancer, microarrays, or DNA chips have found diverse applications, such as monitoring chromosome gains and losses,
tumor classification, drug discovery and development, DNA resequencing, mutation
detection and investigation of the mechanism of tumor development.\textsuperscript{63}

To summarize, a variety of practical techniques used for screening \textit{BRCA} genes in
identifying predisposition mutations has been developed, and this has to date served the
increased request for gene screening by high-risk populations. Nevertheless, there is still
a desperate need to find appropriate antibodies against \textit{BRCA1} to evaluate protein levels
in normal and tumor tissues, but sadly the so-far-raised antibodies against various
epitopes on \textit{BRCA1} have not been proved valuable yet by immunohistochemical (IHC)
analyses due to their low reproducibility.\textsuperscript{15} Moreover, genetic markers may in some cases
not suffice to correctly identify a specific phenotype, especially in the early stages of
tumor development due to the static nature of the cell’s genome. Therefore, it is
desirable to look at protein expression levels because the proteome (entire complement of
proteins expressed by a single cell, tissue or organ) profile is highly dynamic (responds
quickly to external stimuli), and it is the proteins that comprise the proteome that regulate
processes resulting in the phenotype.

In general, screening for protein expression qualitatively is done by Two-
dimensional gel electrophoresis, following extensive sample preparation to isolate
interested protein types.\textsuperscript{64} Two-dimensional (2-D) gel electrophoresis typically involves
carrying out isoelectric focusing based on the isoelectric point of each protein component
along one axis, then sodium dodecylsulfate (denaturing) poly(acrylamide) gel
electrophoresis (SDS-PAGE) along the second axis to further differentiate proteins
according to molecular weights. Following 2-D separation, staining of the gels to label
the separated proteins is achieved using coumassie blue or silver ion. This modality has
been the classic way for protein mapping analysis for years, but it has become less
popular as a result of its limitations of poor dynamic range, the associated intensive labor, long analysis times, and irreproducibility. Moreover, due to the diverse nature of various protein components, this technique requires pretreatment of samples, such as fractionation via isoelectric focusing. Therefore, much effort is being invested into developing automated methods for analyzing proteomes among which are DNA microarray techniques developed recently to study proteomics based on the assumption that mRNA transcription levels correlate well with the corresponding protein translation levels; however, this genomic-based proteomic study has proved to be in poor correlation.

Protein chips, or protein microarrays have replaced DNA microarrays in the study of proteomics and implementation in the development of biologically integrated devices, and thus, they have become a viable alternative to two-dimensional gel electrophoresis on protein expression profiling analysis. Protein microarrays, or protein chips are nothing more than a spatially defined immobilization of proteins on a solid substrate (protein patterning). This technique was originated in the mid 1980s when researchers tried to integrate biomolecules into miniature biological-electronic devices. Commonly used techniques for creating protein arrays (micron or nanometer scale, in 2-D or 3-D) on surfaces include use of conventional photoresist lithography, photochemistry, self-assembled monolayers (SAMs), and dip pen nanolithography. Protein chips are normally analyzed by transduction of detectable fluorescent signals and by use of imaging techniques, such as scanning electron microscopy (SEM), atomic force microscopy (AFM) and time of flight-secondary ion mass spectrometry (TOF-SIMS). Protein chips have also been implemented in detection of tumor
biomarkers,\textsuperscript{87} and protein profiling for differentiating normal or pre-malignant cells from cervical cancer cells.\textsuperscript{88}

Mass spectrometry has recently entered the proteome research arena and it is clear that it has great capability and potential—especially when it is coupled with other conventional or non-conventional techniques—in identification and quantification of protein expression levels from cells, tissues, and other organisms. Traditionally matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) has been combined with two-dimensional gel electrophoresis for peptide fingerprinting after in-gel proteolytic digestion.\textsuperscript{89} A modified version of 2-D separation coupled with mass spectrometry was recently reported in which a 2-D liquid phase separation method composed of isoelectric focusing in one dimension and non-porous silica reverse-phase high-performance liquid chromatography as another, was coupled to electrospray ionization-time of flight mass spectrometry (ESI-TOF MS); this led to successful recognition of potential breast cancer biomarkers from cell lysates of breast epithelial cell lines.\textsuperscript{89}

Not only has mass spectrometry been coupled to 2-D separation techniques in the study of proteome, it has also been combined with protein biochips, and has proven to be an even more powerful tool in identification, differentiation and quantification of proteins, especially biomarkers used in diagnosis and monitoring of cancer diseases.\textsuperscript{88} Protein chips together with surface enhanced laser desorption/ionization (SELDI) mass spectrometry, has successfully detected prostate cancer biomarkers in complex protein mixtures.\textsuperscript{90} In fact, 2-D separation, mass spectrometry, and protein biochips have formed an indispensable technical triade in proteomics;\textsuperscript{91} it will be interesting to see what powerful things it can achieve in years to come.
While a large fraction of the aforementioned strategies allow for study of many different target proteins, it is not really necessary in some cases, especially when investigations are carried on readily defined biomarkers, whether they be nucleic acids or proteins. It is more appropriate to target only those biomarkers for diagnosing and monitoring diseases; large-scale screening does not actually add any significant information. As such, the single-element biosensor platform has become important in the arsenal of tools for diagnosing cancers and monitoring its treatment. This type of biosensor system is typically comprised of a specific biosensing film on which a biological probe is immobilized on the support, and a measurable signal (transduction) is generated upon interaction of the probe with a target molecule. For instance, in an immunoassay (in forward format), an antibody (Ab) is attached on a solid substrate to capture its specific antigen (Ag), and the signal readout will be analyzed upon introduction of a secondary Ab that carries a fluorescent dye, or another type of tag, such as an enzyme or electrochemically active groups. In a protein sensor, the target protein can also be differentiated from interfering protein components by the differing transduced signal intensity in a protein assay. In the case of DNA sensors, a nucleic acid probe (typical length of 24 mer) is chemically tethered to the sensor surfaces and hybridized to its target sequence; the presence of mutations or mismatches in the target and probe sequences result in a signal which can be differentiated from the fully matched hybridization sequence pair.

For breast cancer, a number of biosensor platforms for detection of and monitoring biomarkers have been developed. For example, Wang and co-workers reported a silicon-based ultrasonic immunosensor for detection of breast cancer antigens CA15.3 in the sera of breast cancer patients. The sensor is composed of a flexural plate
wave gravimetric platform where CA15.3 antibodies were immobilized on the plate surfaces. Upon association with CA15.3, a mass increase resulted. Wang and Kawde developed a pencil-based biosensor for label-free electrochemical detection of DNA hybridization events.\textsuperscript{93} In this work, a tiny electrode (pencil lead) was electrochemically loaded with the DNA probe which consisted of an inosine-substituted E908X-WT oligonucleotide to detect point mutations in \textit{BRCA1} gene. The authors demonstrated that they were able to discriminate between fully matched and single-base mismatched duplexed DNA by measuring the electrochemical signal generated from the sensor system.

1.5 STRATEGIES FOR MULTIANALYTE ASSAYS

In order to provide the ability to accurately detect early onset of breast cancer, a sensor system which is able to simultaneously determine the presence of known biomarkers for this specific cancer is highly desired. Detecting or sensing multiple analytes without a pre-detection separation step has always been an endeavor for researchers in the sensor development arena. The general principle for sensing detection with a recognition probe is based on some type of specific interaction between the target molecules and the capturing probes that are immobilized on a substrate surface. There are two these types of specific assays, namely antibody-antigen interaction-based and DNA-DNA pairing interaction-based, both of which depend on reversible, non-covalent bonding forces (hydrogen bonding, and/or hydrophobic interactions, electrostatic, and van der Waals interactions). Assays that are based on antibody-antigen interactions are called immunoassays, or solid-phase immunoassays. Normally, specific antibodies are immobilized on a solid substrate to capture specific antigens (target molecules in a sample matrix) and analyzed through signal generation methods that employ a trace
antibody, or detection antibody, that are labeled with a fluorescent dye to form a sandwich immunoassay (non-competitive immunoassay), or through a competitive immunoassay format, such as a displacement immunoassay.

Incorporating multi-analyte assays into the same platform has been studied extensively and is being pursued by some research groups summarized in Table 1.4, among which are Ekins, et al., and Kakabakos, et al., who first developed the concept of multianalyte immunoassay (MAIA) using confocal and time-resolved fluorescence microscopy respectively. Plowman and coworkers reported a multiple analyte immunoassay based on an integrated optical waveguide sensor in which multiple antibodies were patterned on a planar waveguide to bind target antigens in the sample matrix. The transduced signals were generated by introducing multiple detection antibodies to form sets of sandwich immunocomplexes. Frances Ligler’s research group at the US Naval Research Laboratory has invested significant efforts in the sensor research arena, especially in regard to a variety of multianalyte biosensor platforms. A typical direction in Ligler’s work is the use of a polydimethoxysilane (PDMS) stamp with parallel channels, was used to immobilize the capture molecules, protein (Ab), or ligand. Another similar PDMS stamp with channels running perpendicular to those in the first stamp was sealed to the substrate surfaces tightly, and targeting agents and fluorescently labeled detection ligands were flowed through these channels. The glass microscope-based waveguide was used in conjunction with a CCD camera, a laser, and a few other optical components to yield a fluorescence reader system capable of reading out fluorescence intensity as a function of positions.

It is important to point out that Ligler’s group not only has extensively researched and applied their biosensor systems to various targeting agents, such as toxins, bacteria,
viral, and proteins, but has also investigated different substrates used for building the biosensor system. The substrate material used for building the waveguide arrays range from the initially used glass-based support to the more recently adopted polymeric material, in which they have been attempting to incorporate the newly evolved polymer-based BioMEMS concept into the multianalyte biosensor system.\textsuperscript{1, 105, 106} Interestingly, all the biosensor work reported from Ligler’s group, whether it be based on single- or multiple-analyte assays, predominantly utilizes fluorescence as the detection methods presumably because that this is the most sensitive analytical techniques employed so far.

Table 1.4 Examples of multiple analyte immunoassays.

<table>
<thead>
<tr>
<th>Transducer</th>
<th>MAIA configuration</th>
<th>analytes</th>
<th>detection method</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOW/IRE</td>
<td>etched wells</td>
<td>IgGs</td>
<td>fluorescence</td>
<td>100s of ng/mL</td>
</tr>
<tr>
<td>IC technology</td>
<td>proposed</td>
<td>fluorescence</td>
<td></td>
<td>n/a</td>
</tr>
<tr>
<td>spatial arrangement</td>
<td>proposed</td>
<td>interferometry</td>
<td></td>
<td>pM</td>
</tr>
<tr>
<td>spatial arrangement</td>
<td>three toxins</td>
<td>fluorescence</td>
<td>ng/mL</td>
<td></td>
</tr>
<tr>
<td>spatial arrangement</td>
<td>ovalbumin, SEB</td>
<td>fluorescence</td>
<td>ng/mL</td>
<td></td>
</tr>
<tr>
<td>deskjet printing</td>
<td>IgG subclasses</td>
<td>fluorescence</td>
<td>ng/mL</td>
<td></td>
</tr>
<tr>
<td>Fiber</td>
<td>encoded microspheres</td>
<td>AP, Av, biotin</td>
<td>fluorescence</td>
<td>N/A</td>
</tr>
<tr>
<td>SPR</td>
<td>spatial arrangement</td>
<td>hcG only</td>
<td>angle of reflection</td>
<td>N/A</td>
</tr>
<tr>
<td>Microscope Slide/others</td>
<td>spatial arrangement</td>
<td>TNF, TSH</td>
<td>confocal microscopy</td>
<td>pg/mm\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>chemometrics</td>
<td>three s—triazines derivatives</td>
<td>reflectometric interference spectroscopy</td>
<td>pg/mm\textsuperscript{2}</td>
</tr>
<tr>
<td></td>
<td>multiple capillary tubes</td>
<td>TNT, RDX</td>
<td>fluorescence</td>
<td>ng/mL</td>
</tr>
<tr>
<td></td>
<td>spatial arrangement</td>
<td>LH, FSH,hcG, PRL</td>
<td>time-resolved fluorescence</td>
<td>100s of ng/mL</td>
</tr>
</tbody>
</table>
In addition to fluorescence there are some other types of optical methods for interrogating biosensing films, including the so called label-free techniques, such as interferometry, resonant mirror, and metallic waveguide, or surface plasmon resonance.\textsuperscript{107} Electrochemical methods are third in popularity in the biosensor arena. Wilson and coworkers reported electrochemical multianalyte immunoassays using an array-based sensor based on eight iridium oxide sensing electrodes possessing four different antibodies, an iridium counter electrode, and a Ag/AgCl reference electrode, all of which were patterned on a glass substrate. Single generation and transduction of protein targets were achieved by ELISA, measuring hydroquinone generated by electrochemical oxidation.\textsuperscript{108} There are still other strategies associated with analyzing multiple targets simultaneously, such as those performed on the microfluidic platforms; these will be discussed in detail after a brief introduction to BioMEMS technology.

In addition, there are also a few commercially available biosensor systems that can perform multianalyte assays and the list is summarized in Table 1.5.\textsuperscript{102}

<table>
<thead>
<tr>
<th>Company</th>
<th>System</th>
<th>Fluidic</th>
<th>Automation</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biacore</td>
<td>SPR</td>
<td>Four</td>
<td>Partial</td>
<td>Mass change</td>
</tr>
<tr>
<td>IGEN</td>
<td>ORIGEN</td>
<td>Free solution</td>
<td>Partial</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Fisons Affinity Sensor</td>
<td>IAsys</td>
<td>Cuvette (2)</td>
<td>Partial</td>
<td>Resonance</td>
</tr>
<tr>
<td>Lifepoint</td>
<td>IMPACT</td>
<td>Minicolumns (10)</td>
<td>Partial</td>
<td>Fluorescence</td>
</tr>
</tbody>
</table>

1.6 MULTIANALYTE ASSAYS ON MICRODEVICES

The area of miniaturized devices more commonly known as lab on a chip, or micro total analysis systems (\(\mu\)TAS) has seen rapid growth both in academia and industry in recent years. The first analytical miniaturized device was actually developed as early as the 1970s when a silicon-based gas chromatographic analyzers were reported by
researchers from Stanford\textsuperscript{109, 110} and IBM.\textsuperscript{111, 112} These reports did not initially attract much attention from the research community, probably there was not the need for miniaturized separation science methods then, people did not anticipate the significant breakthroughs possible as a result of miniaturized devices. It was not until 1990 when Manz proposed the miniaturized total chemical analysis systems concept\textsuperscript{113} that the area of miniaturized devices reemerged; subsequently this area has rapidly developed into a field of its own as a result of the efforts of various research groups all over the world. μTAS, or lab-on-a-chip has seen a dramatic increase in growth especially after the mid 1990s when more research groups joined the endeavor to develop this area. The applications of such “chip-based” devices have also broadened, such as the dominant application of separating nucleic acids, amino acids or/and other species, the more specialized separations,\textsuperscript{114, 115} cell manipulation,\textsuperscript{116} bio- and chemical reactors,\textsuperscript{117-123} and even miniaturized mass spectrometers.\textsuperscript{124} Biological microelectromechanical systems (BioMEMS), which fall among the commonly known category of “lab-on-a-chip”, or μTAS, resulted from the marriage between integrated circuits and biotechnology, on which both electrical and mechanical components (typically in micrometer size) are integrated and directed for biochemical and chemical assays.

Microfabrication of BioMEMS devices has mainly accomplished using chemical etching and lithography because the substrate used was predominantly glass or silicon at the early stage of the BioMEMS area. More diverse substrate materials have come into this field in recent years, and polymers (plastics) have attracted more and more attention because there are a variety of microfabrication technologies amenable for polymers that make device fabrication via mass production a tractable option. Microfabrication in polymers consists of hot embossing,\textsuperscript{125} injection molding,\textsuperscript{126} imprinting,\textsuperscript{127} laser
ablation,\textsuperscript{128} and soft lithography.\textsuperscript{129, 130} Initial applications of polymer-based BioMEMS were in the separation field and gradually expanded to many other areas such as immuosensor systems.\textsuperscript{131} Immunoassays can be performed in microfluidic chambers by immobilizing antibodies covalently or by adsorption on the surface of the microdevice, flowing in the targeting proteins, and tranducing the signal with optical, electrochemical, or other techniques. Multianalyte assays using microfluidic platforms have also been made possible, especially with the advent of soft lithography technologies. In the 2005 review of microfluidic techniques in microarray applications by Grodzinski and others,\textsuperscript{132} several strategies were summarized that target multianalyte assays mainly directed towards nucleic acid analysis. In one strategy, multiple nucleic acid probes were patterned by a soft stamp usually made of PDMS, followed by hybridization with their complementary sequences introduced by another perpendicularly aligned PDMS stamp, followed by detection with either optical or electrochemical methods. Using these strategies, high density arrays can be made or high complexity can be achieved to analyze many different targets. Depending upon the needs of the device with respect to array density, multiple nucleic acid probes can in fact be directly spotted onto the microchannel before sealing the device, and then interact with a mixture of targeting sequences introduced into the sealed channel. Patterning proteins or antibodies on microfluidic devices to perform multianalyte assays or specifically multianalyte immunoassays have also been achieved in the same concept as that with nucleic acids.\textsuperscript{131}

1.7 HYBRID MULTIANALYTE ASSAYS IN MICRODEVICES

As can be seen from the previous section, all the multianalyte assays are directed towards the simultaneous detection of the same type of analytes—either proteins or nucleic acids. There has not been much investigation of the hybrid multianalyte assays, in
which different type of targeting agents can be analyzed on the same platform simultaneously, due to the fact that there exist significant biases among different targets in terms of surface immobilization methods, choice of biological buffer systems, and reaction conditions.

In the following research work, a novel strategy to simultaneously analyze both protein antigens and gene sequences will be presented. In this strategy, a universal DNA microarray is fabricated using the mature PDMS patterning technique. Gene mutated materials and the protein antigen target are captured by hybridization interactions, and then signals are generated by near-IR, laser-induced fluorescence. Pre-treatment of both gene and protein targets are carried out; gene target materials are prepared using PCR/LDR combined assays which incorporate the complementary Zipcode DNA sequence and the near-IR fluorescent tag into the target sequence, while protein target materials are semi-synthesized to form a complex that carries a complementary Zipcode DNA sequence and a sandwich immunoassay.

1.8 REFERENCES


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CHAPTER 2. PHOTOCHEMICALLY PATTERNED POLY(METHYL METHACRYLATE) SURFACES USED IN THE FABRICATION OF MICROANALYTICAL DEVICES*

2.1 INTRODUCTION

Biological microelectromechanical systems (BioMEMS) have attracted significant interest in recent years due to their potential impact on biological analysis, drug development, and medical diagnostics.1-5 The promise of BioMEMS in instrument development and assay methodologies is associated with the intrinsic advantages of BioMEMS, such as small footprint and easy multiplexing, and with the potential for integration of various components into the system, as well as mass production of the devices at an attractive cost. Since the first fabrication of micro-electrophoresis devices in glass substrates using photolithography and wet chemical etching,5, 6 various microdevices have been constructed and used for bioanalytical applications, such as PCR amplification of oligonucleotides,7, 8 separation of single-stranded9-11 and double-stranded DNA,12-15 and protein analysis.15, 16

Most of the early BioMEMS devices were made of glass, quartz, or silicon because of the well-established micromanufacturing techniques and surface chemistry of silicon and silicon oxides, their rigidity, and optical properties.17, 18 The use of polymer substrates in the construction of BioMEMS devices has become increasingly important because microstructures, including those with high aspect ratios, can be readily produced in polymers using rather straightforward processing methods.19, 20 In addition, massive fabrication of most parts of polymer-based BioMEMS devices can be achieved using various techniques such as laser ablation,21 injection molding,22 hot embossing,23

imprinting,\textsuperscript{24} and soft lithography.\textsuperscript{25, 26} Moreover, the relatively low glass transition temperature ($T_g$) of most polymers should make it possible to attach biomolecules before devices are assembled (thermal annealing), which cannot be achieved in SiO$_2$-based devices because of the high glass transition temperature ($T_g > 600$ °C) of this material. Poly(methyl methacrylate), PMMA, a thermoplastic polymer, is one of the primary polymeric materials that has been used for fabrication of microstructured devices.\textsuperscript{19, 27, 28}

Due in part to the high surface-to-volume ratio, surface characteristics, such as wettability, surface topography, and interfacial charge density and distribution, are crucial factors that govern the functional capabilities of BioMEMS devices.\textsuperscript{29-32} Chemical modification techniques for SiO$_2$-based devices are well established using silane-based chemistry,\textsuperscript{17} but a wide variety of routine, simple, and well-defined surface modification protocols for polymers used in BioMEMS applications is needed.\textsuperscript{28, 33, 34} Preformed PMMA microchannels have been modified using a pulsed UV excimer laser to reduce band broadening effects in electrophoretic separations,\textsuperscript{34} and poly(carbonate) microchannels have been made “more hydrophilic” by UV irradiation.\textsuperscript{35} PMMA surfaces have also been made more water wettable by direct amination,\textsuperscript{29} vapor-phase deposition of organic films,\textsuperscript{36} copolymer grafting,\textsuperscript{37} or by formation of molded devices via photo- or thermo-polymerization of co-monomers.\textsuperscript{38, 39} In addition, it has been shown that PMMA surfaces can be readily patterned through techniques such as photo-directed electroless deposition,\textsuperscript{39} photoinitiated grafting,\textsuperscript{40} and exposure to deep UV sources.\textsuperscript{41, 42} Electroosmotic flow was also controlled by either UV laser ablation in preformed PMMA microchannels,\textsuperscript{34} or PEG grafting after PMMA surfaces were activated by oxygen plasma treatment.\textsuperscript{43} Continued expansion of surface modification routes is essential to the development of BioMEMS technologies based on polymer substrates.
Reported here is a photochemical surface modification protocol for PMMA, a well-known substrate material for the fabrication of BioMEMS devices. It has been found that surface carboxylic acid groups are formed upon UV exposure of PMMA surfaces in the atmosphere (surface photochemical modification), and the resulting surface carboxylic acids allow for further functionalization of PMMA-based BioMEMS devices. Surface properties of the PMMA for each step of treatment process are systematically investigated—qualitatively and quantitatively—by water contact angle measurements, scanning force microscopy, X-ray photoelectron spectroscopy, fluorescence microscopy, and specific functional group labeling. In addition, the effect of UV modification on electroosmotic flow in hot-embossed PMMA microchannels is demonstrated. Finally, based on the outcomes from deconvolution of X-ray photoelectron spectra of pristine and UV-exposed PMMA surfaces, a possible mechanism for the photochemical carboxylic acid formation is discussed.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL

2.2.1.1 GENERAL

Poly(methyl methacrylate) sheets, Plexiglass or Lucite, were purchased from Goodfellow and AIN and were machined to various-sized pieces. Before any type of experimental analysis was carried out, PMMA pieces were sonicated in isopropanol (IPA) for 15 min, then rinsed with IPA and subsequently dried with a flow of house nitrogen; scanning force microscopy did not indicate any increases in surface roughness as a result of this cleaning protocol. HPLC-grade isopropanol (IPA) and all other chemicals were obtained from Aldrich and used without any further purification unless noted otherwise. The UV light source used here is a low-pressure mercury lamp possessing an emission spectrum.
spanning the 240 nm to 425 nm range; the 254 nm band is the strongest with an intensity of 15 mWcm\(^{-2}\), while that of all others is less than 1.5 mWcm\(^{-2}\) at a 1-cm distance. The microfluidic electrophoresis devices were fabricated using a method previously developed.\(^4\) In brief, a metal molding die, which consists of raised microstructures electroplated from Ni on a stainless steel base plate, was made using the X-ray LIGA technique.\(^1\), \(^4\) PMMA microstructures were embossed in a system that consisted of a PHI Precision Press (model number TS-21-H-C (4A)-5; City of Industry, CA), in which a vacuum chamber was installed to produce low pressure (<0.1 bar) for complete filing of the Ni molding die. During embossing, the molding die was heated to 150 °C and pressed into the sheet PMMA at 1000 lb for 4 min. The PMMA part was then cooled to 85 °C for demolding. The embossed PMMA substrate (0.5-cm thick) was annealed to a piece of PMMA cover plate (0.05-cm thick) to enclose the microfluidic channel, which was achieved by clamping both parts with glass plates and heating at 105°C in a convection oven for 15 min. To make UV-modified (carboxylic acid-modified) PMMA microchannels, the embossed PMMA substrate was first exposed to UV light for 30 min then rinsed with IPA. The cover plate was also processed in a similar fashion, and both PMMA pieces were then annealed at 98 °C for 15 min in the oven described above. The microfluidic electrophoresis device consists of cross-flow channels with a separation channel length of 4 cm, a channel depth of 100 \(\mu\)m, and channel width of 50 \(\mu\)m.

### 2.2.1.2 CONTACT ANGLE MEASUREMENTS

Sessile water contact angle measurements were used to probe the effect of UV exposure on the surface hydrophilicity of PMMA. Contact angle values on PMMA surfaces were obtained with a VCA 2000 contact angle system equipped with a CCD camera (VCA, Billerica, MA). Approximately 2 \(\mu\)L of deionized water (18 MΩ cm)
were placed on the PMMA surface using a syringe, and the contact angle of the water droplet was measured immediately using the software provided by the manufacturer. The measurements were repeated at least five times at separate positions on a given substrate, and the values here are reported as the mean ± one standard deviation.

2.2.1.3 SCANNING FORCE MICROSCOPY (SFM)

The surface topographies of the pristine/unmodified and UV-modified PMMA surfaces were assessed using a Digital Instruments Nanoscope III multimode scanning force microscope in non-contact (Tapping) force mode. The images presented were treated by use of the flatten algorithm using the Nanoscope software. RMS roughness was calculated using the software provided by the vendor.

2.2.1.4 CHEMICAL MAPPING OF THE FUNCTIONALITIES INDUCED BY PHOTOLYSIS ON PMMA SURFACES

The protocol is outlined in Scheme 2.1 and is as follows: a piece of PMMA sheet (2.54 cm x 7.62 cm x 0.05 cm from AIN Plastics) was ultrasonicated in isopropanol for 15 min, rinsed with isopropanol (IPA), and dried with house N₂. The sample was placed on a microscope slide and a 2000 mesh Ni grid (SPI; hole size of 7.6 μm and wire size of 5 μm) held on the sample with an Al dumbbell-shaped mask. The piece of PMMA was placed under the UV lamp for 30 min. Next, the PMMA was rinsed with isopropanol (IPA) and dried with house nitrogen.

The UV-modified PMMA sheet was immersed in 0.5 mM N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) + 0.5 mM fluoresceinyl glycine amide (Molecular Probes) in pH 7.0, 100 mM phosphate buffer. Then, the immersed PMMA was placed on a slow shaker to react overnight. The PMMA sheet was rinsed with pH 7.0 phosphate buffer and dried by tilting onto a Kim Wipe. Fluorescence images were then
immediately taken with a Nikon Photoshot FxA fluorescence microscope. The excitation and the emission filters used were 488 nm and 520 nm, respectively.

**Scheme 2.1** Chemical mapping of carboxylic acid functionalities with fluoresceinyl glycine amide. PMMA was first exposed to UV light through a photomask (2000 mesh Ni grid, with square holes of 7.6 μm x 7.6 μm). The groups formed as a result of exposure were derivatized with fluoresceinyl glycine amide in the presence of EDC to produce fluorescence.

**2.2.1.5 DETERMINATION OF SURFACE CARBOXYLATE CONCENTRATION**

As is illustrated in Scheme 2.2, a protocol taken from Pierce Biotechnology, Inc. was used to measure the surface coverage of carboxylic acid groups.

This protocol of course assumes 100% efficiency for each step (amine coupling and trityl-carboxylic acid coupling). The procedure can be described as follows: samples
were prepared by ultrasonication of the PMMA sheets in IPA for 15 min, rinsing with IPA, and then drying under house N₂ flow. The cleaned PMMA samples were then exposed to UV light in the atmosphere for different periods of time, rinsed with IPA, and dried under house N₂ flow. Each UV-modified PMMA sheet was placed in a capped vial, and 2 mL of EDC solution (5 mM in pH 7.0, 0.1 M phosphate buffer) and 30 μL of ethylenediamine were added to each vial.

**Scheme 2.2** Quantification of carboxylic acid groups on UV-modified PMMA surfaces using trityl cation method. PMMA sheets were first exposed to UV radiation and then the induced functional groups were derivatized with ethylenediamine in the presence of EDC to yield an amine-terminated surface. The amine-terminated surfaces were then reacted with s-SDTB (sulfo-succinimidyl-4-O-(4,4′-dimethoxytrityl)-butyrate), and then subsequently exposed to perchloric acid to release into solution the trityl cation from the PMMA surface, which absorbs strongly at 498 nm.
All vials were placed on a shaker table to react for 3 h, after which each sample was rinsed thoroughly with 18 M\(\Omega\) cm water, then placed in a precleaned glass vial containing 2 mL of freshly prepared sulfo-succinimidyld-4-O-(4,4’-dimethoxytrityl)-butyrate (s-SDTB) solution (0.1 mM), and reacted on a shaker for 30 min. (51.4 mL 70% perchloric acid + 46.0 mL 18 M\(\Omega\) cm), and ultrasonicated for 15 min. At this point, each sample sheet was rinsed thoroughly with 18 M\(\Omega\) cm water, placed in a precleaned vial containing 2 mL of perchloric acid solution (51.4 mL 70% perchloric acid + 46.0 mL 18 M\(\Omega\) cm), and ultrasonicated for 15 min. The absorbance of the solution in each vial was then measured at 498 nm with a Cary 50 UV-Vis spectrometer.

### 2.2.1.6 ELECTROOSMOTIC FLOW MEASUREMENTS

PMMA microchannels (4-cm long × 100-\(\mu\)m tall × 50-\(\mu\)m wide) were formed by hot-embossing with the embossing system (vide infra) and the microchannel was enclosed by thermal annealing as described above.\(^{47}\) The electroosmotic flow (EOF) was measured by the well-established current monitoring method\(^{48}\) described as follows: the microchannel and both reservoirs were first filled with a low concentration buffer, one reservoir was subsequently emptied and filled with a higher concentration buffer, or vice versa. The ionic strengths of the buffers were kept to within 10% of each other (18–20 mM) so as to minimize double layer compression effects.\(^{48}\) Electrodes were placed in the reservoirs, then a high voltage (600 V) was applied across the 4-cm channel, and the current was monitored by following—with a multimeter (Fluke 189)—the voltage drop across a 10 K\(\Omega\) resistor connected into the circuit. EOF values were measured using 10.0 and 20.0 mM acetate buffer in the pH range of 4–6, 10.0 and 20.0 mM phosphate buffer in the pH range of 7–8, and 10.0 and 20.0 mM borate buffer in the pH range of 9–10. In
these measurements, the voltages from the multimeter were uploaded in real time to a computer in which the Flukeview 2.0 program (Fluke) was installed.

**2.2.1.7 ELEMENT-SPECIFIC LABELING OF PMMA SURFACES AND ANALYSIS WITH X-RAY PHOTOELECTRON SPECTROSCOPY**

As previously shown\(^4\) and is outlined in Scheme 2.3, Tl(OEt) will specifically react with carboxylic acid groups while not ester groups, so the amount of Tl coupled to the polymer surfaces will indicate the amount of accessible carboxylic acid groups on the polymer surfaces.

Tl(OEt) exposure of pristine PMMA, UV-modified PMMA and poly(methacrylic acid), PMAA (Scientific Polymer Products, Inc., \(M_w = 87,100\) and \(PDI = 1.02\)) was accomplished by placing the polymer spin-coated glass microscope slides in an Ar-filled glove box (Vacuum Atmospheres) and covering them with neat Tl(OEt) liquid (Strem Chemical, Inc.) for 30 s, rinsing with absolute ethanol, and drying completely. The Tl-exposed slides were then analyzed with an Axis 165 X-ray Photoelectron Spectrometer (Kratos Analytical) using a monochromatized Al K\(\alpha\) (1486.6 eV) X-ray source with a power of 150 W. Survey and high-resolution spectra were obtained using pass energies of 160 eV and 20 eV, respectively. The neutralizer was turned on during the analysis to compensate for any possible charge effects on the insulating polymer surfaces. Core level binding energies for C1s and O1s were corrected according to Beamson et al. referencing the methyl carbons to 285.0 eV and carbonyl oxygens to 532.2 eV, respectively.\(^5\)

Curve-fitting of the high-resolution spectra of the Tl-labeled pristine and UV-modified PMMA was used for mechanistic analysis of the possible photochemical reactions described herein. The curve-fitting was performed using a sum of Gaussian-Lorentzian profile peak shapes after subtraction of a linear background.\(^5\)
2.2.2 THEORY OF X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)

XPS is one type of electron spectroscopy operated under high vacuum with its preeminent practical application in surface analysis, namely nondestructive surface analysis; it is conventionally also called Electron Spectroscopy for Chemical Analysis (ESCA). The principle that is followed to produce surface chemical information is the Einstein equation (Equation 2.1).

**Equation 2.1**  
\[ h\nu = \frac{1}{2} mv_c^2 + E_b + q\Phi \]

Where \( \nu \) is the frequency of the incoming photon, \( h \) is Planck’s constant, \( \frac{1}{2} mv_c^2 \) is the kinetic energy of the outgoing electron of mass \( m \) and velocity \( v_c \), \( q \) is a reference charge, \( \Phi \) is the work function of the emitting material, and \( E_b \) is the binding energy, which is the energy against the Fermi level of the material. As is shown in Figure 2.1, electrons from the inner shell of a substrate atom will be ejected and will possess a characteristic binding energy if a quantum amount of energy (demonstrated in Equation 2.1) is absorbed upon exposure to X-ray source. There are three main types of spectra: survey, high-resolution or region, and valence-band spectra, each of which employs different operating
parameters in terms of pass energy and energy resolution. In general, 160 eV pass energy was used for survey spectra, 20 eV pass energy was adopted for high-resolution spectra, while 40 eV pass energy was applied for valence-band spectra acquisition.

The instrumentation setup is shown in Figure 2.2 in which the main components are the magnetic and electrostatic lens, hemispherical analyzer and multiple-channel detector. For insulating polymer samples, the charge neutralizer is also a very important component in the instrument. A monochromatic X-ray source (Al K_{\alpha}) was used to achieve high spatial resolution and acute sensitivity, a neutralizer was adopted to ensure a proper surface potential when analyzing insulating samples, and the incidence of X-ray employed was always set at 90° against the normal to the sample surface to obtain

![Diagram of photoelectron generation from inner shell of sample surfaces by X-ray source.](image)

**Figure 2.1** Diagram of photoelectron generation from inner shell of sample surfaces by X-ray source.
maximum intensity of photon flux. The spectrometer was always operated in hybrid mode, a combination of magnetic and electrostatic lenses.

Figure 2.2 Schematic layout of the XPS instrumentation.

2.2.3 THEORY OF FLUORESCENCE\textsuperscript{51, 52}

Luminescence is one of the oldest analytical techniques, and it has been discovered and applied to various areas for almost five hundred years. Based upon different resources of the excitation energy obtained by the luminescent molecules, luminescence can be categorized into photoluminescence, chemiluminescence, bioluminescence, triboluminescence, cathodoluminescence, and thermoluminescence.
Photoluminescence is generated when luminescent molecules are excited by UV and visible photons (refer to the diagram of electromagnetic spectrum in Figure 2.3); it can be further divided into fluorescence and phosphorescence according to the paths of energy release from excited states to ground states.

As is illustrated in the typical schematic energy level diagram or Jablonski diagram, valence electrons in molecules at electronic ground states \((S_0)\) are excited upon absorption of a quantum amount of electromagnetic radiation energy (photon) in about \(10^{-15}\) second and raised to a singlet excited electronic energy states \((S_1)\); there are several vibrational levels for each electronic state among which energy dissipation occurs to reach the lowest vibrational level, a process named internal conversion. When no more excess energy is left in the electron, the electron is now at the lowest vibrational level of the first singlet electronic state \((S_1)\), and will return to the ground electronic state upon emission of energy. This phenomenon is called fluorescence; because there is energy lost during the 0.1 second duration of the electron staying at the electronic excited state, the wavelength of fluorescence is longer than that of the excitation light. When the electron

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**Figure 2.3** Electromagnetic spectrum

<table>
<thead>
<tr>
<th>Type of transition</th>
<th>Spectral region</th>
<th>Nuclear Core-level electrons</th>
<th>Valence electrons</th>
<th>Molecular vibrations</th>
<th>Molecular rotations: Electron spin</th>
<th>Nuclear spin</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-ray</td>
<td>X-ray</td>
<td>UV</td>
<td>Vis</td>
<td>IR</td>
<td>Microwave</td>
<td>Radio wave</td>
</tr>
</tbody>
</table>

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at the lowest vibrational level of the first excited electronic state (S\(_1\)) undergoes an intersystem crossing or transition from the singlet to the triplet state (T\(_1\)), phosphorescence will occur; the electron at the lowest vibrational level of the triplet state will return to the ground electronic state and emit excess energy.

![Jablonski diagram](image)

**Figure 2.4** Jablonski diagram

There are mainly three types of fluorescence: Stokes fluorescence, anti-Stokes fluorescence and resonance fluorescence. Stokes fluorescence refers to that when emission of fluorescence is at a longer wavelength (shorter frequency) or the emitted photons are at a lower energy level, and this phenomenon is normally observed in solution. Anti-Stokes fluorescence occurs when thermal energy is added to an excited
state or if a compound has many highly populated vibrational energy levels, which causes the emission to occur at shorter wavelength or longer frequency than that of the absorption. This scenario is seen in dilute gases at high temperatures. The third type is called resonance fluorescence and occurs when fluorescence photons possess the same energy as that of the absorbed photons. It may occur in gases, crystals, and atomic fluorescence but never in solution because of interactions between the solvent.

Interfering emission of light is also commonly observed during fluorescence processes especially at shorter wavelengths of excitation; among these is Rayleigh scattering, a phenomenon when an electron is excited to a higher vibrational level instead of electronic transition, emits light at the same wavelength as that of the absorbed photons within $10^{-15}$ seconds, and then returns to the original lower vibrational level. The intensity of Rayleigh scattering is inversely proportional to the fourth power of the wavelength of the excitation light, so its effect can be minimized by working at a longer wavelength. Another form of scattering is called the Raman effect, which appears at higher and lower wavelengths (although the former is more common) than the Rayleigh scattering peak in fluorescence spectra and are satellites of the Rayleigh scattering peaks with constant frequency difference from the exciting radiation.

Typical instrumentation associated with fluorescence methods include fluorometers and spectrofluorometers, the type used is determined by how the excitation and emission signals are selected; in a fluorometer, the excitation and emission signals are selected by absorption or interference filters, while in a spectrofluorometer, a monochromator is used to select the excitation and emission wavelengths. Shown in Figure 2.5 is a typical instrumentation layout of molecular fluorescence, characteristic with the emission detector 90 degrees to the excitation light source.
2.2.4 THEORY OF SCANNING PROBE MICROSCOPY (SPM)

Scanning probe microscopy (SPM) is defined as a family of microscopy forms where a sharp probe is scanned across a surface and the interactions between the probe and the sample surface are recorded and monitored. It consists of two major types: scanning tunneling microscopy (STM) and atomic force microscopy (AFM) (also called scanning force microscopy (SFM)). In the work described here, AFM or SFM is the form of SPM used for characterizing pristine and/or modified solid substrate surfaces. Atomic force microscopy (AFM) was developed in the mid 1980s; it can profile surface features and produce three dimensional images with magnifications of up to 1,000,000X. It originated from stylus profilers and was advanced in 1986 when Binnig and Quate demonstrated an atomic force microscope that can achieve extremely high resolution by using an ultra small tip at the end of a cantilever.\textsuperscript{53}
There are three operational modes for AFM: contact mode, non-contact mode and Tapping Mode. Due to the soft nature of the polymeric materials used for the described work here, Tapping Mode AFM is the only choice for surface characterization of the substrate materials. A typical SFM instrumentation setup is illustrated in Figure 2.6.

![Figure 2.6 Schematic of Tapping Mode scanning force microscope.](image)

As is displayed in Figure 2.6, the major components in a scanning force microscope consist of a diode laser which emits 632.8 nm red laser light, a scanning probe (usually silicon nitride probes for Tapping Mode SFM) or a force sensor made of an integrated assembly of cantilever and tip, a ceramic piezoelectric tube scanner whose geometry changes when applying electrical voltages across it, a mirror, and a position-sensitive, split-photodiode detector. The sample amounted on the piezoelectric tube scanner is moving in the X and Y directions resulting from the electrical voltages applied across the
piezoelectric scanner tube. The position changes of the tip by the sample surface is sensed by monitoring deflections of the laser light off the backside of the cantilever which are directed by the mirror to the detector, a position-sensitive, split-photodiode. The forces that occur between the tip and the sample in question in a Tapping Mode SFM experiments are mainly Van der Waals forces and columbic forces. Overall, the SFM can be simply designated as an electromechanical sensor, in which an electrical change (applied voltages) causes mechanical changes in the piezoelectric scanner tube, which is further reflected by position changes of the laser on the photodiode detector.

### 2.2.5 THEORY OF WATER CONTACT ANGLE MEASUREMENTS

Water contact angle studies have been used as a quick and qualitative technique for characterization of surface wettabilities or surface energies (surface tension); the underlying principle can be described by the Young’s equation, Equation 2.2 which describes the relationship between the free energy of the interface and a unique contact angle $\theta$.

**Equation 2.2**

$$\gamma_{SV} - \gamma_{SL} = \gamma_{LV} \cos \theta$$

In Young’s equation, $\gamma_{SV}$ refers to the free energy of the interface between solid and vapor phases, $\gamma_{SL}$ refers the free energy of the interface between solid and liquid phases, $\gamma_{LV}$ refers to the free energy of the interface between liquid and vapor phases, and $\theta$ is the water contact angle. A typical representative schematic for Young’s equation is demonstrated in Figure 2.7.

![Figure 2.7 A liquid drop on a solid surface.](image)
Water contact angle goniometry can be used to study unknown substrate surfaces by comparison to known reported data, or in the study of modification processes by comparison of contact angle values before and after surface modifications. Water contact angle measurements include sessile drop static water contact angle measurements and dynamic water contact angle measurements. Water contact angle values may qualitatively reflect the chemical compositions of a surface, but they are also affected by surface roughness, contamination, and water evaporation effects. The volume of water used to make the water droplet also plays a role in the outcomes, so it is recommended to consistently use the same volume when making water contact angle measurements and also make the measurements immediately after the water droplet is formed to avoid any possible water evaporation and contaminations.

2.2.6 THEORY OF ELECTROOSMOTIC FLOW (EOF)

Electroosmotic flow (EOF) refers to the bulk flow of liquid in a channel; the normal flow direction of EOF is defined as that from anode to cathode, while the opposite is called reversed EOF. The phenomenon of EOF was first reported as early as 1809 when Reuss demonstrated in an experiment that water was made to percolate through porous clay diaphragms by applying an electric field.\textsuperscript{54} Electroosmotic flow (EOF) has been a crucial factor in many areas especially in the field of chemical and/or biological separations when EOF was either an ally by improving separation efficiency and providing method of transporting reagents,\textsuperscript{55} or an enemy by causing band broadening in the case of a distorted flow profile induced by variations in the conductivity, zeta potential, and viscosity of the fluid.\textsuperscript{56, 57}

To fully discuss and understand the origin of EOF, one has to understand one key electrical phenomenon, the formation of the electrical double layer (EDL) depicted in a
model called the Gouy-Chapman-Stern (GCS) model, as illustrated in Figure 2.9. Because no EOF will come into being without formation of an electrical double layer, it is necessary to address the EDL thoroughly.

![Representation of EOF of a fluid in a glass capillary.](image)

Figure 2.8 Representation of EOF of a fluid in a glass capillary.

When a solid substrate containing a surface charge is in contact with a liquid electrolyte, there will form a certain density of net surface charges within a certain thickness to the solid substrate plane, and counterions from the solution will adsorb on the substrate surface to shield the net surface charges, thus form several layers in the
solution. The inner layer is relatively immobile, and is called the compact, Helmholtz, or Stern layer, which is that between the inner Helmholtz plane (IHP) and the outer Helmholtz plane (OHP), while the very outer layer is called the diffuse layer and is defined as that between the OHP and the solution reference plane, beyond which is the bulk solution.

![Schematic of electrical double layer (EDL).](image)

**Figure 2.9** Schematic of electrical double layer (EDL).

The value of EOF ($\mu_{eo}$) is determined by several parameters: the dielectric constant of the liquid ($\varepsilon$), the zeta potential of the EDL ($\zeta$), and viscosity of the liquid ($\eta$). The relationship is defined in Equation 2.3.

**Equation 2.3**

$$\mu_{eo} = -\frac{\varepsilon \zeta}{\eta}$$

It is important to point out that as demonstrated in Figure 2.9, the zeta potential refers to the potential in the not-very-well-defined position in the diffuse layer called the shear plane.$^{58}$
2.3 RESULTS AND DISCUSSION

2.3.1 FLUORESCENCE MICROSCOPIC MAPPING OF CARBOXYLIC ACIDS

Chemical labeling of carboxylic acid groups on PMMA surfaces followed by their examination with fluorescence microscopy was used to identify the presence of carboxylic acid groups formed during the photolysis process on the PMMA surfaces. As can be seen from Scheme 2.1, fluoresceinyl glycine amide, in the presence of carbodimide coupling agent, specifically reacts with carboxylic acids on the PMMA surface to form amide bonds. This dye has been used previously in mapping carboxylic acid sites in acrylate-based polymer microfluidic devices.\(^5^9\) Shown in Figure 2.10 is a fluorescence image \((\lambda_{\text{ex}} = 488 \text{ nm}, \lambda_{\text{em}} = 520 \text{ nm})\) of fluoresceinyl glycine amide dye-labeled PMMA surfaces after being patterned by UV-modification using a 2000 mesh Ni grid (hole size of 7.6 \(\mu\text{m}\)) mask for 30 min. As outlined in the experimental section, the

![Figure 2.10 Image of photo-patterned PMMA surface; here a 2000 mesh Ni grid was used as a mask, exposure time is 30 min. Fluorescence image \((\lambda_{\text{ex}} = 488 \text{ nm}, \lambda_{\text{em}} = 520 \text{ nm})\) of specific chemical labeling with fluoresceinyl glycine amide.](image)
green squares in Figure 2.10 are where the UV modification was performed and therefore the green dye should be present. Control experiments on UV-exposed PMMA without EDC did not lead to observation of fluorescence signal. The images obtained from these experiments indicate that carboxylic acid groups are formed on UV-modified PMMA surfaces using the protocol described here.

### 2.3.2 SURFACE TOPOGRAPHIC ANALYSIS OF PRISTINE AND UV-MODIFIED PMMA USING SFM

Surface topography is an important issue for substrate support materials used in applications such as biosensors and microanalytical devices, to name a few.\textsuperscript{32, 60} In particular with microfluidic devices, small changes in substrate surface roughness upon chemical treatment are desired due to the size of the device features (μm). In this study, SFM was used to analyze both pristine and UV-modified PMMA surfaces.

UV-modified PMMA surfaces are slightly rougher than the pristine PMMA surfaces, as can be noted from inspection of Figure 2.11 and Table 2.1. Figure 2.11(A) represents a typical 20 μm x 20 μm SFM image of the surface of pristine 0.5-mm-thick PMMA sheets (Goodfellow), with the surface being relatively uniform and smooth; the RMS surface roughness is 18.0 nm, and the roughness factor, $R$, is 1.014 ($R$ refers to the ratio of the surface cross-sectional distance to the horizontal distance\textsuperscript{29}). Shown in Figure 2.11(B) is a typical 20 μm x 20 μm SFM image of a 0.5-mm-thick Goodfellow PMMA sheet that was UV modified for 30 min under ambient laboratory atmosphere, subsequently rinsed with IPA, and then dried with house N\textsubscript{2}.

The RMS surface roughness for the 30-min, UV-modified PMMA surface is 27.5 nm, which is about 50% higher than that of the pristine PMMA, and the roughness factor $R$ is 1.067. This increased surface roughness is attributed to photo-induced scission
reactions of the polymer chains on the PMMA surfaces.\textsuperscript{61} Preliminary mass spectrometry studies of 1° standard PMMA photochemically modified under the conditions described here confirms this scission pathway is operative. However, under the conditions used here (15 mW cm\(^{-2}\), \(t < 2\)h), the photochemical surface modification route (carboxylic acid formation) is dominant, as noted by the relatively small change in roughness values. For the longest exposure time, we find an RMS roughness of 32.3 nm and \(R = 1.094\). We currently do not understand the origin of the high roughness values for the 10 min exposure. All in all, the observed roughness increase upon photochemical surface modification is quite small, particularly in light of the dimensions of the microchannels.

\textbf{Figure 2.11} Tapping Mode SFM images of A. pristine PMMA and B. UV-modified PMMA (30 min). The scan range for both images is 20 \(\mu\)m x 20 \(\mu\)m, and the Z-range is 200 nm. Root mean square (RMS) surface roughness is 18 nm for pristine PMMA while 27.5 nm for the UV-modified PMMA. The surface area correcting factor R value for the pristine PMMA is 1.014, while it is 1.067 for the 30-min, UV-exposed PMMA.
The average contact angle for pristine PMMA surfaces using water as the probe was found to be 70° ± 2° (5 replicates), in good agreement with the literature value of 67°. After photochemical surface modification in air, but before IPA rinsing, the water contact angle is decreased to about 24° at 30 min exposure time, see Figure 2.12. When the photochemically modified surfaces were rinsed with IPA, the water contact angle values increased, possibly as a result of increased surface roughness caused by IPA removal of low-molecular-weight polymers on the PMMA surfaces formed during the photochemical modification process. When PMMA surfaces are exposed to UV light, the radical ends resulting from bond breaking events in the irradiated volume can be trapped by oxygen in the atmosphere to form initial oxidation products which can undergo further

### Table 2.1 Values of carboxylic acid surface coverage resulting from photochemical surface modification of PMMA as a function of modification time (three replicates).

<table>
<thead>
<tr>
<th>UV modification time (min)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>121</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS surface roughness (nm)</td>
<td>18.0</td>
<td>14.8</td>
<td>41.1</td>
<td>27.5</td>
<td>33.0</td>
<td>32.3</td>
</tr>
<tr>
<td>Correction factor (R) for surface area</td>
<td>1.014</td>
<td>1.008</td>
<td>1.317</td>
<td>1.067</td>
<td>1.042</td>
<td>1.094</td>
</tr>
<tr>
<td>Surface coverage of COOH (10⁻¹⁰ moles·cm⁻²) before correction for surface roughness</td>
<td>0.47±0.01</td>
<td>3.02±2.80</td>
<td>8.57±0.48</td>
<td>13.97±0.55</td>
<td>14.13±0.99</td>
<td>16.83±0.28</td>
</tr>
<tr>
<td>Surface coverage of COOH (10⁻¹⁰ moles·cm⁻²) after correction for surface roughness</td>
<td>0.46±0.01</td>
<td>3.00±2.78</td>
<td>6.61±0.36</td>
<td>13.12±0.53</td>
<td>13.44±0.95</td>
<td>15.53±0.26</td>
</tr>
</tbody>
</table>

2.3.3 SESSILE-DROP WATER CONTACT ANGLE MEASUREMENTS OF PRISTINE AND UV-MODIFIED PMMA SURFACES

The average contact angle for pristine PMMA surfaces using water as the probe was found to be 70° ± 2° (5 replicates), in good agreement with the literature value of 67°. After photochemical surface modification in air, but before IPA rinsing, the water contact angle is decreased to about 24° at 30 min exposure time, see Figure 2.12. When the photochemically modified surfaces were rinsed with IPA, the water contact angle values increased, possibly as a result of increased surface roughness caused by IPA removal of low-molecular-weight polymers on the PMMA surfaces formed during the photochemical modification process. When PMMA surfaces are exposed to UV light, the radical ends resulting from bond breaking events in the irradiated volume can be trapped by oxygen in the atmosphere to form initial oxidation products which can undergo further
photolysis to give smaller fragments.\textsuperscript{61} We hypothesize that the nanoscale roughness causes the increase in contact angle in a fashion similar to that recently observed for nanostructured polymer surfaces that mimic biological surfaces.\textsuperscript{64}

Figure 2.12 Effects of UV modification on the water contact angle on PMMA surfaces. Water contact angle was measured on PMMA surfaces that were UV-modified in air for different UV exposure times both before rinsing with IPA (solid squares) and after rinsing with IPA (solid stars). The water contact angle was measured immediately after each treatment to avoid any possible contamination from the atmosphere.

2.3.4 DETERMINATION OF SURFACE COVERAGE OF CARBOXYLIC ACID GROUPS ON PMMA BY VISIBLE DYE LABELING

UV-Vis spectrometry coupled to carboxylic acid group-specific labeling was used to quantify the surface coverage of carboxylic acid functionalities on UV-modified
PMMA surfaces, Scheme 2.2. Before photochemical surface modification, there exist a small number of carboxylic acid sites on the pristine PMMA, see Table 2.1 and Figure 2.13. This is attributed to hydrolysis of the surface PMMA ester groups by either the manufacturer production or post-manufacturer production conditions of the polymer before its purposeful photochemical modification. The surface concentration of carboxylate groups on PMMA surfaces was found to increase consistently with increases in the photochemical modification time up to 30 min, and then it tended toward a limiting value, as seen in Figure 2.13. The surface coverage of carboxylic acid sites on PMMA surfaces reaches 1.68 nmol cm\(^{-2}\) at a modification time of 2 h, which upon correction for surface roughness (Table 2.1, \(R\) value) is 1.55 nmol cm\(^{-2}\), a value that is \(~1.5–2\) times that of a close-packed alkane monolayer.\(^{65}\) This higher-than-monolayer coverage is most likely due to the formation of carboxylic acid groups below the PMMA surface, an expected outcome based on the photochemical modification protocol (vide supra).

### 2.3.5 EFFECT OF PHOTO-INDUCED CARBOXYLIC ACID PRESENCE ON ELECTROOSMOTIC FLOW IN PMMA MICROCHANNELS

Electroosmotic flow has been discussed recently in regards to its magnitude and direction for chemically modified PMMA microchannels made through machining\(^{29}\) and by laser-ablation.\(^{24}\) To investigate the effect on EOF of carboxylic acid groups resulting from photochemical surface modification, a microchannel (100-\(\mu\)m deep, 50-\(\mu\)m wide, 4-cm long) was hot embossed in PMMA and subsequently irradiated as described here for 30 min, rinsed with IPA, dried with house \(N_2\), and then the microchannel was enclosed by thermal bonding in a convection oven with a piece of pristine PMMA cover plate to form a fluidic conduit.\(^{18, 45, 66}\)
As is seen in Figure 2.14, the EOF in both pristine and UV-modified PMMA microchannels is positive (from anode to cathode) and changes with the pH value of the buffer used. For example, the EOF values increased at higher pH for both photochemically modified (4.67 x 10^{-4} cm^2V^{-1}s^{-1} at pH = 9.0 while 1.88 x 10^{-4} cm^2V^{-1}s^{-1} at pH = 4.0) and pristine PMMA microchannels (3.02 x 10^{-4} cm^2V^{-1}s^{-1} at pH = 9.0 while 1.72 x 10^{-4} cm^2V^{-1}s^{-1} at pH = 4.0), and this is attributed to the fact that the accessible surface carboxylic acids are deprotonated at high pH and protonated at low pH values. In general, the EOF values in photochemically modified PMMA microchannels are higher than those in pristine PMMA microchannels, an observation that is consistent with the presence of a higher surface density of solution-accessible carboxylic acid moieties. This

**Figure 2.13** Surface coverage of carboxylic acids as a function of UV exposure time for PMMA surfaces in ambient air. Values uncorrected (solid squares) and corrected (solid triangles) for SFM-determined roughness are reported.
increased amount of carboxylic acid sites results from the photochemical modification process. Thus, the photochemically modified PMMA surfaces possess more ionizable functional groups than their pristine counterparts. In addition, the significant change in EOF with pH change occurs at a lower pH value for UV-modified PMMA microchannels (~pH = 6.0), while this is observed at a higher pH value for pristine PMMA microchannels (~pH = 7.0). This observation is most likely related to the issue of surface pKa and its dependence on ionizable group surface density, although further work is needed to gain a better understanding of this phenomenon.

2.3.6 MECHANISM FOR THE PHOTOCHEMICAL SURFACE MODIFICATION OF PMMA — XPS STUDIES

As a powerful surface analysis technique, XPS is able to provide information on the chemical changes in the very top layer of the PMMA sample where photochemical reactions occur. The XPS data in Table 2.2 demonstrate that the ratio of O/C on PMMA surfaces increases when the UV modification time of PMMA surfaces in air is increased, which indicates that introduction of oxygen into the polymer occurs during UV-modification of PMMA surfaces as described here. This observation is also consistent with the results from the chemical labeling (trityl cation, Scheme 2.2) of the carboxylic acid functionalities of photochemically modified PMMA surfaces, as it was observed that more carboxylic acid sites were formed with increased modification time in Figure 2.13.

To identify the presence of and provide a semi-quantitative evaluation of the amount of carboxylic acid sites on the UV-exposed PMMA, we employed a carboxylic acid-selective labeling method, Scheme 2.3. Thallium ethoxide has been shown to be effective for semi-quantitatively labeling carboxylic acids on polymeric surfaces. Here, neat thallium ethoxide was used to probe the presence of surface carboxylic acid groups.
on pristine PMMA, UV-modified PMMA and poly(methacrylic acid), PMMA (as a reference) surfaces, and the atomic ratio of Tl/O was obtained for each surface.

**Figure 2.14** Electroosmotic flow measurements in a hot-embossed PMMA microchannel (4 cm × 100 μm × 50 μm). Plot of EOF vs. pH for pristine (solid triangles) and 30-min UV-modified PMMA (solid circles). The electroosmotic flow measurements were carried out using pH values of 4-10. Three different buffers were used; acetate buffer (pH = 4.0, pH = 5.0, and pH = 6.0); phosphate buffer (pH = 7.0 and pH 8.0); and borate buffer (pH = 9.0 and pH = 10.0). The concentration of buffer was held between 18 mM and 20 mM, while the field strength used was 150 V cm⁻¹. For the modified materials, the microchannels of the embossed PMMA were exposed prior to chip assembly through use of a mask.
The Tl/O values were used to obtain approximate carboxylic acid surface coverage values upon comparison to a surface that should possess the maximum number of carboxylic acid sites, namely, poly(methacrylic acid), PMAA, which we arbitrarily assign as a 100% carboxylic acid layer. These surface coverage values are only a rough estimate and should not be compared to those from the trityl-labeling route above, for they do not reflect the labeling efficiency and the tacticity of the polymeric material used (the PMMA is mainly a syndiotactic isomer based on our preliminary NMR results, whereas the PMAA possesses a tacticity that is somewhere between isotactic and

<table>
<thead>
<tr>
<th>Exposure Time (min)</th>
<th>C1s</th>
<th>O1s</th>
<th>O1s/C1s</th>
<th>Average of O1s/C1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77.17</td>
<td>22.83</td>
<td>0.296</td>
<td>0.292±0.009</td>
</tr>
<tr>
<td></td>
<td>77.77</td>
<td>22.23</td>
<td>0.286</td>
<td></td>
</tr>
<tr>
<td></td>
<td>77.31</td>
<td>22.69</td>
<td>0.293</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>75.12</td>
<td>24.88</td>
<td>0.331</td>
<td>0.335±0.007</td>
</tr>
<tr>
<td></td>
<td>74.81</td>
<td>25.19</td>
<td>0.337</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74.74</td>
<td>25.26</td>
<td>0.338</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>74.71</td>
<td>25.29</td>
<td>0.339</td>
<td>0.340±0.003</td>
</tr>
<tr>
<td></td>
<td>74.47</td>
<td>25.53</td>
<td>0.343</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74.69</td>
<td>25.31</td>
<td>0.339</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>73.75</td>
<td>26.25</td>
<td>0.356</td>
<td>0.356±0.002</td>
</tr>
<tr>
<td></td>
<td>73.82</td>
<td>26.18</td>
<td>0.355</td>
<td></td>
</tr>
<tr>
<td></td>
<td>73.72</td>
<td>26.28</td>
<td>0.356</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>74.17</td>
<td>25.83</td>
<td>0.348</td>
<td>0.349±0.007</td>
</tr>
<tr>
<td></td>
<td>74.34</td>
<td>25.66</td>
<td>0.345</td>
<td></td>
</tr>
<tr>
<td></td>
<td>73.93</td>
<td>26.07</td>
<td>0.353</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 XPS quantification data of elemental composition of carbon, oxygen and the ratio of these two elements in accordance to UV modification time on PMMA surfaces (three replicates, confidence interval=90%).
syndiotactic), and the depth to which the Tl(EtO) penetrates is possibly different for PMAA and PMMA. Furthermore, as pointed out by Batich et al., the method works best for surface coverages of carboxylic sites that are less than ~30% of a monolayer.\textsuperscript{49} As noted in Table 2.3, the photochemically modified PMMA surface possesses a much higher carboxylic acid surface coverage compared to its pristine counterpart (about 3.5 times), in general agreement with the trend observed with the trityl-labeling route (Scheme 2.2, Table 2.1).

\textbf{Table 2.3} Comparison of XPS data on different polymer films treated with Tl(OEt).

<table>
<thead>
<tr>
<th>Atomic Ratio</th>
<th>C1s/O1s</th>
<th>Tl4f/O1s</th>
<th>Coverage of COOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tl/Pristine PMMA</td>
<td>2.79 ± 0.20</td>
<td>0.040 ± 0.002</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Tl/UV-modified (30 min) PMMA</td>
<td>2.60 ± 0.07</td>
<td>0.14 ± 0.01</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Tl/Standard PMAA</td>
<td>1.98 ± 0.11</td>
<td>0.36 ± 0.02</td>
<td>100 ± 2</td>
</tr>
</tbody>
</table>

In order to provide insight to the photochemical surface modification mechanism – that is identification and quantification of oxygen-containing species possessing carboxylic acid functionalities – analysis of the deconvoluted O1s region of the X-ray photoelectron spectra for pristine and 30-min UV-modified PMMA surfaces was undertaken (Table 2.4 and Figure 2.15). The O1s signal denoted by \textit{b} in Figure 2.15 corresponds to the carboxylic oxygen (associated with Tl\textsuperscript{+} from labeling), that by \textit{c} the O1s for the carbonyl oxygen, and that by \textit{a} the O1s of the methoxyl oxygen. It is clearly seen that the amount of methoxyl oxygen (\textit{a}) decreased (from 50.2\% to 36.1\%) while the carboxylic oxygen (\textit{b}) increased (from 3.4\% to 19.4\%) upon photochemical modification,
indicating that the methoxyl group is cleaved, and the free carboxylic acid is formed during UV modification.

A proposed PMMA photolysis mechanism for the conditions described in this report is shown in Scheme 2.4 and is based on others proposed in the literature.\textsuperscript{67,68} As is illustrated in Scheme 2.4, PMMA is first raised to the excited state upon absorption of UV light, and then it can undergo three possible pathways to form different radical products. These three pathways may occur simultaneously. In Path I, carbonyl and methoxyl radicals are formed, the latter which will extract hydrogen (from water or the polymer itself) to yield methanol, while the carbonyl radical will form a peracid in the presence of oxygen and UV light, which in turn will lead to carboxyl radicals that react with water to yield a polymer-bound carboxylic acid. In Path II, methyl and carboxyl radicals are formed, which react with water to yield methane and a polymer-bound carboxylic acid. In Path III, tertiary carbon radicals are produced directly from the excited state or when the carboxyl radicals in Path II release carbon dioxide. Then, in the presence of oxygen, the tertiary carbon radicals may further form alkoxyl radicals (through a peroxide intermediate), which in turn lead to polymer chain scission with the formation of acetone and alkyl radical on the scissed polymer chain; the latter may possibly undergo polymer chain branching reactions.\textsuperscript{67,68} It is clear that Path III is operative to some degree in the work at hand, as noted by the small increase in PMMA surface roughness with exposure time (removal of scission polymer product with IPA rinsing) and preliminary mass spectrometry data (vide supra), however it is not the major path. Both Path I and II lead to the formation of carboxylic acid sites, but it is currently unclear if a given path is dominant. The surface labeling and XPS data support the
carboxylic acid formation Paths I and II, but not the specific intermediates. Previous work supports the latter.\textsuperscript{67,68}

Table 2.4 Comparison of the deconvoluted XPS spectra of pristine and UV-modified PMMA. Curve-fitting was achieved by application of a Gaussian-Lorentzian peak shape algorithm (70\% Gaussian and 30\% Lorentzian) after subtraction of a linear background. A relative sensitivity factor of 0.780 for oxygen was used in all cases.

<table>
<thead>
<tr>
<th>O1s Peak Label in Figure 2.9</th>
<th>Position BE (eV)</th>
<th>Atomic Concentration %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pristine PMMA</td>
<td>UV-modified PMMA</td>
</tr>
<tr>
<td>\textit{b}</td>
<td>530.50</td>
<td>3.4</td>
</tr>
<tr>
<td>\textit{c}</td>
<td>532.20</td>
<td>46.4</td>
</tr>
<tr>
<td>\textit{a}</td>
<td>533.70</td>
<td>50.2</td>
</tr>
</tbody>
</table>

Figure 2.15 Deconvoluted X-ray photoelectron spectra in the O1s region for pristine and 30-min, UV-modified PMMA. A. pristine PMMA after exposure to Tl(OEt); B. 30-min UV-modified PMMA after exposure to Tl(OEt). There are three components for both spectra, designated as: \textit{c}. carbonyl oxygen; \textit{a}. methoxyl oxygen; \textit{b}. carboxylic oxygen (bound to Tl\textsuperscript{+}).
This report focused on the surface modification of PMMA with simple UV photochemical methods, during which roughly a monolayer of carboxylic functional groups is formed. Formation of the carboxylic acids is a relatively surface non-destructive process from a physical point of view, as judged from scanning force

**Scheme 2.4** Proposed mechanism for the photochemical surface modification reaction under the conditions described here; adapted from previous work.

### 2.4 CONCLUSIONS

This report focused on the surface modification of PMMA with simple UV photochemical methods, during which roughly a monolayer of carboxylic functional groups is formed. Formation of the carboxylic acids is a relatively surface non-destructive process from a physical point of view, as judged from scanning force
microscopy studies, and is a very important characteristic for applications requiring minimal surface damage. The carboxylic acid groups on the PMMA surfaces lead to increases in its water wettability and the pH-dependent electroosmotic flow in hot-embossed microchannels. In addition, the pendant carboxylic groups on PMMA surfaces make it possible to further functionalize PMMA-based BioMEMS devices. A possible photolysis process mechanism was proposed based on literature reports and outcomes from the studies presented here. Polymer-based BioMEMS devices have attracted increased interest because of their intrinsic advantages, such as ease of fabricating high-aspect-ratio features, their low cost, biocompatibility and favorable mechanical properties. However, simple and convenient surface modification techniques are needed to push these microdevices ahead into the needed applications. The work described here provides a new avenue for the “bulk” surface modification of polymer-based BioMEMS devices, and more importantly, the spatially controlled modification of polymer surfaces that are required for protein and nucleic acid arrays, electronic interconnects, and localized fluid control.

2.5 REFERENCES


CHAPTER 3. PATTERNING ANTIBODIES ON POLY(METHYL METHACRYLATE) MICRODEVICE SURFACES

3.1 INTRODUCTION

BioMEMS devices have generated significant interest in the analytical community, particularly with respect to their applications in biochemical sensing, instrument design, and methodology development.1, 2 Compared to their glass- and silicon-based counterparts, polymer-based BioMEMS devices offer a broad spectrum of substrate materials and fabrication avenues for making the microdevice structures,3, 4 in addition to their common intrinsic advantages among microdevices, such as low sample consumption, high throughput, and portability.1, 2, 5-8 Several years after the first demonstration of miniaturized analytical device fabrication in silicon,9 silicon-based miniaturized analytical devices reemerged in 19908 and catalyzed significant breakthroughs in device fabrication in glass, such as microseparation devices for biologically relevant materials.6, 7 For the most part, the routine wet-chemical etching methods used for making silicon- or glass-based analytical microdevices limit the industrial scalability of their fabrication and also the formation of high-aspect-ratio microstructures due to the isotropic nature of the etching process. Polymer-based microdevices are able to address these issues to a significant degree, as there are many different types of polymers and fabrication techniques from which to choose, depending upon the specific application needs.10-18

Regardless of the type of substrate materials chosen for fabrication of microanalytical devices, an essential objective for further application and utilization of these devices is control of the surface properties of the substrate materials via surface modification and functionalization through a variety of methods.19 Compared to the well-
developed siloxane chemistry\textsuperscript{20} widely used in glass-based microdevices,\textsuperscript{21} surface chemistry for polymer substrates is still being fleshed out.\textsuperscript{4, 21-29} Well-defined and straightforward surface modification protocols are urgently needed for pushing forward application of BioMEMS technologies to the field of bioanalysis. For example, once amenable surface functional groups on device surfaces are formed, proteins, antibodies, enzymes, DNA, and carbohydrates can be further tethered to the modified surface for preparation of bio-sensing films using various techniques such as soft lithography, ink-jet printing, photolithography, and array printing.\textsuperscript{30-32} Antibody arrays are among one of the growth-area applications, in which antibodies at a certain density are patterned on region-specific surfaces for parallel and sensitive biospecific analyses.\textsuperscript{33,34} Integration of antibody arrays into microanalytical devices combines the strengths of both techniques, which will therefore add further appealing features to these devices such as specificity/selectivity, low detection limit, low sample consumption, and fast kinetics.\textsuperscript{35}

In this work antibodies, specifically anti-human CEA (CEA is a breast cancer protein biomarker) antibodies, were immobilized onto carboxylic acid-modified PMMA patterns using carbodiimide coupling and protein G chemistries.\textsuperscript{36} The surface of the poly(methyl methacrylate)—a popular thermoplastic polymer used in fabrication of microdevices—was first modified through a surface photochemical modification method to produce COOH functional groups in a defined pattern.\textsuperscript{37, 38} Biochemical activities of the immobilized anti-CEA antibodies were confirmed to be retained through interactions with a secondary antibody or/and a sandwich immunoassay on the surface of planar PMMA substrates or PMMA microdevice channels. Surface carboxylic acid patterns resulting from the photochemical modification were characterized by scanning probe
microscopy (SPM), while attachment of antibodies on UV-modified PMMA surfaces was confirmed by elemental analysis in the survey spectra from X-ray photoelectron spectroscopy (XPS) studies. Surface ligand densities of the immobilized antibodies, either oriented or random, were measured based on a calibration curve constructed by near-IR laser-induced fluorescence scanning of standard antibody samples and subsequent analysis with a home-built image analysis program.35

3.2 MATERIALS AND METHODS

3.2.1 GENERAL

Poly(methyl methacrylate) sheets, Plexiglass or Lucite, were purchased from Goodfellow and were cut to various-sized pieces. HPLC-grade isopropanol (IPA) and other chemicals were obtained from Aldrich and used without any further purification. Photochemical modification of poly(methyl methacrylate), PMMA, surfaces to yield carboxylic acid functional groups was accomplished by exposure of PMMA pieces to a UV light source having a low-pressure mercury lamp possessing an emission spectrum spanning the 240 nm to 425 nm range; the 254 nm band is the strongest with an intensity of 15 mW cm\(^{-2}\), while that of all others is less than 1.5 mW cm\(^{-2}\) at a 1-cm distance. PMMA pieces were first ultrasonicated in IPA for approximately 15 min, rinsed with IPA, and then dried with house nitrogen (oil free). The cleaned PMMA was then exposed to UV light for 30 min at a distance of 1 cm from the UV lamp, rinsed with IPA, and dried with nitrogen. All PBS buffer solutions were prepared in Nanopure water (Barnstead, 18 M\(\Omega\) cm) and were further filtered through MF75 series filter units from Nalgene (0.2 \(\mu\)m-pore, surfactant-free cellulose acetate membrane or cellulose nitrate membrane).
3.2.2 CHARACTERIZATION OF PMMA SURFACES BEFORE AND AFTER UV MODIFICATION

Described here is a typical scanning force microscopy (SFM) topographical analysis of PMMA surfaces with patterns resulting from UV modification through a contact photo mask. We recently reported a surface photochemical modification protocol on PMMA using UV light with the specifications above; characteristics of PMMA surfaces before and after UV modification were investigated in aspects of surface wettability, surface roughness, surface functionality on plain PMMA sheets, and also electroosmotic flows in the PMMA microfluidic conduit.\(^{37}\) As is outlined in Scheme 3.1 (Step I), a piece of sheet PMMA (2.54 cm × 7.62 cm × 0.05 cm from AIN Plastics) was ultrasonicated in 2-propanol (IPA) for 15 min, rinsed with IPA, and dried with house nitrogen. The sample was then placed on a microscope slide and a 2000-mesh Ni grid (SPI; hole size of 7.6 \(\mu\)m and wire size of 5 \(\mu\)m) held on the sample with a dumbbell-shaped Al mask. The piece of PMMA was exposed to the UV light for 30 min, then rinsed with IPA and dried with house nitrogen. A representative portion of PMMA was cut from the UV-modified PMMA piece and then its surface topography was evaluated by Tapping Mode SFM. The resulting images were flattened and analyzed using the software provided by the SFM instrument manufacturer.

3.2.3 PATTERNING ANTIBODIES ON UV-MODIFIED PMMA SURFACES

As is illustrated in Scheme 3.1 (Step II), a primary antibody (Ab1) (Chemicon; mouse anti-human CEA monoclonal antibody, clone 1105) was covalently attached to the carboxylic acid patterns resulting from UV modification through a contact photo mask (SPI; 500 mesh Ni grid with hole size of 39 \(\mu\)m and wire size of 11.4 \(\mu\)m) by carbodiimide coupling chemistry.
The procedure can be briefly described as follows: the primary antibody was first diluted to a desired concentration (12 $\mu$g mL$^{-1}$) in PBS buffer, $N$-Ethyl-$N'$-(3-dimethylaminopropyl)carbodiimide (EDC, Pierce Biotechnology) was then added to the diluted antibody solution so as to give a final concentration of 40 mM. The solution was mixed well and immediately sandwiched between two pieces of PMMA with carboxylic acid patterns produced in Scheme 3.1 (Step I). The sandwich was placed in a humidified, light-proof chamber and incubated at 4 °C for overnight. After incubation, the pieces of PMMA were rinsed thoroughly with PBS-Tween20,0.05% and PBS a minimum of four times each to remove possible non-specifically adsorbed antibody molecules from the PMMA surfaces. The PMMA pieces were further rinsed with Nanopure water to remove

**Scheme 3.1** Approach for patterning antibodies on UV-modified PMMA surfaces and the resulting interactions between a dye-conjugated secondary antibody with the immobilized primary antibody.
any rinsing buffer content and subsequently dried with house nitrogen for surface elemental analysis with X-ray Photoelectron Spectroscopy (XPS); for interaction with a secondary antibody (Chemicon; goat anti-mouse polyclonal antibody conjugated to FITC dye that absorbs at 488 nm and emits at 525 nm, Step III in Scheme 3.1), the pieces of PMMA were dried gently either with Kimwipe tissues (blotting) or a slow flow of house nitrogen. The secondary antibody (Ab2) was diluted to an intended concentration (12 μg/mL) in PBS buffer and sandwiched between two pieces of PMMA that had on its surface immobilized primary antibody. The sandwich was then incubated at room temperature for two hours in darkness in a humidified chamber. The PMMA pieces were rinsed again with PBS-Tween 200.05% and PBS thoroughly and blotted dry with a Kimwipe or left wet for fluorescence microscopic analysis. A control IgG (bovine IgG conjugated with FITC dye, Cortex, 12 μg/mL) was also sandwiched between another two pieces of PMMA having immobilized primary antibody on their surfaces, subsequently incubated at room temperature for two hours, rinsed with PBS-Tween 200.05% and PBS, and imaged with the same fluorescence microscope (Nikon MicroPhot FxA fluorescence microscope). All images were analyzed by Leica software to evaluate the pattern intensities.

### 3.2.4 XPS SURFACE COMPOSITIONAL ANALYSIS OF PRISTINE, UV-MODIFIED, Ab1-IMMOBILIZED PMMA

Pieces of pristine PMMA, UV-modified PMMA, and PMMA modified with the primary antibody were placed into the sample transferring chamber (STC) in the X-ray photoelectron spectrometer (Axis 165, Kratos Analytical) and the STC then pumped down overnight. The samples were then transferred into the sample analyzing chamber (SAC) which has an operating vacuum around 10⁻⁹ torr. A monochromatized Al Kα
(1486.6 eV) X-ray source with a power of 150 W was used for generating X-ray photoelectron spectra during the surface analysis; survey and high-resolution spectra were obtained using pass energies of 160 eV and 20 eV, respectively. The neutralizer was turned on during the analysis to compensate for possible charge effects on the insulating polymer surface. Values of core level binding energies for C1s, N1s and O1s were corrected for charge effects according to Beamson et al. using the binding energy of C1s at 285.0 eV.39

3.2.5 FABRICATION AND UV-MODIFICATION OF PMMA MICROCHIPS BEARING MICROSTRUCTURES IN SIMPLE “T FORMAT”

The PMMA microfluidic device was fabricated using a method previously described.35 In brief, a metal molding die bearing raised microstructures that were electroplated from Ni on a stainless steel base plate, was prepared by the X-ray LIGA technique. PMMA microstructures were embossed in a system comprised of a PHI Precision Press (model number TS-21-H-C (4A)-5; City of Industry, CA) installed with a vacuum chamber in order to produce low pressure (<0.1 bar) for complete filling of the Ni molding die. During embossing, the molding die was heated to 150 °C and pressed into the sheet PMMA at 1000 lb for 4 min. The PMMA part was then cooled to 85 °C for demolding. The embossed 0.5-cm thick PMMA microchip bears a simple T format microstructure with channel dimensions of 4 cm × 80 μm × 20 μm (referring to channel length, depth, and width dimensions). Before any further use, the PMMA microstructured device was first rinsed with IPA, ultrasonicated in 2% micro cleaning solution (major components include water, glycine and others) for 30 min, rinsed thoroughly with Nanopure water (18 MΩ-cm), and dried with house nitrogen. The cleaned PMMA microchip was then exposed to UV light for 30 min, rinsed with IPA, and dried with
house nitrogen. The UV-modified PMMA microchip was thermally bonded to a piece of pre-cleaned pristine PMMA cover plate (0.05-cm thick) to make the microfluidic conduit and was achieved by clamping both parts with glass plates and heating in a convection oven at 98 °C for 15 min. It is important to point out that the glass transition temperature ($T_g$) of PMMA (105 °C for pristine PMMA) decreased after UV-modification, so that the thermal bonding temperature which is determined by the $T_g$ was also adjusted correspondingly.37

3.2.6 IMMOBILIZATION OF ANTIBODIES ON PMMA MICRODEVICE SURFACES

Immobilization of a primary antibody (anti-CEA monoclonal antibody, mouse IgG1, Chemicon, clone 1105) in enclosed microchannels with channel dimensions of 4 cm $\times$ 80 $\mu$m $\times$ 20 $\mu$m (length $\times$ depth $\times$ width) was carried out by diluting the antibody to a specific concentration with PBS (pH 7.40), mixing with 40 mM EDC, and immediately adding to the microchannel with a clean disposable plastic syringe. The microdevice with antibody solution in the microchannel was then incubated at 4°C for overnight in a humidified chamber protected from light. After incubation, the microchannel was rinsed with PBS-Tween20$_{0.05%}$ using a clean disposable plastic syringe, then with PBS from another clean disposable plastic syringe; this was repeated at least four times for each step to remove any possibly non-specifically adsorbed primary antibody molecules on the PMMA microchannel surface. An FITC-labeled secondary antibody (goat anti-mouse polyclonal antibody, Chemicon) was added to the microchannel with a clean disposable plastic syringe and then incubated at room temperature in a humidified chamber protected from light for two hours. The microchannel was rinsed again with PBS-Tween20$_{0.05%}$ and PBS, respectively, at least four times for each step to remove any non-specifically
adsorbed secondary antibody molecules. The device was kept wet, and the microchannel was immediately observed and imaged with a fluorescence microscope (Nikon MicroPhot FxA).

3.2.7 FABRICATION OF A SANDWICH IMMUNOASSAY ON PMMA MICRODEVICE SURFACES

A section of a pre-cleaned PMMA microdevice with dimensions of 4 cm × 100 μm × 50 μm (channel length × depth × width) was exposed to UV light for 20 min through a contact photo mask (750 mesh Ni TEM grid; SPI) held by a quartz plate on top of the embossed PMMA microchip. The UV-modified PMMA microchip was then rinsed with IPA and dried with house nitrogen. The cleaned PMMA microchip was then thermally bonded to a thin sheet of pre-cleaned pristine PMMA (Goodfellow, 0.5-mm thick) at 98°C for 15 min in a convection oven. Immobilization of the capture antibody (anti-human CEA monoclonal antibody, mouse IgG1, clone 1105; Chemicon) in the microchannel was carried out exactly the same way as described in section 3.2.6. It was then rinsed in the same manner as in section 3.2.6. and dried gently with a clean disposable plastic syringe. Human CEA antigen (Chemicon, 12 μg/mL) was then applied to the microchannel with a clean disposable syringe and then incubated at room temperature for three hours in a humidified, light-proof chamber. And again the microchannel was rinsed the same manner as in section 3.2.6., and dried gently with a clean disposable plastic syringe. The detection antibody (mouse anti-human CEA monoclonal antibody conjugated with FITC, clone CB-30; Chemicon) was applied into the microchannel, and incubated at room temperature for 2 hours in a humidified chamber protected from light. After incubation, the channel was rinsed in the same manner as in section 3.2.6., left wet and imaged with the same fluorescence microscope.
The schematic of fabrication of the sandwich immunoassays is demonstrated in Scheme 3.2.

**Scheme 3.2** Schematic of a sandwich immunoassay prepared on patterned, UV-modified PMMA microchannels. The capture antibody is Ms anti-human CEA monoclonal antibody (clone 1105), the antigen is human CEA, and the detection antibody is Ms anti-human CEA monoclonal antibody (clone CB-30) that is conjugated to FITC dye.

3.2.8 SURFACE LIGAND DENSITY MEASUREMENT WITH NEAR-IR, LASER-INDUCED FLUORESCENCE SCANNING MICROSCOPY

Surface ligand density was measured based on a calibration curve constructed with a model antibody (rabbit anti-mouse IgG monoclonal antibody, conjugated with IR800 dye; Licor). A series of dilutions of the antibody were made at the concentrations of 0, 0.4, 1, 2, and 5 μM in PBS buffer. Then 5 μL of each was spotted on the UV-
modified PMMA surfaces and scanned by a home-built, near-IR fluorescence scanner (excitation at 780 nm, emission at 825 nm) as described in previous work. Briefly speaking, the scanner is composed of a diode laser (PicoQuant GmbH, model 800, Berlin, Germany), counting electronics (PicoQuant GmbH, model SPC 430, Berlin, Germany), and single-photon avalanche diode (SPAD, EG&G Optoelectronics, model SPCM-PQ, Vaudreuil, Canada). The microscope has a 40x objective (Nikon, Natick, MA) and a high numerical aperture (NA = 0.85). The fluorescence images were achieved by scanning the sample attached to a microtranslational stage which was controlled by step motors. The step resolution of the acquired fluorescence images was 101.6 μm and the integration period was at 100 ms. Fluorescence images were obtained and analyzed using a home-built program called HarpPlayer, in which fluorescence intensity of each pixel on the image was recorded during fluorescence scanning of the standard fluorescence spots. To be consistent, fluorescence intensity values reported here for each concentration used for construction of the calibration curve was an average of intensities from seven representative pixels within the fluorescent spot; background subtraction was achieved by using the average fluorescence intensity from the 0-nM antibody spot.

Direct or random immobilization of the antibody was carried out with the same procedure described in section 3.2.3. or 3.2.6., while oriented immobilization was accomplished using protein G, a Fc receptor of the antibody. Protein G was first immobilized using the same procedure as that of antibodies; bovine serum albumin (BSA) was used as a blocking agent, and then the antibody was spotted on a protein G-immobilized area and incubated at room temperature for two hours in a humidified chamber that was protected from light. Afterwards, all rinsing steps were the same as that
in section 3.2.3. Both devices were imaged immediately after preparation to avoid any possible quenching.

3.3 RESULTS AND DISCUSSION

3.3.1 SFM SURFACE TOPOGRAPHY ANALYSIS OF CARBOXYLIC ACID PATTERNS RESULTING FROM UV-MODIFICATION OF PMMA

In general, UV-modified PMMA surfaces are rougher than pristine PMMA surfaces. Shown in Figure 3.1 is an SFM image of a patterned, 30-minute UV-modified PMMA surface and the corresponding cross-section. In Figure 3.1(A), areas that are darker in color (UV light transmitted area) are physically lower in height than those areas that are lighter in color (UV light blocked area); the cross-sectional analysis in Figure 3.1(B) demonstrates the depth difference between pristine PMMA surfaces and UV-modified PMMA surfaces to be less than approximately 90 nm. This height difference indicates that material is removed from the polymer surface during the UV-induced formation of COOH functional groups. As we have shown previously, this damage can be minimized by using shorter exposure times that still yield sufficient carboxylic acid functional group density. Importantly, the surface damage incurred during the surface modification protocol is negligible in comparison to the dimensions of built-in micrometer-scale features of microfluidic devices. We have used longer exposure times here to produce patterns that are readily observable by SFM.

3.3.2 CONFIRMATION OF ANTIBODY IMMOBILIZATION ON COOH-MODIFIED PMMA SURFACES USING XPS AND FLUORESCENCE MICROSCOPY

XPS is a powerful surface analysis tool for monitoring compositional changes on surfaces resulting from chemical reactions. Pristine PMMA, UV-modified PMMA and
Figure 3.1 A. Tapping mode SFM image of sheet PMMA that was UV modified through a photomask (2000 mesh Ni TEM grid, with square holes of 7.6 μm × 7.6 μm); and B. Cross-sectional analysis of the patterned image in A. Point a in the image and line trace denotes where there is no UV-modification, while point b points to where UV-modification has occurred. Z-range in A is 100 nm and scanning scale is 50 μm x 50 μm.
Figure 3.2 Confirmation of the immobilization of antibody molecules on UV-modified PMMA surfaces by X-ray Photoelectron Spectroscopy. Shown are XPS survey spectra of: A. antibody-immobilized PMMA; B. 30-min, UV-modified PMMA; and C. pristine PMMA. There are only two components in the spectra for both pristine and UV-modified PMMA surfaces, namely carbon and oxygen, while there is an additional nitrogen peak in spectrum C indicating the immobilization of antibody molecules.
primary antibody (Ab1)-modified PMMA were evaluated with XPS to compare the surface compositional changes at each step of functionalization. As is shown in the survey spectra in Figure 3.2, there are only two elemental components in both pristine and UV-modified PMMA, namely carbon and oxygen; the C/O ratio is different as a result of the presence of carboxylic acid groups for the UV-modified PMMA. In the spectra of the antibody-immobilized PMMA surfaces, the signal for nitrogen at 399.8 eV is clearly evident and indicates the successful biofunctionalization of the UV-modified PMMA surface by direct linkage of the amine residues of the CEA antibody to the carboxylic acid groups of the COOH-PMMA. XPS cannot provide any information on the biochemical activity of the immobilized primary antibody, so we turned to fluorescence microscopy.

Shown in Figure 3.3 (Upper) is a fluorescence microscopic image of a patterned Ab1-PMMA surface, which was obtained as outlined in Scheme 3.1. Briefly, this was achieved by carbodiimide immobilization of primary antibody (Ab1) on a COOH-patterned PMMA surface, followed by exposure to a secondary antibody (Ab2) that is specific for Ab1 and is conjugated with a green fluorescence dye (FITC). The bright green squares in Figure 3.3 (Upper) are where the secondary antibody was introduced and corresponds to where the UV-modification occurred so as to produce COOH functional groups capable of forming amide bonds during the carbodiimide coupling. The control image in Figure 3.3 (Lower) is used to determine the possible existence of non-specific interaction events between the secondary antibody and the primary antibody; the patterned Ab1-PMMA surfaces were exposed to solutions of bovine IgG that should not interact with the primary antibody. The images were analyzed using fluorescence
microscopic imaging software LCS (Lica), and the signal-to-noise ratio (SNR, defined as \((\text{signal} - \text{background})/(\text{background})^{1/2}\)) in the image in Figure 3.3 (Upper) is seven times higher than that of the control in Figure 3.3 (Lower). In summary, the fluorescence images support the conclusion that the immobilized primary antibody is not denatured and is selectively recognized (lack of non-specific interactions).

Figure 3.3 Fluorescence microscopy is used to investigate the biochemical activity of the primary antibody (mouse anti-human CEA IgG antibody) immobilized after patterned UV modification. A secondary antibody (goat anti-mouse IgG) that is conjugated to FITC dye was introduced to interact with the immobilized primary antibody. A non-specific IgG molecule (bovine IgG) that is conjugated to FITC was used as a negative control to address the non-specific interaction issue.
3.3.3 BIOFUNCTIONALIZATION OF THE SURFACE OF PMMA MICROFLUIDIC DEVICE FEATURES AND CHARACTERIZATION OF ANTIBODY ACTIVITY

In order to investigate the effects of microfluidic device feature nature (planar surface versus channel structure) on the immobilization of antibodies, the methods in Scheme 3.1 and 3.2 were used for hot-embossed PMMA channels of 80 μm depth, and 20 μm in width. First, the impact of immobilization of the anti-CEA antibody in the microchannel was investigated. Upon following the protocol in Scheme 3.1 and subsequent fluorescence microscopy examination of the PMMA microchannel, it was found that the immobilized Ms anti-CEA antibody was recognized by the FITC-labeled goat anti-mouse antibody, see Figure 3.4(A), indicating that as in the case of the planar PMMA the antibody has not been substantially altered by its immobilization.

**Figure 3.4** Biofunctionalization of microfluidic channel surfaces and fluorescence microscopic evaluation of biochemical activity of the immobilized mouse (Ms) anti-human CEA antibody through: A. interaction with a secondary antibody (goat anti-Ms polyclonal antibody) that is conjugated to FITC dye; and B. a sandwich immunoassay wherein CEA is captured by the immobilized Ms anti-human CEA antibody and then reported using the FITC-labeled Ms anti-human CEA antibody. Channel dimensions in A are 80 μm depth × 20 μm width and 100 μm depth × 50 μm width in B. Device is fully functional in B (sealed channel with PMMA cover plate).
Depicted in Scheme 3.2 is the sandwich immunoassay protocol which involves immobilizing anti-CEA antibodies on COOH-modified PMMA surfaces at 4°C overnight, then capturing the antigen CEA by incubating at room temperature for 3 h, and the CEA capture event being detected through another anti-CEA antibody that is labeled with FITC. Patterned fluorescence signal was observed in the PMMA microchannel (depth is 100 μm and width is 50 μm) that has undergone the sandwich immunoassay procedure as shown in Figure 3.4(B). Within the microchannel, there are green fluorescence stripes that are around 25 μm long which correlates to the hole size of the patterning mask, the 750 mesh Ni grid, and this therefore indicates that the primary anti-CEA antibodies were immobilized on the location where COOH functional groups were produced through UV modification, CEA antigen was subsequently captured and detected by another different clone anti-CEA antibodies that are pre-labeled with FITC dye.

3.3.4 MEASUREMENT OF SURFACE LIGAND DENSITY USING NEAR-IR DYE LABELED IgG ANTIBODY

A calibration curve was constructed using the average fluorescence intensity obtained from spots of dried antibody solutions possessing different IgG concentrations. Examination of the spots on PMMA substrates using a scanning microscope capable of imaging the NIR dye-labeled IgG and subsequent integration of spot intensity with HarpPlayer software leads to the plot in Figure 3.5, which demonstrates the linear relationship between the amount of fluorescently-labeled IgG antibody present and the fluorescent signal. This calibration curve was used to determine the surface density of randomly immobilized and oriented IgG antibodies on PMMA. The surface density of randomly immobilized IgG antibodies is 0.38±0.02 pmol cm⁻², while that for the IgG
immobilized using a protein G layer is 0.21±0.01 pmol cm\(^{-2}\), both of which are lower than the theoretical value for one monolayer of IgG molecules. A monolayer of IgG assuming a flat arrangement of the protein on the surface is 1.1 pmol cm\(^{-2}\).\(^{40}\) The lower-than-theory values most likely result from steric hindrance events during the immobilization.

Figure 3.5 Determination of surface antibody density using near-IR, laser-induced fluorescence scanning microscopy. A model antibody that is conjugated to an 800-nm emitting near-IR dye is used to construct the calibration curve. The intensity values reported are the average of seven representative pixels within the same fluorescent spot; error values are ± one standard deviation. Surface ligand densities of random and oriented immobilization of antibodies were both determined using the calibration curve.
3.4 CONCLUSIONS

Antibodies specific for carcinoembryonic antigen (CEA), a breast cancer biomarker, have been successfully patterned on poly(methyl methacrylate)-based microanalytical device surfaces. The patterning method is very simple and straightforward and combines carbodiimide coupling chemistry with the direct, spatially-selective formation of carboxylic acid groups induced by exposure to UV light through a contact mask. Retention of the biochemical activity of the patterned, polymer-bound antibodies was confirmed, and surface ligand density measurements of immobilized antibodies yielded values characteristic of a submonolayer of antibody, with the surface density being 80% larger for randomly immobilized antibodies in comparison to their protein-G oriented counterparts. Considering the fact that there is a large interest in developing disposable, polymer-based microdevices for potential applications in biomedical and clinical diagnosis of diseases that will require the use of immunological recognition agents in array-based formats, the work reported herein sheds some light on promising methods for the patterned immobilization of biologicals that are simple to utilize.

3.5 REFERENCES


CHAPTER 4. PREPARATION OF BREAST CANCER GENE BIOMARKER BRCA1 MUTATIONAL MATERIALS

4.1 INTRODUCTION

4.1.1 BRCA1 AS THE TARGET GENE FOR DIAGNOSIS OF BREAST CANCER SUSCEPTIBILITY

Breast cancer is one of the most common and fatal cancer diseases that affects women worldwide (Breast Cancer Information Core (BIC), http://research.nhgri.nih.gov/projects/bic/). As is true with most other cancer diseases, early detection of breast cancer is very crucial for proper medical treatment because treatment of advanced breast cancer will be much more difficult and inconsistent, often futile and disfiguring.¹

Around 10% of breast cancers are caused by inherited genetic mutations (or abnormal genetic sequences), among which are mutations in the two most commonly involved genes, namely Breast Cancer Type I Gene (BRCA1) and Breast Cancer Type II Gene (BRCA2), with a higher risk with BRCA1 abnormalities. People who inherit mutations in this gene will have a higher risk of developing breast cancer (50-85% chance of developing breast cancer in the lifetime²) as well as types such as ovarian cancer. The BRCA1 gene, which expresses a specific protein in breast and ovarian tissue, has been found to be a DNA damage repair and tumor suppressor gene,³ meaning that at its normal stage, it maintains normal cell growth of breast tissue and suppresses tumor formation through the process of protein expression. Since it was initially mapped to chromosome arm 17q in the early 90s,⁴ ⁵ considerable efforts have been devoted to BRCA1 research, including development of reliable, inexpensive, accurate, and sensitive testing methods, either by a combination of PCR amplification and 2-D electrophoresis⁶ or by using fluorescence-assisted mismatch analysis for screening.⁷ Despite the existing
techniques available to research and clinical implementations, there is still an urgent need to
develop robust, rapid, point-of-care diagnostic tools for breast cancer and cancers in general.

4.1.2  CELL LINES AS EFFECTIVE BREAST CANCER STUDY MODEL SYSTEMS

Cell lines can be established such that they preserve the similar properties to that of the
tumor from which they were derived. Furthermore, cell lines derived from either primary or
metastatic cancers can provide unlimited sources of cells that can further be distributed to help
comparative studies among different labs. Specifically, for breast cancer cell lines, there are
currently only a few tens that have been characterized and widely used. Moreover, most of the

Figure 4.1 Homo sapiens genome view and the location of BRCA1 gene in chromosome 17.
available and widely studied breast cancer cell lines are metastatic cell lines, such as MCF-7 breast cancer cell lines. In order to develop effective techniques for early detection of breast cancer, primary breast cancer cell lines are required. In the reported work, HCC1937 (Hamon Cancer Center), a primary breast cancer cell line that was established at Southwestern Medical Center and deposited at American Tissue Culture Center (ATCC) by Gazdar and coworkers,8,9 was used as a tumor model system from which the genomic BRCA1 gene was isolated and further analyzed via PCR/LDR combined assays. A primary colon carcinoma cell line (HT-29, ATCC) was used as a wild-type control for the breast cancer cell line (HCC1937).

4.1.3 POLYMERASE CHAIN REACTION (PCR)/LIGASE DETECTION REACTION (LDR) COMBINED ASSAYS

Polymerase chain reaction (PCR) was first discovered by Kary Mullis and his colleagues at Cetus Corporation in the early 1980s.10 Combined with the breakthrough of isolation and purification of a thermal stable polymerase11 and the birth of programmable thermal cyclers, PCR has since dramatically reshaped the structure of experimental molecular biology, providing researchers with a superior tool that combines high sensitivity and specificity with great flexibility. It is not surprising that Kary Mullis was therefore awarded the 1993 Nobel Prize in Chemistry less than ten years after the invention of the PCR technique.

Since its discovery, there have appeared dozens of versions of PCR reactions, such as real-time PCR and reverse-transcriptional PCR. A typical PCR reaction involves gently mixing all the necessary reaction components, performing the amplification reaction in a thermal cycler, and testing the fidelity of the PCR product. The PCR components are of water, reaction buffer, MgCl2, dNTPs, forward primer, reverse primer, target DNA, and polymerase, each of the components needing to be chosen and designed carefully. Normally, the water used in the PCR reaction is deionized and sterilized and is commercially available. The PCR buffer is intended to
provide optimal pH and monovalent salt environment for the final reaction; commercial PCR buffer (pH 8.4) is usually a 10X concentrated solution which includes 200 mM Tris-HCl, 500 mM KCl, and 15 mM MgCl₂ (cofactor for the polymerase). The final concentration of MgCl₂ in the PCR reaction can also be further adjusted to obtain optimum result.

Deoxynucleotides (dNTPs) are added as the bricks for the PCR product, the doubly stranded DNA. Moreover, they provide energy for the reaction because the β and γ phosphates in the dNTPs are the only energy source among all the reaction components. The amount of DNA template used in the cycling is somehow dependent on how much DNA is available and how easy it is to replace it; it should also be free from contaminants, such as other DNA sources. The key player that contributes to the automation of PCR is the thermally stable enzyme or the DNA polymerase that can actually function at high temperature and beyond. There are several commonly used DNA polymerases, including the “gold standard” used in PCR, Taq DNA polymerase which was initially isolated from Thermus aquaticus from the outflows of the thermal pools of Yellowstone National Park. Other thermally stable polymerases isolated from other thermophilic bacteria consist of Pfu, Tth, Tfl, Tli, and others.

Each of these thermally stable polymerases possesses specific attributes in terms of processivity, fidelity, and persistence, and their choice in a given amplification is based on these attributes. Most of the aforementioned components can be ordered from commercial sources and used as is except the DNA template. Particular efforts are mostly invested in the designing of PCR primers and optimizing the annealing temperature in order to acquire the optimal PCR amplicon. As is depicted in Figure 4.2, a typical PCR thermal cycling comprises three steps: denaturing the DNA template at 94°C, annealing at appropriate temperature, and extending the strand at 72°C.
Figure 4.2 Schematic of Polymerase Chain Reaction (PCR)
Automatic PCR has been a powerful tool in the molecular biology field, while another automatic thermal cycling process called the ligase detection reaction (LDR) has further revolutionized DNA diagnostics. LDR has evidenced in discrimination of single-base difference between the wild type and the mutated type genetic materials since it was invented by Barany and coworkers. LDR itself is able to amplify DNA templates in a proportional manner, but it can be raised to an exponential amplification scale if it is coupled with another set of adjacent oligonucleotides, complementary to the first set and the target. Shown in Figure 4.3 is schematic of a typical LDR thermal cycling reaction.

**Figure 4.3** Schematic of Ligase Detection Reaction (LDR).

In LDR, the cloned thermally stable enzyme or LDR ligase specifically links two adjacent oligonucleotides when they are hybridized at 65°C to a complementary target and only when the nucleotides are perfectly base paired at the junction. Therefore, a typical LDR
thermal cycling process is composed of three steps: denaturing the double-stranded DNA template at 94°C, the specific ligation at 65°C, and last linear amplification at 65°C.

The LDR reaction mechanism involves the formation of a phosphodiester bond between a 3'-hydroxyl group from one of the two primers, namely the discriminating primer, with the phosphate group on the 5' end of another primer called the common primer. The key molecular driving force for the reaction comes from a very specific ligation enzyme while the energy source is from the enzyme cofactor nicotinamide adenine dinucleotide (NAD⁺) for this thermodynamic reaction.

When PCR is coupled to LDR, the exquisite specificity inherent in LDR will make multiplexing possible as different primer sets are able to simultaneously ligate along the same DNA template without the interference commonly seen in PCR-based assays. It will further increase the throughput of the assay when DNA arrays (gene chip), which normally utilize hybridization events between the immobilized probe and the targeting sequence, are incorporated into the multiplexed assay. Thanks to the series of efforts invested by Barany and related laboratories, the Zipcode universal DNA array format has been further increasing the sensitivity and detection limit of the multiplexed assay due to the fact that the universal array format uncouples mutation detection from array hybridization and, therefore, is capable of detecting frame shift, deletions and single point mutation that are not always achievable during direct hybridization.

4.1.4 SLAB-GEL ELECTROPHORESIS (SGE)

New technologies such as capillary-gel electrophoresis and microdevice electrophoresis have emerged in recent years and have played important roles in biomacromolecule analysis. Due to the fact that traditional slab-gel separations offer high parallel profiling and easy
accessibility, SGE has not been completely replaced and is still commonly used in most biological research labs for biomacromolecule separations for assessment of impurities and identification of unknown compositions.

There are two types of slab-gel electrophoresis: agarose gel electrophoresis and polyacrylamide gel electrophoresis. Agarose gel electrophoresis is mainly employed to separate nucleic acid molecules or very large proteins. The gel is made of agarose, a compound derived from seaweed, which is made of a linear polysaccharide of galactose and 3,6-anhydrogalactose. The commonly used concentrations of agarose gel include 0.8%, 1.0%, and 3.0% (the percentage of agarose in the buffer added during casting) and they are able to separate nucleic acids ranging from 20bp to 20,000bp.

Poly(acrylamide) gel electrophoresis is widely used for separation of protein and small nucleic acid molecules. Poly(acrylamide) is a polymer made of the monomer acrylamide and crosslinker such as bis(anylamide). Depending upon the proportion of crosslinker added during polymerization, there will form different pore-sized poly(acrylamide) gels. Poly(acrylamide) gel electrophoresis can be categorized into native or non-denaturing poly(acrylamide) gel electrophoresis (PAGE) and denaturing poly(acrylamide) gel electrophoresis or sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate is the only component difference between PAGE and SDS-PAGE.

PAGE is employed when the activity of biomacromolecules needs to be retained during separation, while in SDS-PAGE, the strong ionic detergent SDS denatures secondary, tertiary, and quaternary structures by binding to hydrophobic regions of protein, and its binding also confers net negative charges on the protein, which results in a constant charge-to-mass ratio. Therefore, the proteins are separated on the gel in the electrical field by their mass. Buffer
systems used in protein separation with poly(acrylamide) gel electrophoresis include sample buffer and running buffer; the commonly used sample buffer for SDS-PAGE is Laemmli buffer named after the researcher who invented the buffer system (pH 6.8), which comprises 62.5 mM Tris-HCl, 2% SDS, 25% glycerol, and 0.01% bromophenol blue. In PAGE for large-molecular-weight protein separation, the sample buffer (pH 6.8) typically consists of 62.5 mM Tris-HCl, 40% glycerol, and 0.01% bromophenol blue. For peptide and small protein denaturing gel separation, the sample buffer (pH 6.8) consists of 200 mM Tris-HCl, 2% SDS, 40% glycerol, and 0.04% coomassie blue G-250. Components in the running buffer (pH 8.3) for protein denaturing slab gel separation can be either 2.5 mM Tris, 19.2 mM glycine, and 0.01% SDS for large proteins, or 10 mM Tris, 10 mM Tricine, and 0.01% SDS for peptides and small proteins.

As is true with protein separations, there are also different buffer systems for nucleic acid separations depending upon specific applications of the electrophoresis using either agarose or poly(acrylamide) gels. There are two types of sample buffers: TBE-urea (pH 8.0) buffer and TBE (pH 8.0) buffer. The former is used for denaturing ssDNA and RNA separations and consists of 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, 7 M urea, 12% Ficoll, 0.01% bromophenol blue, and 0.02% xylene cyanol FF. The latter is used for nondenaturing and dsDNA separations and comprises 50 mM Tris-HCl, 25% glycerol, 5 mM EDTA, 0.2% bromophenol blue, and 0.2% xylene cyanol FF. The running buffer typically used for nucleic acid electrophoresis is either TBE or TAE buffer; TAE buffer offers faster electrophoresis but lower resolutions than TBE buffer.

The electrophoresis system can be set up to run either vertically or horizontally. Biomacromolecules (proteins or nucleic acids) always migrate from the cathode (−) to anode (+).
in the electrical field. Representative electrophoresis setups (Bio-rad), including both horizontal and vertical for ready gel precast gel applications, are illustrated in Figure 4.4.

![Typical slab gel electrophoresis experimental setups and results. At upper left is a typical vertical electrophoresis setup for ready gel precast gel electrophoresis, while at lower left is a typical horizontal setup for ready gel precast gel electrophoresis (Bio-Rad). On the right is a schematic of a working slab gel with eight wells where samples and sizing standard are injected.](image)

**Figure 4.4** Typical slab gel electrophoresis experimental setups and results. At upper left is a typical vertical electrophoresis setup for ready gel precast gel electrophoresis, while at lower left is a typical horizontal setup for ready gel precast gel electrophoresis (Bio-Rad). On the right is a schematic of a working slab gel with eight wells where samples and sizing standard are injected.

On the slab gel, biomacromolecules can be either qualitatively identified by comparison with a well-defined step ladder, or quantitatively measured by using a precision sizing standard run on the same gel as the target sample, an imaging device, and some image analysis software, such as ImageQuant. Once identified or quantified, target samples in the gel can be further purified by cutting the specific band out of the gel and then subsequently treating it using
chemical methods (dissolving the gel and extracting the product) or others (e.g. freeze & squeeze) to expel the pure target product out of the slab gel.

4.2 MATERIALS AND METHODS

4.2.1 GENERAL

Illustrated in Figure 4.5 is an overview of the strategy used for analysis of $BRCA1$ gene isolated from a primary breast cancer cell line; a PCR/LDR multiplexed assay is adopted to target the single mutation in the $BRCA1$ gene.

![Diagram](image)

**Figure 4.5** The adapted PCR/LDR combined assay for detection of single insertion mutation in the $BRCA1$ gene.

The breast cancer cell line (HCC1937) used in the assay was purchased from American Tissue Culture Center (ATCC) and stored in a liquid nitrogen tank for later use. The cell line,
deposited by Gazdar and coworkers from University of Texas Southwestern Medical Center, bears a single insertion C at position 5382 in exon 20 of the germ-line BRCA1 gene. The cell line was further cultured using the protocol provided by ATCC. Culture reagents include cell culture medium (RPMI 1640 medium, Sigma), 10% fetal bovine serum, and 1:500 of antibiotic/growth medium. Major facilities include a liquid nitrogen tank for storage of the cell line, a laminar-flow hood equipped with UV lamp for dealing with the cell line, microanalytical centrifuges, water bath, and incubation oven (37ºC, 5% CO2).

Isolation of genomic DNA from the cultured cells was accomplished with a kit from Sigma (Genelute Mammalian Genomic DNA Kit). The PCR amplification kit was ordered from Promega, while the primers were customer synthesized product from Integrated DNA Technologies (IDT). The LDR reaction kit, including the reaction buffer and ligase, was purchased from New England Biolabs (NEB). The discriminating and common primers were customer synthesized product from IDT, and the water used is nanopure water produced in the research lab. The agarose gel electrophoresis setup (Bio-Rad) is a horizontal setup specifically designed for ready gel precast mini-gels, while that for the polyacrylamide gel electrophoresis of the LDR product is connected to a 700-nm near-IR detector (Licor). All the slab gels, including agarose gels and polyacrylamide gels, are ready gel precast mini-gels purchased from Bio-Rad Corporation. All the buffers, either running buffer or sample buffer, are from Bio-Rad. The staining agent, or the DNA intercalating dye ethidium bromide (EtBr), is also from Bio-Rad. The 25bp DNA step ladder was purchased from Promega, while the 50-350bp concentrated sizing standard used in the analysis of LDR product fidelity is a product of Licor Biosciences and is composed of 14 IRDye700-labeled DNA fragments with equal banding intensities in 90% formamide solution with bromophenol blue. Procedures of using this near-IR sizing ladder are as
follows: denaturing the solution at 95°C for 1-2 min, placing on ice for 10 min, and then loading 0.2 μL for any well or comb. The imaging system for either agarose gels or poly(acrylamide) gels is a Kodak imaging system equipped with epi-white light and UV transmission light. Analysis of gel images was done with imaging software such as ImageQuant.

4.2.2 CELL CULTURE OF PRIMARY BREAST CANCER CELL LINE

The procedure for the cell culture can be described briefly as follows: first of all, a sufficient amount of cell growth medium (100 mL in total) was prepared, which includes 10 mL of fetal bovine serum, 0.2 mL antibiotic, and 90 mL RPMI1640 medium (2 mM L-glutamine, 1.5 gL⁻¹ sodium bicarbonate, 4.5 gL⁻¹ glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate). The growth medium was then sterilized by passing through a filter unit (150 mL PES filter unit with 0.22 μm size pore, Corning Inc.) using a sterile syringe. Next, the cell line was added to a centrifuge tube, 12 mL of the prepared growth medium was pipetted into the tube and mixed with a sterile syringe. 4 mL of the cell line in the growth medium was added to each of the three p60 petridishes which have a bottom surface area of 25 cm². The cells were observed under a microscope to ensure the proper density of the cells, and then placed in the incubation oven (37 °C, 5% CO₂). Finally, a daily check on the cell growth was performed in order to make sure the cells were growing normally. The medium was also changed.

When the cell line grew to a good degree of confluency, it was ready for splitting. The old growth medium was removed with a sterilized disposable syringe by gentle vacuuming, then 1-mL of trypsin was added to the petridish to detach the cells from the bottom surface, then cells removed with a sterilized disposable syringe by gentle vacuuming, and then another 1 mL of trypsin was added to the same petridish and incubated in the incubation oven for 2-5 min to help achieve complete detaching.
For splitting the cells, 8-mL of the growth medium (already warmed in a 37 °C water bath) was added to the p60 petridish and mixed well with a sterilized pippet three times, and the 9 mL of the cell suspension (1 ml of the added trypsin plus 8 mL of the added growth medium) was evenly split into three new p60 petridishes and mixed thoroughly by a set of shakings. The split cells can be further cultured in the incubation oven or used for other application.

4.2.3 ISOLATION OF GENOMIC DNA FROM THE CULTURED CELLS

The isolation of genomic DNA from the cultured cells was carried out with an isolation kit from Sigma as is depicted in Figure 4.6. Generally, the procedure is as follows: the cells were first harvested by detaching them from the petridish bottom surface with trypsin, 5 mL of the growth medium was then added, and the detached cells were transferred to a plastic tube. The cells were then pelleted at 300 x g for 5 min and the growth medium was carefully removed with a wide-pore pipette tip. The cell pellet was resuspended thoroughly in 200 μL of the resuspension solution, 20 μL of the proteinase K solution and 200 μL of lysis solution C were then added. The new cell suspension was then agitated on a vortex mixer for about 15 sec and incubated at 70 °C for 10 min. 500 μL of the column preparation solution was added to each pre-assembled GenElute Miniprep binding column and centrifuged at 13,000 x g for one min. Finally, 200 μL of absolute ethanol was added to the lysate and mixed well by agitation on the vortex; the lysate was then transferred with a wide-pore pipette tip (100-1000 μL) to the pretreated binding column and centrifuged at 7,300 x g for one min. The flow-through liquid was discarded and the binding column was now placed in a new 2 mL collection tube. 500 μL of pre-diluted washing solution was added to the binding column, and the column was centrifuged at 7,300 x g for one min. A second wash can be carried out with the same washing solution. At last, 200 μL of the elution solution was added directly to the center of the binding column, incubated at room temperature
for five min, and centrifuged at 7,300 x g for one min to elute the genomic DNA. A second elution might be performed with the procedure.

**Figure 4.6** Schematic of isolation of genomic DNA from a primary breast cancer cell line. (GenElute mammalian genomic DNA miniprep Kit (Sigma))

### 4.2.4 POLYMERASE CHAIN REACTION (PCR) AND OPTIMIZATION OF THE REACTION CONDITIONS

The genomic sequence of exon 20 (68917-69000) from Homo Sapiens *BRCA1* gene (ensemble gene ID: ENSG00000012048) is shown in green color in the following: 19′ 5′ CAT GAT TTT GAA GTC AGA GGA GAT GTG GTC AAT GGA AGA AAC CAC CAA GGT CCA AAG CGA AGA GAA TCC C CAG GAC AGA AAG GTA AAG CTC CCT CCC TCA AGT TGA CAA AAA TCT CAC CCC ACC ACT CTG TAT TC 3′. While the specific
fragment sequence around exon 20 in the BRCA1 gene that was extracted from the nucleus of the primary breast cancer cell line (HCC1937, ATCC) has a single insertion C at position 5382 (in red color in italicized font). For the PCR reaction, the extracted genomic DNA was used as the DNA template, in either a purified format or as was isolated from the cell line. The two primers for the PCR reaction were: 5'- AGG AGA TGT GGT CAA TGG AAG AAA -3' (upper primer, \( T_m = 56.2^\circ C \)) and 5'- GAA TAC AGA GTG GTG GGG TGA GAT -3' (lower primer, \( T_m = 58.1^\circ C \)). Thus, the expected PCR product was a 121 bp DNA sequence: 5' A GGA GAT GTG GTC AAT GGA AGA AAC CAC CAA GGT CCA AAG CGA AGA GAA TCC CCAG GAC AGA AAG GTA AAG CTC CCT CCC TCA AGT TGA CAA AAA TCT CAC CCC ACC ACT CTG TAT TC 3'. The PCR reaction included making a cocktail of all the necessary reagents at certain concentrations as is shown in Table 4.1, mixing thoroughly with a pipette tip, and dividing into five 200-\( \mu L \) PCR vials (Daigger & Company, Inc).

**Table 4.1** A typical PCR cocktail used for the amplification of the BRCA1 gene sequence.

<table>
<thead>
<tr>
<th>PCR components</th>
<th>Composition or Concentration (( \mu M ))</th>
<th>Volume (( \mu L ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 ( \mu M/dNTP )</td>
<td>2 ( \mu L/dNTP )</td>
</tr>
<tr>
<td>Upper primer (24bp)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lower primer (24bp)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PCR water</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>DNA template</td>
<td>Genomic DNA (HCC1937 cell line)</td>
<td>1</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>Taq DNA polymerase</td>
<td>1</td>
</tr>
</tbody>
</table>
All five vials were then placed into the metal block of the thermal cycler and a drop of mineral oil was added onto each cap of the five vials to protect the solution from evaporation during the heating process. Thermal cycling was achieved with a preset program based on parameters listed in Table 4.2.

**Table 4.2** The optimized thermal cycling parameters for PCR amplification of *BRCA1* gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>First denaturing</td>
<td>94 ºC</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94 ºC</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>63 ºC</td>
<td>40 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 ºC</td>
<td>40 sec</td>
</tr>
<tr>
<td>Last extension</td>
<td>72 ºC</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Fidelity of the PCR product was examined by analysis of the product with agarose gel electrophoresis (3%), followed by ethidium bromide (EtBr) staining and visualization with UV light at 254 nm. Optimization of the thermal cycling parameters was carried out by adjusting the annealing temperature and by using purified genomic DNA acquired through the solid-phase reverse immobilization (SPRI) technique. The integrity and purity of the PCR product was further improved by carrying out a secondary PCR reaction with a purified 1st PCR product as the DNA template. Purification of the 1st PCR product was accomplished with a purification kit called SpinPrep PCR clean-up kit (Novagen). This kit includes three major steps: binding,
washing, and eluting, and it can be described briefly as the following: 17 μL of the PCR product was transferred to a clean 1.5-mL microcentrifuge tube, 68 μL of SpinPrep binding buffer was added and mixed well by agitation on a vortex mixer. The mixture was then transferred into a spin unit comprising a SpinPrep PCR filter inserted in a 2-mL receiver tube, and the assembly was then centrifuged at 12,000 rpm for 1 min. The filter unit was removed and inserted into another new 2-mL receiver tube, then 400 μL of the SpinPrep binding buffer was added, and the assembly was centrifuged at 12,000 rpm for 1 min. The filter unit was again removed and placed into another 2-mL receiver tube, then 500 μL of reconstituted SpinPrep washing buffer was added, and the assembly was centrifuged at 12,000 rpm for 1 min. The residual SpinPrep washing buffer was further removed by centrifuging the filter unit inserted in a new 2-mL receiver tube at 12,000 rpm for 2 min. The filter unit was finally transferred into a 1.5-mL elution receiver tube, into which was pipetted 50 μL of the prewarmed (70°C) SpinPrep elution buffer onto the filter membrane, the tube and its contents were incubated at room temperature for 3 min, and then the PCR purified product was eluted by centrifugation for 1 min at 12,000 rpm.

4.2.5 LDR FOR SINGLE-INSERTION DETECTION IN BRCA1 GENE

Since its invention in the early 1990s, the highly sensitive, specific, and accurate ligase detection reaction has been extensively applied to cancer disease diagnosis, forensic testing and even coupled to immunoassays to make antigen detection ultrasensitive. Reported here is an example of the ligase detection reaction for sensitive single-mutation detection in the BRCA1 gene from a breast cancer cell line. The two primers designed for the ligation reaction were a modified version of the reported primer pair. The discriminating primer: 5′-cgc aag gta ggt gct gta ccc gca-CAA AGC GAG CAA GAG AAT CCC-3′, in which the sequence in small letters was the complementary Zipcode sequence (cZip11), and the nucleotide cytosine at the
very end of 3’ was an insertion base. The common primer: **5’-P-CAG GAC AGA AAG GTA AAG CTC CCT C-Cy5.5-3’**, in which there was a near-IR dye (Cy5.5) conjugated to the 3’ end and phosphate group at the 5’ end in order for the ligation to occur. The sequence of the expected LDR product is **5’-cgc aag gta ggt gct gta ccc gca-CAA AGC GAG CAA GAG AAT CCC CAG GAC AGA AAG GTA AAG CTC CCT C-Cy5.5-3’**, which is a 70mer ss-DNA sequence with a cZip11 tag on one end (5’), with a near-IR dye on the other (3’).

LDR is very much like PCR except that it is linearly amplified under a thermal stable DNA ligase. The commonly used LDR cocktail components are listed in Table 4.3, while the operating parameters are shown in Table 4.4 according to a typical standard ligase reaction. Two negative control reactions were also carried out to test the specificity of the ligase reaction under the same operating parameters, except that there are slight and unique differences in terms of components of the LDR cocktail; one negative control used a different discriminating primer in which an insertion A instead of C was at the 3’ end, while another negative control consisted of using DNA template extracted from a wild type cell line.

**Table 4.3 Components of the LDR cocktails for the test sample and the two negative controls.**

<table>
<thead>
<tr>
<th>LDR</th>
<th>Negative Control #1</th>
<th>Negative Control #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDR buffer</td>
<td>LDR buffer</td>
<td>LDR buffer</td>
</tr>
<tr>
<td>Nanopure water</td>
<td>Nanopure water</td>
<td>Nanopure water</td>
</tr>
<tr>
<td>Discriminating Primer (w/ insertion C, 50 nM)</td>
<td>Discriminating Primer (w/insertion A, 50 nM)</td>
<td>Discriminating Primer (w/insertion C, 50 nM)</td>
</tr>
<tr>
<td>Common Primer (50 nM)</td>
<td>Common Primer (50 nM)</td>
<td>Common Primer (50 nM)</td>
</tr>
<tr>
<td>DNA template (1.25 nM, 2nd PCR purified product)</td>
<td>DNA template (1.25 nM, 2nd PCR purified product)</td>
<td>DNA template (1.25 nM, gDNA from cell line HT29)</td>
</tr>
<tr>
<td>Taq Ligase</td>
<td>Taq Ligase</td>
<td>Taq Ligase</td>
</tr>
</tbody>
</table>
4.2.6 IMMOBILIZATION OF ZIPCODE11 OLIGO SEQUENCES ON UV-MODIFIED PMMA SURFACES

Immobilization of oligo DNA sequences (Zipcode11: 5′-TGC GGG TAC AGC ACC TAC CTT GCG-(dT)₁₅-(CH₂)₆-NH₂-3′) was tried in different buffer systems and coupled covalently to COOH-terminated PMMA surfaces through carbodiimide chemistry (Scheme 4.1).

**Table 4.4** The typical operating parameters in the LDR reaction (40 cycles) used here.

<table>
<thead>
<tr>
<th>Name</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>First denaturing</td>
<td>94 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>Denaturing</td>
<td>94 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>65 °C</td>
<td>2 min</td>
</tr>
</tbody>
</table>

**Scheme 4.1** Patterned immobilization of amine-terminated oligonucleotides on UV-modified PMMA surfaces.
The procedure can be briefly described as follows: precleaned pieces of PMMA sheet were exposed to UV light (see Chapter 2) through a contact photomask for 30 min, rinsed with IPA, and dried with house nitrogen. Zipcode11 oligo DNA was diluted with MES buffer to intended concentration, mixed with 40 mM EDC, and then immediately sandwiched between two pieces of UV-modified PMMA sheet. The sandwiched chips were incubated at 4°C overnight or at room temperature for 3 h, then rinsed with 2x SSPE buffer, followed by rinsing with nanopure water and gently drying with house nitrogen. Confirmation and optimization of the immobilization by using different buffer systems were monitored by X-ray Photoelectron Spectroscopy (XPS).

4.2.7 HYBRIDIZATION OF LDR PRODUCTS TO IMMOBILIZED ZIPCODE11 SEQUENCES

As was illustrated in Figure 4.5, the LDR product sequence for the test sample that carries a complementary Zipcode sequence (cZipcode 11) at one end should hybridize to the immobilized Zipcode sequence (Zip11), and its presence can be detected by near-IR fluorescence detection. The hybridization conditions were totally dependent on the complementary Zipcode11 sequence and independent of the other part in the LDR product sequence bases, which is why the name of universal Zipcode sequence is used. The protocol for the hybridization, signal detection and analysis was taken from previous reported work and can be generally described as follows: the LDR product was diluted in 5x SSPE buffer to an intended concentration, 5 μL was spotted on the immobilized Zipcode11 area, and the LDR product/Zipcode 11-PMMA was incubated for 3h in a dark, humid container placed in a hybridization oven which had already been preheated and equilibrated at 50°C. After incubation, the surface was rinsed with 2x SSPE buffer, then placed in fresh 2x SSPE buffer, and the surface was thoroughly cleaned by shaking the PMMA sheet in the 2x SSPE buffer on an orbital shaker for at least 15 min twice. The chip
was at last gently dried with house nitrogen and then examined using a fluorescence scanning microscope.

4.3 RESULTS AND DISCUSSION

4.3.1 FIDELITY OF PCR PRODUCT AND OPTIMIZATION OF THE PCR REACTION

Fidelity of PCR products is mostly determined by the design of the specific primers and choice of an optimum annealing temperature. In this work, both primers were chosen and optimized by a program which automatically checks the specificity of primer pairs to avoid any possible formation of self-dimers, hetero-dimers, and hairpins. Therefore, optimization of the PCR reaction was mainly carried out by optimizing the annealing temperature and pre-treatment of the genomic DNA template.

Depicted in Figure 4.7 are agarose gel electrophoresis results of PCR products that were produced under different annealing temperatures. Lane 1 and 8 are 100bp DNA step ladder, Lanes 2 and 3 are PCR products produced under annealing temperature of 53°C, lanes 4 and 5 are PCR products produced under annealing temperature of 59°C, and lanes 6 and 7 are PCR products produced under annealing temperature of 63°C. As is noticed from the gel image, there are least non-specific PCR products (products other than the expected 121bp PCR product) for annealing temperature of 63°C compared to the other two annealing temperatures.

Lanes 3, 5 and 7 are PCR products with purified gDNA as the template while lanes 2, 4 and 6 are PCR products using unpurified gDNA as the template. The gel image also disclosed a fact that purification of the gDNA with SPRI didn’t really help to produce more specific PCR product, but rather eliminated the PCR product completely (see Lanes 3, 5, and 7) although the mechanism behind this phenomenon is still not very clear yet. A possibility that can be drawn
from a preliminary analysis is that the magnetic beads used in SPRI purification probably adsorbed the genomic DNA template or simply damaged it.

The intensity of the expected PCR product was not very strong, therefore a secondary PCR reaction was performed in order to prepare a PCR product of better quantity and specificity. The secondary PCR was carried out using 63°C as the annealing temperature; the purified 1st PCR product was used as the DNA template, and all other parameters were remained the same as that in the first PCR reaction. Shown in Figure 4.8 is a typical agarose gel electrophoresis image of the purified 2nd PCR product.

**Figure 4.7** Agarose gel electrophoresis of PCR products obtained under different annealing temperatures. Lanes 1 and 8 are 100bp DNA step ladder; Lanes 2, 4, and 6 are PCR products using unpurified gDNA as the template; Lanes 3, 5 and 7 with purified gDNA as the template. Annealing temperatures are 53°C for lane 2 and 3, 59°C for lane 4 and 5, and 63°C for lane 6 and 7.
A bright band at 121bp was clearly shown. The quality of the secondary PCR product further determined the quality of the LDR reaction in which the secondary PCR product was used as the DNA template for the ligation.

4.3.2 LDR PRODUCTS MONITORED BY DNA POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Following PCR amplification of the region of interest, the mutation (single insertion C at 5382 position in exon 20 of BRCA1 gene from the genomic DNA of HCC1937 breast cancer cell line) was detected in ligase detection reaction, in which a unique thermal stable ligase joined the pair of adjacent oligonucleotides complementary to the DNA sequence of interest (see Figure 4.5). Ligation occurs only when the nucleotide at the junction between the paired

**Figure 4.8** Agarose gel electrophoresis of the 2nd PCR product. Lane 1 is a 25bp DNA step ladder, while Lane 3 is the secondary PCR product (121bp). There is no injection in Lane 2.
oligonucleotides is exactly complementary to that in the DNA template sequence of interest, therefore LDR is able to discriminate between wild-type and mutant sequences. Covalently attached on the 5' end of one of the paired oligonucleotides (paired primers) is a 24-mer non-genomic oligonucleotide sequence that is complementary to a 24-mer Zipcode oligonucleotide sequence immobilized on a known location on the universal DNA array. On the 3' end of the same primer is the junction nucleotide that is perfectly complementary to that on the DNA sequence of interest. The 3' end of the other primer is labeled with a near-IR fluorescent dye (Cy5.5, $\lambda_{\text{abs}} = 685$ nm and $\lambda_{\text{em}} = 706$ nm) and the 5' end of the same primer is phosphorylated. Therefore, observation of fluorescence signal from the universal DNA array indicates the presence of the single-point mutation only when the ligation is successful.

Fidelity of the LDR product was monitored by DNA poly(acrylamide) gel electrophoresis coupled to a near-IR fluorescence detector. Displayed in Figure 4.9 is a typical PAGE image of LDR products of the test sample and the control samples. As is detailed in section 4.2, the DNA sizing ladder (Lanes 1 and 5 in Figure 4.9) used in PAGE for LDR product analysis are DNA strands that are evenly conjugated to a 700-nm fluorescent dye, and the expected LDR product is also labeled with a near-IR dye on the 3' end of the sequence (see Figure 4.5). For control #1 (lane 3 in Figure 4.9), there is an adenine nucleotide at the junction of the paired primers instead of a cytosine nucleotide; thus no ligation between the 3' end of the discriminating primer and the 5' end of the common primer shall occur during LDR thermal cycling. Therefore no fluorescent DNA band at the 70mer shall be produced, and this is observed.

For control #2 (Lane 4 in Figure 4.9), the DNA template used in the LDR is a wild-type instead of the mutated $BRCA1$ gene, thus the nucleotide at the junction of the adjacent paired primers is not complementary to that on the DNA sequence of the wild type, and no ligation shall
occur. This is borne out by the lack of fluorescence signal at the 70mer position on the PAGE image. It is clearly seen that there is a bright fluorescent band at the 70mer position for the tested LDR sample (Lane 2 in Figure 4.9), which indicates success of the ligation for the C-insertion mutation.

Figure 4.9 Poly(acrylamide) gel electrophoresis of the LDR products from the test sample and control samples. Lanes 1 and 5 are the DNA sizing standard (50-350bp, Licor), Lane 2 is the LDR product with the secondary PCR product as the DNA template, while Lanes 3 and 4 are the two negative controls. Lane 3 is the product using a discriminating primer with an insertion A instead of C in the specific position, and Lane 4 is the product using the DNA template isolated from a wild-type cell line.
4.3.3 XPS ANALYSIS OF IMMOBILIZED ZIPCODE11 OLIGONUCLEOTIDES ON UV MODIFIED PMMA SURFACES

As illustrated in reaction Scheme 4.1, immobilization of the amine-terminated Zipcode11 oligo sequences will introduce nitrogen in the surface elemental composition of the modified PMMA compared to the pristine and UV-modified PMMA surfaces. XPS can be used to probe surface compositional changes and interrogate the surface density of the immobilized oligonucleotides.\textsuperscript{25} XPS analysis was carried out using the same protocol described in previous work (see Chapter 2).\textsuperscript{26, 27} In brief, the pristine, UV-modified, and oligonucleotide-immobilized PMMA chips were all thoroughly rinsed with nanopure water, dried with house nitrogen, and then placed in the sample transfer chamber (STC) and pumped down overnight. The samples were transferred to the sample analysis chamber (SAC) the next day when the vacuum pressure in the STC reached a minimum of $10^{-8}$ torr; survey and high-resolution spectra were acquired with the charge neutralizer turned on to compensate for possible charging effect on the insulating polymer. The peak at 397.3 eV signifies the existence of nitrogen (N1s emission), indicating the successful immobilization of amine-terminated Zipcode oligo sequences. Furthermore, the peak intensity can be used as a parameter to compare different immobilizations of oligonucleotides assuming that surface treatments prior to and after immobilization are the same for each type of immobilization.

Shown in Figure 4.10 are N1s high-resolution XPS spectra from the dumbbell-patterned, UV-modified PMMA surfaces, which includes both the UV-modified and the pristine PMMA areas, resulted from immobilization reactions using the same concentration of Zipcode11 oligonucleotide (10 $\mu$M) and EDC (100 mM), the same buffer composition (PBS buffer), but under different buffer pH values. As is clearly demonstrated in Figure 4.10, the N1s signals from two different immobilization conditions are very close in terms of intensity on the UV-modified
Figure 4.10 XPS spectra of variously-treated PMMA surfaces treated with Zipcode11 oligo sequences with the same buffer system but under different pH values on UV-modified PMMA surfaces (top), and pristine PMMA surfaces (bottom); the latter was to test for non-specific adsorption of Zip11 on the pristine PMMA surfaces.
PMMA sheets, while dramatically different in the pristine PMMA area; there was significant non-specific adsorption resulting from the pH 11.49 PBS buffer system, while a negligible amount of non-specific adsorption was produced under pH 7.38 PBS buffer system. The reason for this non-specific adsorption is still under investigation. However, by a preliminary analysis, it is believed that buffers with different pH values infer different level of charges onto the DNA strands, therefore changing the net surface charges on the DNA strands. Hence the electrostatic interaction between the treated PMMA surface and the charged DNA strand is uniquely different under immobilization conditions.

4.3.4 NEAR-IR LASER-INDUCED FLUORESCENCE SCANNING OF PMMA CHIPS RESULTING FROM HYBRIDIZATION REACTIONS

After rinsing with 2x SSPE buffer and gentle drying with house nitrogen, hybridization chips were immediately scanned with a home-built near-IR, laser-induced fluorescence scanner in a darkroom laboratory. Images were analyzed with a home-built program called HarpPlayer. As is demonstrated in Figure 4.11, there are bright fluorescence spots at locations where the complementary DNA sequences were immobilized and hence the hybridization events occurred.

Figure 4.11 Near-IR, laser-induced fluorescence scanning image of the hybridization event between the immobilized Zipcode11 and the LDR product of the test sample.
In the control experiments where the two control LDR samples were incubated with Zipcode11-immobilized PMMA chips, there also appeared fluorescent signal which was attributed to the non-specific adsorption of the excess common primer (see Figure 4.12).

**Figure 4.12** Near-IR, laser-induced fluorescence scanning images of the hybridization event between the immobilized Zipcode11 and the LDR products of the two control samples.

### 4.4 CONCLUSIONS

A primary breast cancer cell line was successfully cultured and is readily available for future applications. As one of the applications of the cultured cell line, *BRCA1* mutated genomic materials were successfully prepared for future fabrication of hybrid biosensor systems. The detailed outcomes can be summarized and conclusions are drawn as follows. Genomic DNA from the cell line was successfully isolated and *BRCA1* gene was investigated on the single-insertion C-containing specific region of exon 20 through PCR/LDR combined assays. PCR was carried out and optimized to obtain the targeting sequence in exon 20 of *BRCA1* gene, and proved to be in good quantity and quality as was evidenced by agarose gel electrophoresis results. The mutated genomic material was then obtained by LDR reaction using the DNA template sequence prepared by PCR. LDR products were analyzed by PAGE and were shown to
have good integrity. Proof of concept of the array-based assay on the LDR test sample and the
two negative controls showed self-consistent results: the hybridization of the LDR test sample
was successful, but due to the interfering content of the excess fluorescent common primer in the
LDR product, there was no significant discrimination between the test sample and the controls in
terms of fluorescence intensity of the hybridization event; but the expected 70mer LDR product
only appeared in the test sample and not the control samples on the PAGE gel image. Therefore,
it is safe to conclude that the LDR product of the test sample was successfully prepared.

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CHAPTER 5. PREPARATION OF BREAST CANCER PROTEIN BIOMARKER TARGETING MATERIALS

5.1 INTRODUCTION

As is true with most other cancer diseases, it is often futile and disfiguring to treat the cancer when it reaches its advanced stages. Therefore, it is crucial to develop techniques that are capable of detecting breast tumor formation accurately at its earliest stage and subsequently take appropriate measures for treatment.¹

Among the screening and testing methods, tumor markers are one of the targets used for early detection and diagnosis of cancers. Tumor markers are materials produced by tumors or by other cells of the body induced by cancer or certain noncancerous conditions. Usually, these materials may be found in the blood, urine, tumor, and other tissues. Genomic and proteomic-based biomarkers are the most widely studied tumor markers for early detection of cancers; genomic biomarkers are the genetic mutations or alterations in specific genes, while proteomic biomarkers are the changes in protein shape, function, and pattern of expression due to cancerous conditions.

Compared to genomic biomarkers, protein biomarkers are much more complicated due to the huge total number of proteins in the cell, variation of proteins among individuals, different cells, and even different developmental stages of the same cell type. Basically, proteomic biomarkers are very dynamic while genomic biomarkers are relatively static. Protein biomarkers have also been identified as important factors in diagnosing and monitoring cancer diseases. Changes in protein structure, expression patterns, and function may indicate predisposition to cancerous conditions, body response to drug treatment, and relapse of cancer diseases. Moreover, genetic biomarkers may, in some cases, not suffice to correctly identify a specific phenotype, especially in the early stages of tumor development due to the static nature of the cell’s genome.
Therefore, it is desirable to look at protein expression levels, because the proteome (entire complement of proteins expressed by a single cell, tissue, or organ) profile is highly dynamic (responds quickly to external stimuli); it is the proteins that comprise the proteome which regulate processes resulting in the phenotype. Specifically for breast cancer, protein biomarkers have proven to be valuable in diagnosis, prognosis, and monitoring of disease therapy. Strategies for early diagnosis of breast cancer or breast cancer predisposition are: screening for genetic mutations in genetic biomarkers especially for high-risk populations and examining proteomic biomarkers such as Her2/neu, CEA, MUC-1, and mammoglobin; and accurate and early staging of breast cancer diseases by analysis of micrometastasis of breast cancer.

As it is known that there are genetic, proteomic, and metabolic biomarkers for a specific cancer disease, it is desirable to simultaneously assay all different types of biomarkers at the same platform in order to build a comprehensive diagnosis of the disease. There are a number of reported techniques for screening and examining each type of biomarker: 2-dimensional gel electrophoresis after isolation of the protein content from cells, mass spectrometry, and the newly developed biochip technologies. Simultaneous assaying of different types of biomarkers on the same platform is still at early stages. The concept of DNA-directed protein assay is to convert a DNA chip into a protein chip, thereby it is possible to assay both DNA and protein analytes on the same DNA-chip platform. Conjugation of a protein with an oligonucleotide is the first step to achieve this hybrid assay. There are several strategies for preparing protein-DNA conjugates. One is directly utilizing the cysteine residue on the protein and links the protein to a thiol-reactive oligonucleotide sequence.\(^2\)\(^,\)\(^3\) However, there are very few free cysteines on secreted recombinant proteins such as antibodies, due to the oxidizing nature of the subcellular environment.\(^4\) The second strategy is to use bifunctional cross-linkers, either small molecule
such as sulfo-SMPB or biotin-streptavidin interaction,\textsuperscript{5-10} to covalently link the amino group or carboxylic acid group on the protein to a derivatized oligonucleotide. The disadvantage of using this strategy is that it is difficult to control the number and topology of the conjugation sites, hence resulting in heterogeneity of the conjugates. The third strategy is to create a peptide bond between a protein and an oligonucleotide via a process named “expressed protein ligation”,\textsuperscript{11, 12} during which a fusion protein with an intein moiety and a cellulose-binding domain (CBD) for purification is expressed, and then the protein is cleaved from the intein-CBD partner under reducing and low-temperature conditions, thereby generating a purified protein with a C-terminus \( \alpha \)-thioester bond. This bond can be linked directly to thiol-derivatized oligonucleotide sequences. The most drawback of this strategy is that it is time-consuming, often taking days for splicing the proteins and preparing the conjugates.

Among the arena of researching protein-DNA conjugates are those by Niemeyer and coworkers who have invested significant efforts in developing and applying the concept of DNA directed protein assays since mid 1990s,\textsuperscript{13} in which biotin labeled antibody molecules are linked to biotin labeled ss-DNA molecules through a cross-linking molecule called streptavidin (STV) which has four biotin-binding domains. Then the antibody-DNA complex is hybridized to its complementary oligonucleic acid sequences arrayed on a DNA chip (see Figure 5.1). The assay outcome is transduced through color changes in the enzymatic reaction upon the formation of a traditional Enzyme Linked Immunosorbent Assay (ELISA) comprised of the capture antibody-DNA complex, antigen, and the enzyme linked detection antibody. The main theme in the reported DNA-directed protein assays including those by Neimeyer and coworkers, though is to convert the DNA chip into a protein chip, thereby to analyze the targeting protein materials in a
much more economical way, especially when lower protein concentration and smaller array spots are used.

Figure 5.1 Schematic diagram of the DNA directed assay. STV refers to streptavidin, a protein with four monomer domains. Biotin is a small organic molecule that recognizes the monomer domain in the streptavidin molecule. AP refers to alkaline phosphatase, an enzyme specifically for the oxidation of alkaline phosphate-containing molecules.

The DNA-directed protein assay allows economical analysis of protein targets, but in principle it also provides new ways of assaying both genomic and protein targeting materials simultaneously on the same platform. To fabricate such a platform, the first functional layer would be the specifically designed oligonucleotide sequences addressed in microarray spots which in concept are similar but in size much smaller than the microtiter plates used in Neimeyer’s work. Instead of only assaying protein materials using the DNA titer plate, in the
work reported here both genetic mutated materials and protein biomarker antigens—each incorporated with a specific oligonucleotide sequence that is complementary to one of those sequences in the microarray spots—are to be analyzed in a so-called hybrid assay format, through the base-pair hybridization reactions on the microfluidic platform. Signal generation and transduction are through the near-IR lased-induced fluorescence detection method because it is one of the most sensitive detection method so far reported. A simplified representation of the hybrid assay is shown in Figure 5.2.

**Figure 5.2** Representation of the hybrid bioassay on a microfluidic platform. As a model system, the genetic mutational material is related to the *BRCA1* gene single point mutation (see Chapter 4) while the protein targeting material used here is a breast cancer protein biomarker CEA.
As is illustrated in Figure 5.2, on the microfluidic platform, there are arrays of Zipcode oligo DNA sequences covalently immobilized on the polymeric substrate. Then a mixture of both the genetic and proteomic targeting materials will be introduced in the sample reservoir and flowed through the microchannels. Hybridization interaction between the Zipcode probes and the targeting materials will be read out dynamically or offline through fluorescence imaging methods. Preparation of the materials for the genetic targets have been discussed in Chapter 4; hence, in this chapter, preparation of the protein targets will be detailed spanning the semi-synthetic process and qualitative analysis of the product at each reaction step with a variety of complementary techniques.

5.2 MATERIALS AND METHODS

5.2.1 GENERAL

The ultimate goal of the materials preparation is to covalently link single-stranded DNA sequences (cZipcode1) with anti-breast cancer biomarker antibodies (IgG), and to use this complex as a capture-based probe of breast cancer protein biomarker (antigen) detection on a hybrid biosensor system using a microdevice platform. The ss-DNA sequence (thiol-cZipcode1, 24 bases, 5'-/5ThioMC6-D/GCT GAG GTC GAT GCT GAG GTC GCA -3') was customer synthesized by Integrated DNA Technologies (IDT) with standard desalting treatment. Mouse anti-human carcinoembryonic (CEA) monoclonal antibodies (IgG molecules in PBS buffer) were purchased from Fitzgerald Industries International, Inc., and used with proper dilution and buffer system (phosphate buffered EDTA; PBE). The heterobifunctional link molecule, sulfo-SMPB (MW 458.38Da, spacer length of 11.6 angstroms), was a product of Pierce Biotechnology, Inc., received in solid powder, and used in appropriate buffer system. The D-Salt Dextran Desalting Columns (MWCO: 5,000Da) was ordered from Pierce Inc. and used according to the protocol.
provided by the company. Cleland’s REDUCTACRYL Reagent,\textsuperscript{14} or the solid-phase reduction agent DTT, was obtained from EMD Biosciences, Inc., and used according to the instructions provided by the company. Millex-GV Filter (0.22 $\mu$m, PVDF, 4 mm, non-sterile) was ordered from Millipore Corporation and used according to instructions accompanying the product. Another type of desalting column called NAP-5 columns are prepacked minicolumns purchased from GE healthcare, and were used as instructed by the company. All the buffer systems, including phosphate-buffered saline (PBS, 20 mM KH$_2$PO$_4$, 150 mM NaCl, pH 7.38), phosphate-buffered EDTA (PBE, 2 mM EDTA in PBS), nanopure water (18.0 $\Omega$.cm) were home prepared or produced and further purified with sterilized filter bottle units (membrane pore size: 0.2 $\mu$m).

The overall reaction scheme of the cross linking is illustrated in Scheme 5.1, in which the lysine residue on the IgG antibody molecule is first linked to the succinimidyl ester group on the heterobifunctional linker sulfo-SMPB by forming an amide bond. The modified IgG antibody molecule, sulfo-SMPB-IgG is now carrying a free maleimidyl functional moiety which is thiol reactive. The thiol-derivatized cZipcode1 oligonucleotide sequence is custom-synthesized product, and received in oxidized form. This sequence is first reduced with solid-phase DTT to release free thiol functional groups, and then the free thiol group on the cZipcode1 sequence is ready to link to the maleimide moiety on sulfo-SMPB-IgG, thus the IgG antibody is cross-linked to the cZipcode1 oligonucleotide sequence through the heterobifunctional linker sulfo-SMPB. The detailed activation reaction of the thiol-derivatized cZipcode oligonucleotide sequences is depicted in Scheme 5.2. The thiol-derivatized cZipcode1 oligonucleotide sequences that carry the DMTO protecting group are reduced by the solid-phase DTT and as a result, the thiol-derivatized cZipcode1 oligonucleotide sequences are converted into two types of SH-terminated molecules:
one is the cZipcode1 oligonucleotide sequence now bearing the free reactive sulfur hydryl group, and another is the small protecting molecule. The reducing agent DTT undergoes ring formation during the reaction: the two free thiol groups on it are oxidized and form disulfide bond, a six-membered ring is thus formed.

Scheme 5.1 The overall reaction scheme of covalently linking IgG with ss-DNA molecules.
5.2.2 MODIFICATION OF IgG MOLECULES WITH SULFO-SMPB AS MONITORED BY UV-VIS SPECTROMETRY

IgG antibodies (1.5 mgmL⁻¹ or 10 μM in PBS buffer) were first diluted with PBE buffer to an intended concentration of 3.8 μM. Sulfo-SMPB was brought to room temperature and then weighed into the diluted antibody solution in at least 50-times molar excess of the antibody. The reaction mixture was then incubated at 4°C for two hours. The mixture was brought to room temperature and desalted with a D-salt dextran desalting column which had already been washed and equilibrated with PBE buffer. Compositional analysis of the collections from the desalting column was monitored by scanning UV-Vis spectrometry in which the absorbance at 280 nm is referred to be the modified antibody component. Concentrations of the collected antibody aliquots were determined based on the standard antibody sample prepared with the received antibody reagent in the same PBE buffer.

Scheme 5.2 Reduction of thiol-derivatized cZipcode1 DNA sequences with solid-phase DTT.
5.2.3 REDUCTION OF THIOL-DERIVATIZED ss-DNA SEQUENCES WITH DTT AS MONITORED WITH UV-VIS SPECTROMETRY

The thiol-derivatized cZipcode1 oligo DNA sequences were first diluted in PBE buffer to an intended concentration of 5 μM in a microcentrifuge tube and mixed well with gentle agitation on a vortex mixer. DTT resin was weighed into tube containing the oligo solution in about 100-times weight excess of the oligo DNA. The mixture was placed on the vortex and agitated for about 15 min at room temperature and then filtered into another new microtube in which desalting was achieved so as to get rid of the small SH-terminated protecting group which resulted from the reduction reaction. In the desalting process, 500 μL of reduction product was applied to the pre-equilibrated NAP-5 column, and around 1.0 mL of desalted reduction product was collected and then analyzed with UV-Vis scanning spectrometry; the absorbance at 260 nm was monitored to determine the presence of the activated cZipcode1 oligo DNA sequences.

5.2.4 CROSS-LINKING OF MODIFIED IgG ANTIBODIES WITH ACTIVATED cZIPCODE OLIGO DNA SEQUENCES

The isolated, activated cZipcode1 oligo DNA sequences were immediately added to the modified IgG antibody solutions in a 1:1 molar ratio and incubated at 4°C for about two hours. Longer incubation of the reaction mixture will not do any harm to the reaction, though. The final cross-linked product then underwent desalting, purification, concentration, or buffer exchange in order for respective analyses to confirm the formation and to characterize the structure of the conjugate.

5.2.5 MONITORING THE INTERMEDIATES WITH UV-VIS SPECTROMETRY

The sulfo-SMPB-modified IgG antibody was desalted in order to get rid of the excess sulfo-SMPB as well as the released NHS ester, and then was analyzed with a Cary50 scanning UV-Vis spectrometer. Data was collected with a scanning range of 400 cm⁻¹ to 200 cm⁻¹ and
collected at an interval of 2 cm\(^{-1}\) at medium survey rate. During the reduction of thiol-derivated cZipcode1 oligo DNA sequences, the reduced oligo was also desalted and monitored by UV-Vis scanning spectrometry in the same mode.

5.2.6 CHARACTERIZATION OF STARTING MATERIALS, INTERMEDIATE, AND FINAL PRODUCT WITH MALDI-TOF MASS SPECTROMETRY

Mass spectrometry has proven to be powerful in the analysis of both proteins and DNA molecules in that this technique is able to identify compositions by providing information on an intrinsic physical parameter, molecular weight of the analytes. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) coupled with a time of flight (TOF) analyzer is a well-known mass spectrometric technique used for analysis of complex mixtures because this technique is tolerant of salt and other impurities thus sample preparations are relatively easy and straightforward. In the reported work here, MALDI-TOF was performed on a Bruker Reflex III (Bruker, Breman, Germany) mass spectrometer operated in linear, positive ion mode with a N\(_2\) laser. The laser power was used at the threshold level required to generate signal, and the accelerating voltage was set to 28 kV. The instrument was calibrated with protein/peptide standards bracketing the molecular weights of the protein samples (typically mixtures of apomyoglobin and bovine serum albumin using doubly charged, singly charged and dimmer peaks as appropriate). Samples were prepared in 0.1% TFA at a concentration of 50 pmol/\(\mu\)L. Sinapinic acid (SA) was used as the matrix for proteins after desalting treatment with a protein trap while the DNA samples were desalted with Ziptip (a 10 \(\mu\)L pipette tip with a bed of chromatography media fixed at the end; Millipore) and spotted with DNA matrix (a mixture of THAP and HPA) on the MALDI target. Allotments of 1 mL of matrix and 1 mL of sample were thoroughly mixed together; 0.5 mL of this was spotted on the target plate and allowed to dry before being sent into the instrument for analysis.
5.2.7 CHARACTERIZATION OF STARTING MATERIALS, INTERMEDIATE, AND FINAL PRODUCT WITH ANIONIC ION EXCHANGE CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is a commonly used analysis technique for purification of mixtures of biomolecules such as DNA and proteins. The key in acquiring nice separation profiles lies in several parameters, including appropriate choice of stationary phase, optimization of the mobile phase composition, and application of optimum pressure and buffer gradients. Described here is a specific type of liquid chromatography, namely anionic ion exchange chromatography, in which the column (DNAPac PA-100 column, Dionex) is packed with a pellicular anion-exchange resin comprised of a 13-μm-diameter nonporous polymeric substrate to which is bound quaternary amine-functionalized microbeads (100-nm, alkyl quaternary ammonium-functionalized latex beads, see Figure 5.3). This microporous structure will result in higher resolution due to the rapid mass transport characteristics associated with the resin. The buffer used in the separation is composed of two buffer systems: buffer A is 20 mM Tris-HCl with 0.3 M NaCl at pH 6.30, and buffer B is 20 mM Tris-HCl with 1 M NaCl at pH 6.30. The structure of the microscale resin used as the packing materials in the ion exchange column is depicted in Figure 5.3, and the buffer gradient in the time table is listed in the following table.

Table 5.1 Time table for the buffer gradient.

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
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<td>100.0</td>
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<tr>
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<td>0.0</td>
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<td>40.00</td>
<td>1.00</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
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<tr>
<td>40.10</td>
<td>1.00</td>
<td>100.0</td>
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<td>1.00</td>
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</tr>
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</table>
Lasers have been used in ion sources to generate gas-phase ions from solid or liquid samples deposited on a sample surface since the mid 1970s.\textsuperscript{16} By controlling the laser power, thermal decomposition of fragile analytes can be avoided. Unfortunately, the maximum mass that could be analyzed prior to 1988 was only about 1000 Da, depending on the laser beam used, because higher laser intensities will destroy the sample. With the pioneering work by Tanaka and coworkers who introduced matrix into the sample, desorption/ionisation of high-mass molecules without inducing fragmentation was achieved.\textsuperscript{17} Hillenkamp and co-workers improved this matrix idea by using organic molecules instead of cobalt as the matrix, significantly increasing the sensitivity of the mass analysis.\textsuperscript{18, 19}
In the work, a Bruker Reflex III MALDI-TOF (Bruker, Breman, Germany) instrument was used for analysis of both antibodies and oligo DNA sequences. This instrument is able to offer low limit of detection (~$10^{-15}$ mole) and high resolution (routinely $>15000$ FWHM for $m/z$ 1000), allowing accurate determination of the molar mass of testing samples. A typical layout of the MALDI-TOF mass spectrometer is illustrated in Figure 5.4 below.

![Figure 5.4 Schematic of MALDI-TOF mass spectrometer.](image)

As is shown in Figure 5.4, the major components in MALDI-TOF MS are the ion source, mass analyzer, and detector. Laser light is used to generate gas-phase ions from the dried mixture of sample and matrix (1:1000 ratio by concentration) that is deposited on the stainless steel MALDI target. The generated ions are then accelerated by a chosen accelerating potential to an electromagnetic field in the TOF mass analyzer. Sample ions with different mass-to-charge ratio will be separated in the analyzer and reach the detector at different times, and thus a mass spectrum is recorded.
5.2.9 THEORY OF ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography is based on the difference of overall charges to separate molecules. It can be used for purification of proteins, oligonucleotides, peptides, or other charged molecules. The analyte of interest must have a charge opposite to that of the functional group attached to the packing resin in the separation column in order to bind. Because this interaction is coulombic, binding must take place under low ionic strength conditions while elution is achieved by increasing the ionic strength to break up the ionic interaction.

Depicted in Figure 5.5 is the block diagram of ion exchange chromatography (upper) which includes four main components: a pump that is used to pump buffers in different gradients, autosampler that can be programmed to draw samples automatically, ion exchange columns that are packed with specific ion exchange materials, and detector that can sense and transduce signals from the separated species. There are different detectors to choose such as fluorescence and UV detector. Here used in the research work is the broad spectrum UV detector. Illustrated in Figure 5.5 (lower) is an anion exchange packing materials, microbeads modified with positively charges species, thus it is capable of exchanging anions from the mobile phase, thereby results in the separation of species bearing different net negative charges on the surfaces.

5.3 RESULTS AND DISCUSSION

5.3.1 UV-VIS SPECTROMETRY

UV-Vis spectrometry is a fast technique for compositional analysis of liquid samples; it is especially informative for samples with very characteristic absorbance profile, such as nucleic acids at 260 nm and proteins at 280 nm. Concentrations of the sample can also be estimated if a
very similar standard sample is used for comparison. In the reported work, UV-Vis absorbance spectra were recorded to identify desalting collections that contain the expected component.

**Figure 5.5** Upper: block diagram of HPLC setup; Lower: schematic of anion exchange chromatography using positively charged microbeads as packing materials in the column.
either the SMPB-IgG or the reduced cZipcode1 oligo DNA sequences (refer Scheme 5.1 and 5.2).

The desalting column used to separate the excess sulfo-SMPB and the released NHS ester from the reaction product sulfo-SMPB-IgG (Scheme 5.1) is called a D-salt dextran desalting columns which has a cutoff molecular weight of 5,000Da. During the desalting, large molecules such as IgG antibodies will come out first, then small molecules such as the sulfo-SMPB and the released NHS ester. As is shown in Figure 5.4 where the UV-Vis spectra of all the collections from the desalting column were recorded spanning absorbance wavelength of 200 nm to 400 nm, there are two set of absorbance peaks: one is at 280 nm (traces of collections 5, 6, 7 and the standard antibody sample in Figure 5.4) which is attributed to the IgG molecules, another is at 269 nm (traces of collection 8, 9 and 10) which probably resulted from the sulfo-SMPB and NHS ester. Concentrations of SMPB-IgG in collections 5, 6, and 7 were also estimated by comparing their absorbance intensities with that of the standard IgG antibody sample which was prepared in the same PBE buffer at a concentration of 1.25 μM. Then the SMPB-IgG with a known concentration was added to the reduced and desalted cZipcode1 oligo DNA sample which has the same concentration to link the IgG with the cZipcode1 sequence.

Desalting of the reduction product of the thiol-derivatized cZipcode1 oligo DNA was carried out with NAP5 columns (MWCO = 5,000Da). There were three collections for each desalting. Based on the instructions of use provided for the NAP5 column, the elution buffer came out into the first collection, the reduced cZipcode1 sequence came out into the second collection, and very small molecules such as the protecting group came out into the third collection. Demonstrated in Figure 5.7 are the UV-Vis spectra of all the collections of the reduction product and the cZipcode1 standard sample.
Figure 5.6 UV-Vis spectra of all collections from the desalting of SMPB-IgG reaction products. The standard antibody (IgG antibody) prepared in PBE buffer was also analyzed with the same spectrometric conditions.
There is clearly an absorbance peak at 260 nm in elution 2 as expected; the concentration of the reduced cZipcode1 sequence was estimated by comparing its absorbance intensity with that of the standard cZipcode1 sample which was prepared in PBS buffer at a concentration of 2 \( \mu \text{M} \).

The cZipcode1 sequence with free sulfur hydryl groups can now readily react with the sulfo-SMPB modified IgG antibodies. All the starting reagents, intermediate product, and the final cross-linking product were further confirmed and monitored by MALDI-TOF MS, a technique that is able to provide accurate mass determinations for each component in the samples.

**Figure 5.7** UV-Vis spectra of elutions of the reduced thiol-cZipcode1 sequence, the standard cZipcode1 sample (2 \( \mu \text{M} \)), and PBS buffer.
5.3.2 MALDI-TOF MASS SPECTROMETRY

MALDI-TOF MS was used to test the integrity of all the starting materials including the thiol-derivatized cZipcode1 sequence and the mouse anti-human CEA monoclonal antibody (IgG). The major peak at 7776.5 m/z in the mass spectrum of Figure 5.6 indicated that the DNA starting material is pure and as stated by the manufacturer.

![MALDI-TOF mass spectrum of the thiol-derivatized cZipcode1 oligo sequence.](image)

**Figure 5.8** MALDI-TOF mass spectrum of the thiol-derivatized cZipcode1 oligo sequence. The oligo was first desalted with Ziptip, mixed with the DNA matrix (a mixture of THAP and HPA), and spotted on the stainless steel MALDI target to dry out prior to mass analysis.
There are three major peaks in the MALDI-TOF spectrum in Figure 5.9: one at an \( m/z \) of 149657 attributed to the molecular ion of the IgG molecule, one at an \( m/z \) of 74777 representing the doubly charged molecular ion, and one at an \( m/z \) of 49934 representing the triply charged molecular ion of the IgG molecule. The spectrum confirms that the antibody is intact and of relatively high purity as a starting material. Intermediate products which include the sulfo-SMPB-IgG and the reduced cZipcode1 sequence were also analyzed by MALDI-TOF to check if they were successfully prepared or not as can be seen in the spectra of Figure 5.8 and Figure 5.9.
Unfortunately, it is hard to conclude if the intermediate was the expected product or not, because the peak in the MALDI-TOF spectrum is very broad and the mass differences between the respective starting material and the resulting intermediate probably are too small to differentiate. The same situation was seen in both intermediate products; the sulfo-SMPB-IgG only has a mass difference of 241.3Da compared to the starting IgG molecule, while the reduced thiol-cZipcode1 sequence only has a mass difference of 405Da compared to the thiol-derivatized cZipcode1 sequence.

**Figure 5.10** MALDI-TOF spectrum of sulfo-SMPB-IgG product. The matrix used was sinapinic acid.
The final cross-linked product IgG-cZipcode1 complex was evaluated using the same mode of MALDI-TOF mass spectrometric analysis despite the non-conclusive result of the intermediate products. The mass spectrum in Figure 5.12 showed a small peak which has mass around 157553Da significantly higher than the starting materials. Interestingly, it is approximately the sum of the molecular weight of the IgG molecule and that of the reduced thiol-cZipcode1 sequence, implying that one oligo was attached to the IgG antibody molecule.

Figure 5.11 MALDI-TOF spectrum of reduced thiol-cZipcode1 sequences.
5.3.3 ANIONIC ION EXCHANGE CHROMATOGRAPHY

Although all the intermediate reaction products were purified to some degree by desalting columns to get rid of small impurities, due to the fact that the MWCO of the desalting column used is 5,000Da, there would still be excess sulfo-SMPB-IgG or reduced thiol-cZipcode1 sequence or some other small molecular weight components present in the final cross-linked product.

Figure 5.12 MALDI-TOF mass spectrum of the final cross-linking product IgG-cZipcode1.
product. HPLC has proven to be a powerful technique in separation of biomolecule mixtures. Thus, HPLC was explored to determine the best conditions for separation and purification of the final reaction product mixture.

The separation conditions were modified as compared to those reported by Niemeyer et al., in which they tried to separate the conjugation product mixture that contains streptavidin, free IgG, and the streptavidin-modified IgG complex. The separation column used is an anionic exchange column that has quaternary charged ammonium ion present on the microsized latex beads which serve as the stationary phase. The Tris-HCl-NaCl gradient buffer system was the same as that used in Niemeyer’s work except that the pH value is adjusted according to the pI of the antibody used (mouse IgG1, from mouse ascites, pI = 6.4-7.6) to pH 9.0 instead of pH 6.3 in the reference. Displayed in Figure 5.13 are the summarized elution profiles of all the starting reagents, intermediate, and final cross-linked product.

Figure 5.13 Ion-exchange chromatographic elution profiles of starting reagents, intermediate, and the final cross-linked product with the cross-linking reaction buffer PBE as the sample carrying buffer.
The sample carrier buffer is PBE, while the running and elution buffers are each Tris-HCl buffer with a given concentration of NaCl. Absorbance of the UV light was used as the detection method. It is observed from Figure 5.13 that oligonucleotides eluted at 18 min, IgG and PBE both eluted at 1.2 min. It is probably because that EDTA in the PBE buffer was charged and interacted with the stationary phase during the separation. To confirm this assumption, all the samples were buffer-exchanged into PBS buffer, and ran HPLC with the same running method as that with PBE buffer as the sample carrier buffer.

The elution profiles of all samples in PBS buffer, including starting reagents, intermediate, and the final cross-linked product, were depicted in Figure 5.14.

Figure 5.14 Ion-exchange chromatographic elution profiles of starting reagents, intermediate, and the final product with PBS as the sample carrying buffer. The inset small figures are the zoom-out profiles of the corresponding elution peak at elution time of 1.2 min.
It is noticed that there was no UV absorbance for the eluted control PBS buffer at elution time of 1.2 min. IgG molecules and its intermediate were still eluted at 1.2 min. Therefore, it confirmed the assumption of EDTA effect on the co-elution phenomenon in Figure 5.13. No peaks were seen in between the IgG molecule and the ss-DNA, as is expected of the final cross-linked product. There are two possibilities: one is that the concentration of the final cross-linked product was too low to be detected, another is that the final cross-linked product might co-eluted with the starting IgG and the intermediate sulfo-SMPB-IgG molecules.

5.4 CONCLUSIONS

In summary, UV-Vis spectrometry was proved to be a straightforward technique for monitoring components of the intermediate product after desalting purification. It was verified by MALDI-TOF mass spectrometry that both starting reagents are of high purity and the final product was successfully prepared using the reaction scheme listed in Scheme 5.1 and 5.2. In order to get relatively pure final cross-linked product, ionic exchange chromatography was explored to ascertain the optimum conditions for separation and purification, and it was found that PBS buffer has to be the choice of carrying buffer instead of the cross-linking reaction buffer PBE because PBE co-eluted with IgG molecules. The pH value of the running buffer has to be adjusted according to pIs of the test samples, e.g. pH 9.0 is a better choice than pH 6.3 such that the IgG molecule may interact with the charged, microporous packing materials in the separation column (the microsized resins covered with the functionalized microbeads), hence the resolved elution of this molecule.

5.5 REFERENCES


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CHAPTER 6. SUMMARY OF CONCLUSIONS AND FUTURE WORK

6.1 SUMMARY OF CONCLUSIONS

This research intended to explore a novel methodology in which breast cancer biomarkers among different classes are analyzed simultaneously on the same platform. It combined the hybrid biosensor system with polymer-based microfluidic devices; thus, by reaching this goal, it will provide a robust analytical diagnostic platform on which biomedically related events are analyzed in high throughput, high sensitivity, and high specificity, while at the same time will obtain low false positive and low false negative diagnostic results, therefore increasing the accuracy of the technique.

Background information on fields that are involved in the multiplexed microfluidic-based hybrid biosensor system is detailed in chapter 1. The overall research motivation, goal, and synopsis were first presented to give a big picture of this multi-faceted project. It was followed by general information on cancer disease, especially that of breast cancer, spanning fundamental terms that will be frequently encountered in the following text, prevention, diagnosis, and general treatment techniques. The concept and importance of biomarkers were then introduced and followed by strategies of discovery and detection for both genetic and protein biomarkers in the format of single-analyte and multi-analyte assays. A brief literature review on the multianalyte assays was then presented. Background on the microfluidic device was an indispensable section because it was the platform of the hybrid biosensor system. Literature was also navigated on the idea of microfluidic-based multianalyte assays in a hybrid biosensor format; hence, the significance of the work was demonstrated.
In chapter 2, a simple and straightforward surface modification method, photochemistry method, on the microfluidid device substrate PMMA was reported. This surface modification produced roughly a monolayer of carboxylic functional groups, which made the surface amenable for further functionalization and applications. Changes on the surface properties induced by the photochemical modification were characterized in aspects of surface composition, surface topography, surface wettability, and effect on electroosmotic flows in the microchannels embossed in the PMMA substrate qualitatively and quantitatively. The surface modification mechanism was also explored in order to give an idea on controllable manipulation of surface properties for future implementations. A variety of analytical techniques were used for the characterization of the photochemical surface modification. Principles and instrumentation setup of these analytical tools were also described and illustrated in the chapter. Work presented in this chapter provided a new avenue for the spatially controllable surface modification of polymer-based BioMEMS devices which have attracted increased interest due to their intrinsic advantages, such as high-aspect-ratio, low cost of fabrication, and scalability.

In chapter 3, an effective antibody patterning method was investigated and characterized on both plain PMMA surfaces and PMMA microchannel surfaces. This method, using antibodies specific for a breast cancer biomarker carcinoembryonic antigen (CEA) as a model system, combines carbodiimide coupling chemistry with the direct, spatially-selective formation of carboxylic acid groups induced by exposure to UV light through a contact mask. Retention of the biochemical activity of the patterned, polymer-bound antibodies was confirmed, and surface ligand density measurements of immobilized antibodies yielded values characteristic of a submonolayer of antibody, with
the surface density being 80% larger for randomly immobilized antibodies in comparison to their protein-G oriented counterparts. Considering the fact that there is a large interest in developing disposable, polymer-based microdevices for potential applications in biomedical and clinical diagnosis of diseases that will require the use of immunological recognition agents in array-based formats, the work reported herein sheds some light on promising methods for the patterned immobilization of biologicals that are simple to utilize.

In chapter 4, a PCR/LDR combined assay was demonstrated in order to prepare breast cancer biomarker *BRCA1* gene related mutational materials. The background information on *BRCA1* gene as a breast cancer biomarker was introduced at the very beginning in order to emphasize the significance of study on this specific gene. Cell line used as the model study system for breast cancer was also validated in theory. Techniques, such as PCR, LDR, and slab gel electrophoresis that were heavily used in the experimental section, were thoroughly explained and displayed. In the following experimental sections, cell culture and isolation of genomic DNA were performed and succeeded. *BRCA1* gene was investigated on the single insertion C-containing specific region of exon 20 through PCR/LDR combined assays. PCR reaction was also optimized to get the targeting sequence in good quantity and quality as was evidenced by agarose gel electrophoresis results. The targeted genomic mutational material was then achieved by LDR reaction on the DNA template sequence prepared by PCR. LDR products were analyzed on PAGE and shown to have good integrity.

Preparation of breast cancer protein biomarker targeting materials was detailed in chapter 5. First of all, the need of preparing protein biomarker targeting materials was
rationalized by briefing the value of applying multiple biomarkers from different classes in the diagnostic of breast cancer. Then, means of incorporating protein materials onto DNA-functionalized surfaces were explored in the literature, and a unique strategy was thus presented for the specific application in the work. Single-stranded DNA (cZipcode1) sequences were cross-linked through a heterobifunctional linker, sulfo-SMPB, to monoclonal antibodies that were raised against breast cancer biomarker CEA. A variety of analytical techniques were adopted to monitor the reaction and to characterize the product. The success of preparation of the final conjugation product, IgG-DNA, paved the way to fabrication of the hybrid biosensor system on which both the genetic mutational materials from BRCA1 gene and the protein targeting materials will be analyzed simultaneously through a universal DNA microarray format on the microfluidic platform.

6.2 FUTURE WORK AND DIRECTIONS

The goal of this research was to fabricate a hybrid biosensor system for multi-analyte detection of breast cancer biomarkers on the multi-modular polymer-based microfluidic devices. Both genetic and protein targeting materials were successfully prepared, and the remaining issue is how to assay these materials, how to configure the hybrid biosensor format. Inspired by the mature technology soft lithography, the microfluidic device will be in a hybrid format, i.e., constructed in both thermal plastic PMMA and the soft material PDMS. The array of universal Zipcode DNA sequences will be produced by patterning lines of Zipcode DNA sequences with a PDMS stencil, and then, targeting materials will be flowed perpendicularly to the DNA lines. Arrays of individual assay will thus be formed as is illustrated in Figure 6.1.
A PDMS stencil with four parallel channels will be fabricated using known techniques. Two types of Zipcode DNA sequences will be patterned covalently (see chapter 4) in parallel lines in quartets; therefore eight lines of DNA are to be immobilized. The two target materials, BRCA1 gene and CEA biomarkers incorporated in respective unique format, will be introduced to interact with each of these eight lines of DNA by another PDMS stencil that is aligned perpendicularly to the DNA lines. Thus, arrays of unique spots will be produced and ready for data analysis by fluorescence scanning imaging.

**Figure 6.1** Schematic of patterning universal Zipcode DNA sequences through PDMS stencil on photochemically modified PMMA surfaces.
An alternative to this aforementioned patterning method is as follows. Arrays of Zipcode DNA sequences are first prepared by simply spotting each sequence onto the PMMA microchannels. The mixture of targeting materials is then flowed into each of the microchannels to interact with each of the DNA spot.

A second phase for this project is to explore the effect of the orientation of the IgG antibodies on the IgG-DNA conjugate. In chapter 5, IgG antibodies were randomly linked to DNA through the amino groups on the lysine residues that are distributed anywhere on the IgG molecule including the antigen binding site CDR (Figure 6.2).

![Figure 6.2 Structure of IgG antibodies.](image)
Thus, this strategy might not be able to reach the maximum binding capacity of the IgG antibodies. It is helpful to explore other functional sites on the IgG molecule, such as the carbohydrate moiety on the Fc of the IgG, disulfide bonds in the hinge region, and other possible receptors to avoid linking close to the two Ag binding regions.

The last phase of the research will be to test the feasibility of the hybrid biosensor system in the settings of breast cancer cells, on which surfaces the antigen protein biomarkers are bound and within which the genetic mutational materials related to the breast cancer are contained.
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Thanks a lot!!
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Suying Wei was born on July 28, 1975 in Zhucheng, Shandong Province, China. She attended Zhucheng Fanhua High School from 1989 to 1992. She was then assigned to Shandong University of Science and Technology (formerly Shandong Institute of Mining and Technology) and got her bachelor’s degree in chemical engineering in 1996. After graduation, she worked for one year at Shandong Yanzhou Coal Bureau. In 1997, she began her graduate study at Beijing University of Chemical Technology (BUCT), and got her master’s degree in applied chemistry in 2000. She then came to the U.S. and intended to study metallic catalysts in University of Missouri-St. Louis, but she ended up rejoining her husband at Louisiana State University to pursue her doctoral degree in analytical chemistry. Suying Wei received the Pfizer Graduate Fellowship in Analytical Chemistry (2005-2006) and the James Robinson Award for Outstanding Research in Analytical Science at LSU. Suying Wei is currently a candidate for the degree of Doctor of Philosophy in chemistry, which will be conferred at the fall 2006 commencement.