Modified plastic surfaces for high performance microfluidic immunoassay devices

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**MODIFIED PLASTIC SURFACES FOR HIGH
PERFORMANCE MICROFLUIDIC IMMUNOASSAY
DEVICES**

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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Summary

The goal of this research is studies of surface modifications and bioconjugations for a plastic microfluidic device and to explore how these enhance the sensitive and specificity of the device in flow-through ELISA.

The protein/poly-(dimethylsiloxane) (PDMS) interaction was studied. A new molecular level approach was developed using AFM to study the conformation and kinetics of the adsorption of protein A on a PDMS surface through investigations of the effects of pH values, protein concentrations, and contact times on the adsorption.

To covalently immobilize protein on PDMS surface, (3-aminopropyl)-triethoxy silane (APTES) was used to add amino groups onto PDMS. Amino groups in the APTES were converted into carboxyl groups by reaction with succinic acid anhydride (SAA), then captured the protein through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Immunoglobulins (IgGs) were covalently immobilized onto the PDMS using the SAA/EDC surface treatments and examined through a well-organized competitive inhibition ELISA.

Poly (vinyl alcohol) or PVA is one of the most hydrophilic polymers with stable chemical properties and good dissolution. A simple but effective technique was investigated to prepare a PVA-functionalized PDMS for suppressing non-specific adsorption of protein. Proteins were covalently immobilized on the PVA- functionalized PDMS surface for the development of a solid surface for immunoassays. A PVA-functionalized PDMS microfluidic ELISA device was fabricated and employed to detect important biomarkers, including HBsAg, HBeAg and α -FP, via a flow-through process. The methodology established in this research can provide a simple, fast and sensitive hepatitis B virus infection diagnoses tool.

Dextran was investigated as a biocompatible polymer for cell repelling biomaterial coatings. PDMS was modified with a dextran solution to enhance PDMS surface hydrophilicity and furthermore to achieve high immobilization efficiency of proteins through dextran's abundant hydroxyl groups. A dextran modified PDMS microfluidic ELISA device was fabricated with unique architecture for eliminating crossover interference. This microfluidic device was

used to simultaneously detect multiple important biomarkers, which included IL-5, HBsAg, and IgG. Moreover, the fabricated dextran-modified PDMS device demonstrated its capability for colorimetric detection of proteins and results could be observed clearly by the naked eye.

Immunoassay devices including microarrays and microfluidic systems were fabricated with an UV-curable resin by a new economic approach, which can simply produce a 3-D patterned structure, but also simultaneously introduce functional epoxide groups for efficient protein immobilization. The epoxy resin formulation was optimized and the protein immobilization kinetics was studied. The performance of the epoxy resin-based microarray was improved by optimization of printing buffer, probe concentration, and immobilization time. The dynamic range and limit of detection (LOD) of protein microarrays fabricated on the epoxy resin film was characterized by using direct immunoassay with goat IgG and anti-goat IgG as model proteins. In addition, detection of IL-5 and IgG was conducted in the epoxy resin-based microfluidic devices. The device was explored to colorimetrically detect proteins via naked human eyes for point-of-care service and/or high throughput screening of infectious diseases.

In general, our research not only provides engineering solutions to important biomedical applications, but also has significance in the surface chemistry science of bioconjugation and probe immobilization for enhanced sensitivity of solid phase immunoassays. This hybrid of an engineering platform and surface chemistry science gave birth to a bioconjugation enhanced microfluidic ELISA for biomedical analytical applications.

Chapter 1 Introduction

1.1. Introduction

Immunoassay, which is the reliance on the immunological detection of organisms, is one of the most sensitive and reliable methods used in diagnostic and drug discovery applications¹. In recent years, its research and development continues to be fuelled by the high demand placed by society on high quality health care, assessment of food quality, monitoring of environmental pollution and biological security. The growing interest in the design of immunosensors aims at fast, economical, portable and sensitive technologies. ELISA has been the mainstay of diagnostic tests used for the detection of organisms, especially that of infectious diseases¹, for more than twenty-five years. Although ELISA analysis is considered part of mainstream clinical diagnostic methods due to its sensitivity and specificity over other analytical methods, there is a need to create new ELISA array platforms which costs less and are environmentally friendly (pollution free).

Over the past decades, many micro-electro-mechanical systems (MEMS) have been developed for a diverse range of applications²⁻⁵. Recently, miniaturization techniques have been migrated from the semiconductor field to the biomedical science fields. Miniaturization is one of the crucial approaches for the development of more efficient research and diagnostic tools. By integrating large parallel arrays of miniaturized fluidic components and sensors into the smallest possible space, such strategies offer faster analysis, smaller sample volumes, and reduced reagent costs, as well as internal referencing mechanisms that lead to more accurate results⁶⁻⁸. The research topics in this area are enormous and clearly hold much promise for both technology developers and users.

The biochip is a hybrid device that contains both mechanical and biological components. Success of a lab-on-chip system greatly relies on the use of new materials, novel architecture, economical fabrication processes and efficient immobilization of biomolecule probes. With the current progress in the development of versatile, inexpensive, and reliable techniques for microfabrication and protein immobilization/stabilization, ELISA continues to represent a significant portion of the biosensor and shows the potential to

produce commercial products in a number of areas. Therefore, this research is motivated by the need to build up microsystems as platforms which are strengthened by surface chemistry innovations to achieve fast, high-throughput and environmentally friendly ELISA.

1.2. Motivations

It is possible to accomplish a simplified methodology to fabricate high performance immunoassay devices, particularly flow-through ELISA (a multi-step assay) devices for fast, high-throughput, real-time and environmentally friendly diagnoses. The main motivation of this thesis project was to study the fundamentals involved in ELISA microfluidics, such as surface science, bioconjugations, material science and microfabrication, and then to construct highly sensitive and high throughput polymeric microfluidic ELISA devices with the guidance obtained from the scientific investigations.

It is important to fabricate low cost microfluidic devices for practical applications. Most of the early devices were made using silicon and glass as the substrates with technologies directly borrowed from the semiconductor industry². However, for applications, particularly in the biomedical field requiring disposable device or batch production, silicon wafer and its MEMS manufacturing process are too expensive. Polymer substrate was studied to fabricate the microfluidic device by simple molding instead of the expensive patterning process^{9,10}. In this work, new and economical approaches are explored to fabrication a low cost polymeric microfluidic ELISA device.

The major concern in fabrication of a sensitive immunoassay lab-on-a-chip is to efficiently immobilize capture proteins (probes) on a solid surface, since the surface concentration of binding sites and bioactivity of the capture proteins have great impact on the sensitivity of the immunoassay. A surface with adequate homogeneity, specificity and biocompatibility could enhance efficient protein immobilization for high sensitivity of plastic microfluidic ELISA devices.

It is highly desirable to fabricate a portable microfluidic device for point-of-care service. Microfluidic devices will lose their advantages if large and heavy desk equipment is required. Various detection strategies are currently being applied for microfluidic immunoassays, such as fluorescence¹¹, chemiluminescence¹² and thermal lens microscopy¹³. These methods require

large and expensive equipment, although they are sensitive and accurate for clinical diagnoses. Bulkiness would be the obstacle to their applications, especially in health point-of-care and high throughput pathogen screening. Thus, it is of great importance for exploring a portable microfluidic ELISA device, such as a lab-on-chip system with a detection mechanism that is visible to the naked eye.

1.3. Objective of study

The objective of this research is to investigate novel surface modifications and bioconjugations in the design and construction of a microfluidic ELISA device. The work not only explores fundamental and scientific insights of the plastic microfluidic system in chemistry and biology to obtain knowledge necessary to improve the device performance. A number of characteristics are important in the evaluation of the suitability of the designed system for a particular application, namely high sensitivity, low-cost and point-of-care detection. These characteristics include properties of the bio-affinity element, sensing surface material, method of immobilization, assay format and mechanism of signal transduction. From the system design, device fabrication, and system integration to the material surface modification, each step embraces innovative characteristics of a next generation immunoassay which will be performed in a fast, sensitive, portable and environmentally-friendly scheme.

1.3.1. Microfluidic ELISA devices

Microfluidic devices are designed and fabricated to conduct ELISA; they are used as biological reaction chambers, as well as serving as a flow pathway. To prepare polymer based microfluidic devices, the fabrication methods were identified according to the properties of the polymeric materials chosen.

PDMS is particularly popular in the fabrication of a microfluidic device since it can be cast in a mold with high fidelity, and exhibit good mechanical and optical properties¹⁴. A delicate PDMS microfluidic device which can automatically and efficiently conduct sampling, incubation, washing and detection within the plurality of test sites for multiple screening immunoassays is fabricated. The novel microfluidic system allows inflow from the inlet and overflow of the fluid to the outlet while maintaining the fluid level in the detection well, thus

eliminating crossover reaction among different detection wells caused by back flow or back diffusion from one channel to another channel.

It is of significance to find a new, simple and economical method for one-step fabrication of microfluidic devices. In this thesis project, a UV-curable epoxy resin is described for simple one-step fabrication of a microfluidic ELISA device using UV-embossing technology.

1.3.2. Polymer surface functionalization

In spite of the prestigious advantages of the PDMS for replication application, its principal limitation in flow-through applications is due to its hydrophobic surface properties. Proteins and other molecules thus tend to adsorb to a pristine PDMS surface; the surface is poorly wetted and prone to forming bubbles in aqueous systems. These limitations can be reduced or eliminated through chemical means, plasma treatment of the surface, or surface modification by the addition of hydrophilic polymers. To enhance the flow-through ELISA performance, a scientific study of innovative bioconjugations to functionalize the PDMS was conducted. Novel methods were used to import functional groups onto the PDMS surface; to make the surface more hydrophilic and to add functional groups to the surface to covalently immobilize probe antibodies or/and antigens.

1.3.3. Efficient immobilization of biomolecule

In a miniaturized immunoassay system, the immuno-sorbent solid surface preparation is of major importance for achieving a high sensitivity, considering the significant shrinkage in sample volumes.

The fundamental mechanism of a protein's passive adsorption on PDMS was investigated by atomic force microscopy (AFM), expanding our knowledge of protein adsorption processes at the molecular level. Although adsorption is ostensibly a simple and convenient method to immobilize biomolecules, covalent immobilization often results in higher biological activity and enhanced stability¹⁵,¹⁶. The application of covalent binding needs a polymer surface with reactive groups, such as NH_2 or COOH . Thus, there is a need to import appropriate reactive groups to the pristine PDMS. Furthermore, the sensitivity and specificity of the immunosensor are expected to be improved through the modification

brought by functional groups which can be used to efficiently immobilize the biomolecules specifically and increase the hydrophilicity of PDMS.

1.3.4. Colorimetric detection scheme

A miniaturized microfluidic device will lose its advantages if the use of large and heavy desk equipment is required. To fabricate a portable microfluidic immunoassay device, a colorimetric detection scheme was established to construct a lab-on-a-chip system that has a detection mechanism that is visible to the naked eye. The colorimetric detection should be accurate and is expected to be used to assess the positive or negative nature of the detected biomarker (with the naked human eye). The colorimetric microfluidic ELISA device thus offers a great potential to fabricate an economical and sensitive lab-on-chip system for applications in screening assays, and also provides an excellent opportunity to develop a portable microfluidic ELISA device that requires only human eye observation of results for point-of-care services.

1.3.5. Biomedical applications of sensitive microfluidic ELISA devices

Infectious diseases can be caused by various pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. The clinical diagnoses of an infectious disease often requires different and tedious, labor-intensive immunoassays to screen various possibilities; some of the common immunoassays are ELISA¹⁷, immunofluorescence¹⁸, Western blot¹⁹ and immunodiffusion²⁰, of which ELISA is the most important method. Advances in microfluidics and MEMS technologies could be used to design an economical, high throughput and real-time diagnostic ELISA tool²¹. Due to our endeavors in the surface functionalization and covalent probe immobilization, significant improvements in the detection of pathogens or disease related biomarkers are expected comparing to the conventional micro-plate based ELISA. To demonstrate its capabilities, the functionalized plastic microfluidic ELISA device has been applied to diagnostic applications.

The hepatitis B virus (HBV) infection is a serious global health problem infecting a billion people worldwide; of these, 350 million are suffering from chronic HBV infection. The most widely used Hepatitis B virus surface antigen (HBsAg) screening tests are ELISAs. In general, it costs 30 to 50 Singapore

dollars per set of blood tests. The patients will have to wait 6-8 hours to obtain the test results. IL-5 is predominantly produced by CD4⁺ T cells²²; it regulates growth, differentiation, activation, and survival of eosinophils²³, and is a marker of disease activity and treatment efficacy in bronchial asthma²⁴. IgG can serve as a specific marker for neuromyelitis optica, an inflammatory demyelinating disease²⁵. The commercially available ELISA kit requires six different reagents and a total of 4.5-hours to conduct a single test. Therefore, a low-cost, simple, instrument and electricity-free screening tests for HBV biomarkers, IL-5 and IgG detection is highly desirable, especially for individual tests, since many blood transfusion services in countries with limited resources can only process a limited number of specimens.

1.4. Organization

This dissertation is devoted to the study of hydrophilic polymer functionalized microfluidic ELISA devices for detection of proteins with high sensitivity and specificity. This dissertation is organized in the following manner:

In Chapter 1, an introduction and overview of the motivation and objective of this PhD research is made.

Chapter 2 reviews the issues pertinent to the microfluidic systems in bio-analytical applications, microfluidic immunoassay, polymer based microfabrication, and polymer surface modification methods, especially the modification of the surface hydrophilic property.

In Chapter 3, the experimental approaches used in this study are described in detail. The modification and characterization methods of the polymer surface and the fabrication process of polymeric microfluidic devices are introduced. Chemicals and instruments used in this study are also listed.

Chapter 4 discusses the adsorption of protein A on a PDMS surface which is quantitatively studied by AFM. The surface protein A concentration was quantified at the molecular level to evaluate the effects of bulk solution concentration, pH, and immobilization time.

Chapter 5 presents a simple method for assembling a silanol layer onto the PDMS surface. The APTES adsorption kinetic was studied. The APTES functionalized PDMS was characterized by FTIR, AFM force curve and fluorescamine measurements. Protein was covalently immobilized on

APTES-PDMS for immunoassays. The competitive ELISA with probe immobilization through EDC had a detection limit of pg mL^{-1} level. The method was proved to be applicable for immobilization of different proteins.

Chapter 6 introduces an effective technique to suppress non-specific adsorption in the development of a solid surface for immunoassays. The hydrophilic polymer, PVA, was covalently immobilized on the PDMS. A PVA-functionalized PDMS was activated with aldehyde groups via glutaraldehyde treatment. Biomolecules can be covalently immobilized to the PVA-functionalized PDMS surface for ELISA applications. A sandwich ELISA on the PVA-functionalized PDMS microfluidic device was demonstrated for the detection of hepatitis B virus related biomarkers, HBsAg, HBeAg, and α -FP.

In chapter 7, the fabrication of a dextran modified PDMS microfluidic ELISA device is described. The dextran functionalization of PDMS was conducted in a simple, economical and fast flow-through process and demonstrated great enhancement of the hydrophilicity and efficient covalent immobilization of proteins on the PDMS microchannel surface. The microfluidic device was used to simultaneously detect multiple important biomarkers, which include IL-5, HBsAg and IgG.

Chapter 8 presents an economical and one-step approach to fabricate protein microarray and microfluidic ELISA device using UV-curable resin. The protein immobilization kinetics on the UV-cured epoxy film was studied. The analytical dynamic range and detection limit of the epoxy-based protein microarray was characterized. The UV-curable epoxy resin was used for simple one-step fabrication of a microfluidic ELISA device by UV-embossing technology. The epoxy-based microfluidic device was explored to flow-through sandwich ELISA for IL-5 and IgG detections.

Finally, in chapter 9, a general conclusion is given and some perspectives on possible future research is summarized and proposed.

Chapter 2 Literature review

This chapter reviews the background information about micro-Total Analysis Systems (μ TAS), microfluidic immunoassay, polymer-based microfabrication, and polymer surface modification methods. The concept, history, advantages and developing trends of micro-total analytical systems as well as the scaling-down effects on the miniaturization of a total analysis system, especially on the performance of a μ TAS, are overviewed in section 2.1. Nowadays, more and more established biological and analytical assays have been translated into miniaturized microdevices considering the superior advantages of μ TAS. Immunoassay (IA) is one of the analytical biochemistry tests which have been miniaturized into a small device. In recent years, the growing interest in the design of immunosensors aims at fast, economical, portable and sensitive technologies. Section 2.2 reviews the current progress in the development of versatile microfluidic immunoassays. The methods and techniques developed for fabricating plastic microfluidic immunoassay platforms are introduced in this section. Section 2.3 focuses on the surface properties of PDMS and the approaches used to enhance its hydrophilicity for microfluidic applications.

2.1. Overview of miniaturization

μ TAS is an abbreviation for micro-Total Analysis System⁷. It describes a chemical system that automates all necessary analytical steps (e.g. sampling, transport, filtration, dilution, chemical reactions, separation and detection). Such a system can shrink a whole laboratory into the size of a chip; they are therefore also known as lab-on-a-chip. Table 2.1 lists the definition of terms in the configuration possible in this field.

Table2.1. Terminology used in this section

Terminology	
Micro total analysis system (μ TAS)	Miniaturization of an overall analytical process from sample preparation through reaction and separation to detection
Microfabricated devices	Microstructures fabricated by methods basically applied in microelectronics, such as micromachining, microlithography, injection molding and embossing
Microfluidic	Transporting and manipulating minute amounts of fluid through microchannels on the chips
Lab-on-a-chip	A microfabricated device, integrating several laboratory processes on a single chip

Since 1990, many advantages in Micro-Electro-Mechanical Systems (MEMS) technologies are helping the envisioned μ TAS come to fruition. Advancements in MEMS fabrication schemes enable the integration of physical sensors, mechanical structures, and wireless communication capabilities on a simple chip. Complete microfluidic systems and control electronics can feasibly be fabricated on a single chip, consequently decreasing overall system cost and incorporating complex microfluidic system capability and control. The miniaturized devices are useful in multi-disciplinary fields, from biomedical, to environmental, and to agricultural communities. One of the main applications of microfabricated devices is in biomedical analytical chemistry^{26, 27}. This branch of chemistry is concerned with gathering qualitative or quantitative information on different kinds of samples, often with only a small amount of sample to work with or with the analytes of interest present in very low concentrations^{28, 29}. Experimentally, there are usually multiple steps to be performed in each assay. It is challenging because in between these steps, much handling and transporting of untreated and treated samples occur, greatly increasing the risk of contamination and therefore the possibility of a less than trustworthy analytical results. It is of interest to miniaturize the overall analytical process, from sample preparation, reaction, and separation to detection. The potential applications almost cover every aspect of bio-analytical science. They have been developed for use in studies of genome to proteome³⁰, on-chip PCR³¹, capillary electrophoresis^{32, 33}, immunoassay^{34, 35}, and affinity chromatography. More and more analytical tests are being translated into miniaturized device form.

2.1.1. Advantages of miniaturization/ μ -TAS

It should be noted that miniaturization inherently contributes many analytical benefits²⁶. Prominently, by reducing the length and dimension of a reaction vessel by one order of magnitude, fewer amounts of sample would be used in the assay. Reduced sample volumes mean that original sample volumes can be divided for multiple analysis protocols, optionally providing more accurate information regarding the analytes, e.g. statistically more “correct” data. Secondly, because the surface area-to-volume ratio increases, the influence of surfaces becomes large and consequently surface-bound chemistry, e.g.

immobilization chemistry, is favored in the micro/nano domain as compared to bulk solution-based reactions. Reaction kinetics and thus the analytical throughput may be increased due to shorter distances for analytes diffusion. In the case of immobilization chemistry, for e.g., an enzymatic digestion of a protein in a microchannel with the protease immobilized on the channel wall, the time for substrate diffusion from the solution in the channel to the catalytic site is reduced by two orders of magnitude as the dimension of the channel is reduced by one order of magnitude. Thirdly, the overall cost of the assay is reduced by minimizing the reagent consumption, since samples and reagents are either expensive or hard to obtain.

2.1.2. Trends in μ TAS

The microstructure design, miniaturized assay scheme, mode of operation and materials used for the fabrication of μ TAS are largely dependent on the application and the intended life-time of the devices. Several trends that emerged in the last decade have an important impact on the design and operation of μ TAS devices. First, the miniaturized devices are increasingly utilizing microfluidic systems for applications³⁶. Using microfluidic devices, the sample usage can be scaled down from μ L to nL. This is another inherent point of a microfluidic system since decreasing the sample size has several advantages such as reduced times for mixing by diffusion, decreased transport time, smaller sample and reagent volumes netting a reduced cost, and others. Secondly, the cost pressures have led to the increasing attractiveness of “disposable” devices, which in turn led to increasing attention being paid to new materials, such as polymers³⁷, as fabrication materials as opposed to “traditionally” used silicon and glass⁴.

2.2. Miniaturized immunoassay

Immunoassay (IA) is one of the core tests of analytical biochemistry - its specific binding abilities of antibodies and antigens are widely used in selective and sensitive measurement of biomolecule analytes in complex samples. In medicine, immunoassays are used in two categories of diagnostic applications: 1) the diagnoses of a disease state or identification of the organism responsible for a disease state, and 2) the management of treatment for a disease, either

through monitoring of the disease state or of the drugs used for therapy. These are vital activities in human health management, as shown in table 2.2.

Table 2.2. The EIA application in clinical field

Application	Share	Analytes type	Concentration
Drug testing	26%	Small molecules	10 nM - mM
Therapeutic	20%		
Drugs of abuse	6%		
Infectious diseases	21%	Bacteria, viruses, large	low
STDs	13%	molecules, antibodies	
Non STDs	8%	and metabolites	
Endocrinology/hormones	33%	Small molecules, large	pM - nM
Thyroid	20%	molecules including	
Non-thyroid	13%	Abs	
		(70% <5 kDa)	
Immunology	7%	Antibodies	pM - nM
Allergy	3%		
Autoimmunity	3%		
Other	1%		
Cancer	5%	Large molecules,	pM - nM
		antibodies	
Other	8%		> nM

Different types of immunoassays have been developed over the last decades. Homogeneous immunoassays work in homogeneous phases (in solution or gels), which do not require separation of unbound complexes from the bound. The distinguish feature of heterogeneous immunoassays is that they require separation of bound and free antibody/antigen. Because the heterogeneous immunoassay has the ability to concentrate molecules from solutions of low concentration onto a surface, the sensitivity and specificity of heterogeneous assays are higher than that of homogeneous immunoassay. Thus heterogeneous immunoassay is widely used for clinical disease diagnoses, environment monitoring.

Conventional heterogeneous immunoassay test is a multistage, labor-intensive process. With the rapid development of lab-on-chip technology since the 1990s, miniaturization of immunoassay has become an area of interest to improve reaction kinetics, reduce expensive reagent consumption and realized automation process³⁸. Towards the miniaturization of immunoassay platforms, there are two approaches, namely, antibody microarray³⁹ and microfluidic immunosensors^{34, 40} (figure 2.1).

Since a report in 2000 established that gene microscopy technologies could be applied to arraying and assaying proteins in a microscopy format, protein chips

have been followed with considerable interest^{41, 42}. Antibody/antigen microarrays are becoming an important tool in proteomics, drug discovery programs, and diagnostics^{43, 44}. The performance of antibody/antigen microarray is dependent on various factors, one of which is the use of an appropriate microarray surface for the immobilization of either antigen or antibody samples. At the first generation protein microarray, the supporting materials used to fabricate antibody/antigen microarrays can be divided into major categories: poly-L-Lysine coated slides adapted from DNA chip technology, glass slides surfaces grafted with functional silane, and slides coated with gels, such as polyacrylamide and agarose. The functionalization of glass surfaces is rooted in the development of silanization chemistry, in which silane with different functional groups has been used. However, the functionalization process is tedious and multi-step chemistry modification process can not guaranty the uniformity of the surface. For the gel-based protein microarray⁴⁵, the density of the printed spots is sacrificed. Later, concepts for slides coatings such as dendrimer and PEG-epoxy were introduced and improved. New supporting materials, such as nano-structured metal plate⁴⁶ and functionalized polymeric materials^{47, 48}, also attracted research attention.

The other type of miniaturized immunoassay, microfluidic immunosensors, has attracted interest since the introduction of microfluidic technology in the early 1990s. Microfluidic technology in general seeks to improve analytical performance by reducing the consumption of reagents, decreasing the analysis time, increasing reliability and sensitivity through automation, and integrating multiple processes in a single device. These features are particularly suitable for immunoassay applications. Enormous amount of effort has been expended towards the development of microfluidic immunosensors that combine the analytical power of microfluidic devices with the high specificity of antibody-antigen interactions^{38, 49-51}.

Figure 2.1 A. Images of the spotting seal and resultant protein microarray⁴¹. (a) The spotting seal, comprising a 25 mm × 55 mm polyvinyl chloride sheet perforated with 48 small holes (2 mm diameter). (b) Images of a protein microarray used for sandwich immunoassay of IL-8. B. Schematic of a rapid diffusion immunoassay in a T-sensor⁵². Reagents are pumped separately through device inlets and meet at the junction. For a competitive binding assay, sample containing antigen to be measured spiked with a fixed concentration of labeled antigen is pumped through one inlet and a fixed concentration of antibody (Ab) through the other. The diffusive transport of antigen is altered depending on the fraction that binds to Ab and is the basis for determination of sample antigen concentration.

In general, a successful construction of a miniaturized immunoassay includes several basic aspects, such as detection scheme, probe protein immobilization and materials and relevant microfabrication methods to fabricate the assay platform. The research progress towards miniaturized immunoassay is reviewed in the following sections.

2.2.1. Detection scheme of miniaturized immunoassay

There are diverse signal transduction pathways to recognize an antibody/antigen binding event. Based on the reaction scheme, the assay can be defined as label-based sensing or label-less sensing. For the label-less scheme, there is no need to label the biomolecule. The presenting techniques are surface plasma resonance⁵³, quartz crystal microbalance⁵⁴, impedance spectroscopy⁵⁵, et al. In this part, topic is limited to the label-based sensing. In most of the existing immunoassay, a label is used to increase the sensitivity of detection. Current labels include metals, redox labels, optical labels and enzymes. Among the different labels that have been applied for the development of immunoassays, enzyme is one of the most frequently used species because of its capability to efficiently catalyze the conversion of a substrate into a detectable product. The catalytic turnover by the enzyme amplifies the signal

and increases the sensitivity of the assay because the number of detectable molecules can be exponentially higher than the number of antigens.

Optical immunosensors can employ many different types of measurement techniques including absorption, fluorescence, phosphorescence, refraction, dispersion spectrometry, and so on, for detections in which chemiluminescence, light absorbance, fluorescence, phosphorescence, light polarization, or rotation are measured.

The most commonly observed form of detection is fluorescence which is based on fluorophore dyes that emit light at specific wavelengths; the presence and enhancement or reduction in the optical signal can indicate a binding reaction^{56, 57}. To detect the fluorescent signal, several different setups have to be used, of which the most common involves a microscope focused on the microchannel and connected to a charge coupled device (CCD) camera or photomultiplier tube. Chabinyk M.L. described a prototype of an integrated fluorescence detection system that uses a microavalanche photodiode (μ APD) as the photodetector for microfluidic devices fabricated in PDMS⁵⁸ (figure 2.2A). The prototype device consisted of a reusable detection system and a disposable microfluidic system. The photodetector, a solid-state μ APD array, was embedded in a thick PDMS slab and placed below the microchannel and orientated orthogonally to the axis of the optical fiber which was used to couple light into the microchannel for the excitation of fluorescent analytes⁵⁹. However, the fluorescence detection generally depends on the properties of the dye label, and so the detection scheme is much susceptible to photobleaching and pH changes⁶⁰. A new type of fluorescence detection that hold promise for microfluidic applications is fluorescence polarization, which detects the changes of anisotropy caused by the slower rotation of a bound vs. free label. Therefore, this detection scheme is less susceptible to the properties of the dye label⁶¹.

One of the most cited paper is the pioneering work presented by Sato¹³. In their work, polystyrene beads pre-coated with anti-CEA antibody were introduced into a microchannel, and then a serum sample containing CEA, the first antibody, and the second antibody conjugated with colloidal gold were reacted successively¹³. They developed a laser-induced thermal lens microscopy (TLM) that was especially useful for ultra sensitive determination in a

micro-space (figure 2.2B). Since the colloidal gold particles have strong absorbance at ~ 500 nm, the resulting antigen-antibodies complex, fixed on the bead surface, could be detected using thermal lens microscopy (TLM). Through this approach, a highly selective and sensitive determination of the amount of CEA in human serum was made possible using a sandwich immunoassay system. The sample containing 0.1 ng mL^{-1} CEA showed an obvious TLM signal and the determination limit was $\sim 0.03 \text{ ng mL}^{-1}$, whereas the cutoff value for serum diagnoses of colon cancer is normally around 5 ng mL^{-1} .

Chemiluminescence is another type of signal which generates light by the release of energy as a result of a chemical reaction. Chemical reactions using synthetic compounds and usually involving a highly oxidized species, such as peroxide, are commonly termed chemiluminescent reaction. The advantages of chemiluminescence detection are similar to those of fluorescence, with the added benefit that there is no requirement for an external light source. Prototype device based on chemiluminescence have been reported by Richard Davidsson¹².

A colorimetric signal is attractive since it can be read either by eye to assess whether tests have worked²⁸. Colorimetric detection alleviates many of the autofluorescence, photobleaching and other complexities generally associated with fluorescence detection schemes and is especially favorable for low cost, on-spot detection and home screening applications. Jaisree Moorthy introduced a microfluidic prototype for detecting botulinum neurotoxins directly from whole blood⁶². For detection of the toxoid, biotinylated antibodies were immobilized on streptavidin-functionalized agarose gel beads. The gel beads were introduced into the device and were used as readouts. Enzymatic reaction between alkaline phosphatase and BCIP/NBT produced an insoluble, colored precipitate that coated the beads thus making the readout visible to the naked eye. However, the capability to quantify the amount of antigen is limited by availability of imaging and analysis tools. Frank Y.H. Lin reported a method to quantify the color product⁶³. They transformed the red-green-blue values of color digital images to a perceptually uniform color space. Background pixel values were determined by using two narrow strips each of five-pixel width, one on the top and the other one on the bottom of the images. The difference between the value of each pixel within the channel and the background was

then calculated. The average difference was computed across the whole channel, which was normalized against channel length. This algorithm was subsequently adopted for calculating the color difference of the circular dots in the conventional dot-blot ELISA.

Besides optical signals, electrochemistry is the second most commonly used detection method. Electrochemical detection techniques are well suited for miniaturized devices since electrochemical techniques measure the results of the biological recognition in terms of electrical parameters⁶⁴⁻⁶⁶. They can be directly interfaced to an electronic computational system for further signal processing as well as to an integrated microfluidic biochemical detection and analysis system⁶⁷. The inherent good sensitivity may be improved by using an amplified electrochemical transduction with systems that allow enzyme substrate regeneration or by modifying electrodes for an effectively enzymatic product accumulation⁶⁸. The three main types of electrochemical signals usually used to indicate the antigen-antibody interaction are potentiometric, amperometric and conductometric signals.

One of the examples is presented by Jong Soo Ko⁶⁹. In their polymer-based microfluidic device, gold electrodes for electrochemical bio-sensing were patterned onto the PMMA bottom substrate. A PDMS top substrate patterned with micro-channels was firmly bonded with the PMMA and hermetically sealed. As model immuno-sensing tests for the microfluidic device, functionalization of ferritin and biotin to the sensing surfaces on gold electrodes and their bio-specific interactions with anti-ferritin antiserum and streptavidin have been investigated. An electrochemical detection method, voltammetric, which tracks the precipitation of insoluble products onto the sensing surface and their subsequent decrement in the electrode area, was chosen for signal registration. Changming Li developed a novel method with dual-ring electrodes and a sensing cavity for electrochemical enzyme immunoassay⁷⁰. The method is suitable for miniaturization and integration with microfluidic devices. The enzyme conversion from home-synthetic PAPP to p-aminophenol (PAP) was examined by differential pulse voltammetry. A competitive inhibition ELISA resulted in a very promising detection limit of 118 fg mL^{-1} of goat IgG and a dynamic range of 118 fg mL^{-1} to 1.18 ng mL^{-1} . The other screening printed EC immunosensor reported by Dr. Li is shown in figure 2.2C.

Figure 2.2. A. An integrated fluorescence detection system in PDMS for microfluidic applications⁵⁸. (a) The light from a blue LED was coupled into the optical fiber to excite fluorophores in the channel. The isotropically emitted photons were detected by the actively quenched μ APD. The photocounts from the quenching circuit were counted with an HP universal counter interfaced with a personal computer. B. Glass microchip for immunosorbent assay¹³: (a) overview, (b) cross section, (c) photography of the microchip and microscopy photograph of the beads in the microchannel. C. Thick-film fabrication of microfluidic biochip⁷¹. (a) Section view of reaction portion. (b) Section view of contact pad

2.2.2. Microfluidic immunoassay

Microfluidic immunoassay device possesses remarkable features such as high surface-to-volume ratio and nano-liter volume of microchannel. This microchannel can serve as immuno-reactor chamber that leads to significant decrease in analysis time from hours to minutes and with minimal sample/reagent utilization as compared to micro-well technology⁷². In the following section, platforms preparations in the development of microfluidic immunoassays were summarized.

2.2.2.1. Microfluidic immunoassay platforms

Silicon

Much of the early microfluidic work was done using silicon as a substrate since the technology was developed in the electronics industry and other MEMS fields⁸. Apart from the fabrication aspect, the surface chemistry of silicon, or rather the microscopic layer of silicon dioxide that forms upon contact with air has been studied extensively and many techniques exist to covalently attach molecules to the reactive silanol groups. Therefore, successful research has demonstrated the immunoassay on the silicon fabricated channels. Julia Yakovleva covalently immobilized affinity proteins, protein A and protein

G, on silicon microchips with overall dimensions of 13.1×3.2 mm, comprising 42 porous flow channels of 235 μm depth and 25 μm width to develop microfluidic immunosensors. Different hydrophilic polymers with long flexible chains, such as polyethylenimine, dextran, polyvinyl alcohol, and aminodextran, were employed to modify silicon substrate to achieve a more sensitive and specific immunoassay⁷³.

However, despite these advantages, silicon has been gradually replaced by glass and polymers for many applications in the biomedical field. These materials and the associated production methods are too expensive, or their material properties often induce problems, such as lack of optimal clarity, low impact strength and poor bio-compatibility.

Glass

For many immunoassay applications, particularly those involving optical detection, glass is the standard material. Glass remain the material of choice for some applications and commercial devices despite higher cost due to better optical clarity, well-understood surface chemistry, and advanced fabrication technologies adapted from the microelectronics industry. Recently, Peter Åsberg reported an elegant micro-contact printing (μCP) approach to functionalize glass surface. This novel patterning method can anchor biomolecule and noncovalent assemble conjugated polyelectrolyte/biomolecule complexes to a chip surface⁷⁴. Poly (vinyl alcohol) is another hydrophilic polymer most used to functionalize glass solid phase^{75, 76}. Most recently, Shaurya Prakash developed one very interesting technique named “click” chemistry on silica surface⁷⁷. In this work, microfluidic channels fabricated in glass were explored using a chemical surface modification method for covalent attachment of various polymers by using silane-based “click” chemistry which is defined as a class of robust and selective chemical reactions with high yields, and are tolerant to a variety of solvents, functional groups and air. This method relies on silane chemistry as the starting step and follows with terminally functionalized liner and dendritic polymers covalent attachment. To control the reaction, an alkyltrichlorosilane is used to set up the modular reaction by providing a site for nucleophilic substitution. The “click” modification attaches desired functionality to the glass surface.

Polymer/PDMS

The microfluidic devices have been developed on standard polymeric materials such as polyethylene terephthalate (PET), polycarbonate, polyethylene (PE), polystyrene and polyimide. These polymers allow getting microstructures with various surface properties such as hydrophobicity, conductivity, reflectability, roughness or crystallinity⁶⁷. One particular polymer of interest is poly (dimethylsiloxane) or PDMS which is a transparent, elastomeric polymer that can be fabricated rapidly with features having dimensions as small as 10 nm. The elastomeric nature of PDMS makes it seal well, and often the adhesion due to conformal surface contact with a smooth, flat surface is enough to seal microchannels for low pressure applications. Pristine PDMS is a hydrophobic material⁷⁸. Researchers would like to directly immobilize proteins since it has high avidity for proteins, even better than polystyrene which is the most popular material used to build ELISA micro-plates. One microfluidic immunoassay for the detection of *Helicobacter pylori* infection was reported by Frank Y.H. Lin. In this work, bacterial lysate antigens were passively adsorbed on PDMS and the *H. pylori* antibody detected *H. pylori* antigens in quantities as low as 10 ng mL⁻¹.

Currently, various surface chemistry approaches are developed to modify PDMS for microfluidic applications, in that proteins and other molecules tend to adsorb to a pristine PDMS surface. Furthermore, this surface is poorly wetted and prone to forming bubbles in aqueous systems. A number of methods for surface modification have been reported to generate a hydrophilic surface. The methods include polymer grafting⁷⁹, chemical fictionalization⁸⁰, polyelectrolyte coating, and plasma oxidation⁸¹. Apart from those approaches, adsorption of polymers^{82, 83} or biomolecules^{84, 85} on the PMDS surface, followed by covalent immobilization of desired biomolecules on the adsorbed material, such as three-layer biotin-neutravidin sandwich coating, polyelectrolyte multilayer have been reported (detailed in *section 2.3*).

Table 2.3 summarizes the characteristics of the materials used in microfluidic immunoassays. For bioanalytical microdevice application, polymers are better suited for fabricating μ TAS than silicon⁶⁹ because many types of polymers show better biocompatibility. Furthermore, polymer microstructures can be

mass produced at significantly lower cost than silicon and glass microstructures¹⁴. Thirdly, plastics come in various forms and shapes. It is often possible to find a polymer that has the optical qualities desired for a given application. For example, some polymers, such as PDMS, are transparent in the ultraviolet region of the electromagnetic spectrum, and most thermoplastic polymers, such as PMMA and PC, are transparent in the visible region.

Table 2.3. Summary of the materials used in miniaturized immunoassay platforms

<i>Materials</i>	<i>Silicon</i>	<i>Glass</i>	<i>Polymers</i>
cost of raw materials	expensive (~1 dollar/ cm ²)	less expensive (20 ~40 cents/cm ²)	cheap (~1cent/cm ²)
ease in fabrication	clean-room, involves series of processes and chemicals	clean-room, involves wet chemical etching geometry limitation due to isotropicity	high processability (replication, laser etching, embossing et al.) no need clean-room
mass production	yes, slow process, High cost	yes, slow process. high cost	yes (not well-developed fast process, low cost
optical	opaque	transparent	transparent
biocompatibility	usually need surface modification	Ok	depends on polymers application

2.2.2.2. Plastic microfluidic immunoassay device

Plastics are classed into three major groups: elastomers, thermoplastics, and thermosetting plastics. All three types can be used in fabricating μ TAS. The properties and fabrication methods of this polymer will be specifically addressed in the following section.

Plastic microfluidic platform fabrication

The two major ways of machining polymers are via replication from a master and direct machining. Direct machining methods remove small amounts of polymer in places where microstructures such as microchannels or micro wells should be located. Replication produces a microstructure by allowing a polymer workpiece to form from an inverse copy of a mold⁸⁶.

Laser micromachining is a direct machining method. It is based on the removal of polymer material using intense UV or infrared radiation generated by a laser. The radiation wavelength used affects the removal mechanism⁸⁷⁻⁸⁹. If infrared lasers are used, the irradiated material is heated and decomposed, leaving a void in the polymer materials. If UV radiation is used, the irradiated polymer decomposes, presumably by a mixture of two mechanisms: thermal

and direct bond breaking⁹⁰. In general, laser micromachining is a localized, non-contact machining technique. Industrial applications of laser machining include drilling, cutting engraving, marking and texturing. Most significantly, laser machining can remove materials in small amounts with a small heat-affected zone⁹¹. Micromachining with controlled accuracy can be achieved. When the laser beam driven by stepper motors moves across the substrate surface, it engraves a microchannel into the substrate and the cross section of the microchannel depends on the shape of the laser beam, its moving speed, the laser power and the thermal diffusivity of the substrate material. A further attractive advantage of laser machining compared to other micromachining techniques is the possibility of low-cost rapid prototyping. In most cases, laser micromachining is used in fast prototyping of devices.

There are at least three different replication methods that can be used to make a polymeric device: hot and UV embossing, injection molding, and casting. These methods are common in that a very soft or even liquid form of polymer is poured or pressed into a mold, after which the material is hardened and removed from the mold.

Casting is one of the most used techniques in research laboratories for fast production of moderate numbers of prototypes. It is a rather straightforward process which rapidly yields microstructures. The smallest feature size depends mainly on the quality of the mold which can be made at the nanoscale. Casting uses chemical processes to harden the polymer. Two components, a base and a hardener or cure, are mixed just prior to use. The chemical curing process starts immediately upon mixing and, after a certain amount of time, results in hardening of the polymer. In the case of hot embossing and injection molding, these two methods both use heating and cooling cycles to soften and structure the polymer on a mold.

Challenges of polymer materials

Currently, an important research direction in microfabrication is the manufacture of plastic microchips, which are easier to manufacture at lower cost than glass or silicon-glass chips and, additionally, may provide greater flexibility in design. However, polymer microdevice fabrication methods are not well established and most devices are developed by trial-and-error. To prepare

polymer based microdevices, fabrication methods have to be identified according to the physical and chemical properties of the polymeric materials in use. As a result, there is a considerable potential for establishing fabrication procedures and improving existing processes through further technical understanding and innovation.

The other concern for polymeric materials in most biomedical devices applications is their low biocompatibility. The term ‘biocompatible surfaces’ is used to define surfaces that are introduced in the human body without causing any allergic or rejective reactions. For example, blood-compatible materials are required to eliminate, or largely reduce, the adsorption of blood proteins, to avoid surface-induced thrombosis. In the molecular level, the bio-adhesion all begins with the rapid adsorption of proteins to the device surface therefore makes the surface for the subsequent adhesion of other molecules and cells⁹²⁻⁹⁴. Factors that affect protein adsorption include surface energy/tension, surface roughness, surface charge, and so on⁹⁵. Therefore, surface modification is generally considered as an applicable method to improve the biocompatibility of a substrate surface through proper procedures⁹⁶.

2. 3. PDMS surface modification

In spite of the advantages for replication application, the principal limitation of PDMS for flow through applications is due to its hydrophobic surface properties. Thus, proteins and other molecules tend to adsorb to an untreated PDMS surface and result in strong non-specific bindings for bio-fouling^{78, 97}. Tethering of polymers to solid surfaces via chemical bonding provides interesting ways to modify the surface properties and, therefore, is of significant interest in several applications, such as wettability, nonfouling surface technology, and biomedical science⁹⁸. Various approaches have been developed to improve the hydrophilicity of PDMS for various applications. The methods are summarized below.

2.3.1. Exposure to energy sources

Energy sources such as plasma^{99, 100}, ultraviolet light¹⁰¹, and corona discharges have been used to create chemical groups on polymer surfaces by oxidation. When a corona or plasma is applied to a polymer surface, the surface

is simultaneously subjected to many high-energy species which includes electrons, ozone, radiation, and ions. In the case of PDMS, the current knowledge of the effects of the corona or plasma exposure are: 1) the formation of an SiO_x silica like layer with higher oxygen content than that of bulk PDMS, and 2) the disappearance of the silica like structure which is replaced by low molecular mass and medium-sized PDMS groups. However, the surfaces of oxidized PDMS are known to recover its hydrophobicity after oxidation^{102, 103}. Owen's group gave a summary of the possible causes of hydrophobicity recovery¹⁰⁴: 1) reorientation of surface silanol groups into the bulk polymer, 2) cracking of the SiO_x structure, which create voids for the movement of free PDMS chains from the bulk phase to the surface, 3) condensation of silanol groups at the surface, and 4) changes in surface roughness. The positive aspect of the energy treatment, especially oxygen plasma treatment, is that various functional methods can be developed on this oxidized surface, such as the silanization process discussed below.

However, energy sources that are commonly used to create chemical groups on polymer surfaces by oxidation tend to roughen the surface and this leads to physical damage to the surface of the PDMS producing “cracks” or other undesirable microstructures on the surface^{105, 106}.

Figure 2.3. A. AFM images of O_2 -plasma treated PDMS surfaces in a RIE reactor after being exposed to different radiation doses¹⁰⁵ (a)-(d). For (a) and (b) images a $1 \times 1 \mu\text{m}^2$ scan area was used, while in (c) and (d) a scan area of $5 \times 5 \mu\text{m}^2$ is shown. B. SEM images of PDMS¹⁰⁶. (a) SEM of pristine PDMS at lower resolution. (b) SEM of pristine PDMS at high resolution. (c) SEM of oxygen plasma treated PDMS at lower resolution. (d) SEM of oxygen plasma treated PDMS at high resolution.

2.3.2. Silanization

Surface modification by a silanization reaction is established in numerous fields of bionanotechnology, especially in the field of biomaterials, where cellular adhesion and proliferation often need to be promoted for better biocompatibility^{107, 108}. Thin films of APTES are commonly used to promote adhesion between silica substrates and organic or metallic materials with applications ranging from advanced composite to biomolecular micro-devices^{109, 110}. To date, silanization studies are focused on glass and silicon¹¹¹. Usually, devices fabricated in glass, fused silica or silicon (Si/SiO₂) are conveniently used for exploring silane-based surface modification chemistries. In the case of APTES modified glass, the reaction is self-catalyzed by the amine group of APTES¹¹². For this case, rapid hydrolysis of APTES produces ethanol and trisilanols. The Si-C bond is hydrolytically stable and the aminopropyl group will not be cleaved, while the transient silanol groups will condense with other silanols to yield aminopropyl-functional resins. Recently, polymer surface was modified with APTES¹¹³. Because there is limited silanol on pristine PDMS, following the trend of APTES deposition on glass or silicon, special treatment (such as oxygen plasma or UV irradiation) would be done to generate silanol groups on the PDMS. For example, irradiation in air with UV light at 184 and 254 nm leads to in-situ generation of ozone and oxygen atoms that oxidizes the PDMS to produce a short-lived, highly hydrophilic glasslike surface¹¹⁴. A similar approach involves the generation of ozone using a corona discharge followed by UV photolysis at 254 nm¹⁰⁰. Probably one of the most successful approaches involves the generation of hydroxyl groups on the surface using oxygen plasma treatments, followed by reaction with trialkoxy-based silane reagents. However, energy sources such as plasma, ultraviolet light, and corona discharges that are commonly used to create chemical groups on the polymer surface by oxidation tend to roughen the surface and lead to physical damage to the surface of PDMS, producing “cracks” or other undesirable microstructures on the surface. Moreover, those methods are very difficult to modify the surfaces of microfluidic channels in an assembled device due to its deeply embedding in PDMS slabs. Therefore, ambient methods are highly desired. Gerardo A. etc. demonstrated a simple and

economical method to functionalize PDMS with APTES after exposure to ozone without the need for photolysis¹¹⁵. It is suitable for more homogeneous oxidation of three-dimensional structures of PDMS without the need to irradiate from different angles to achieve a homogeneous oxidation.

Figure 2.4. A. APTES modifies glass/silicon surface¹¹¹ (a) glass surface, (b) $\text{H}_2\text{O}_2/\text{H}_2\text{SO}_4$ treatment brings Si-OH groups onto glass surface, (c) silane covalently bound to the oxide surface, and (d) thermal curing of the film, which, in cross-linking the free silanol groups. B. APTES modifies polymer surface¹¹³. (e) initial adsorption via hydrogen bonding, (f) subsequent lateral bond formation to the nearest neighbors, and (g) multilayer formation.

2.3.3. Polyelectrolyte multi-layers

As a comparatively milder method, layer-by-layer technique was quickly developed to assemble biocompatible or other functional surfaces. For polymer surface modification, some performed layer-by-layer electrostatic self-assembly of polyelectrolyte with chemical cross-linking on the PDMS surface as a way of imparting lasting hydrophilicity to the surface¹¹⁶. Polyelectrolyte multilayers (PEMs) are thin composite films that are fabricated by alternately exposing a surface to cationic and anionic polyelectrolyte in solution. An important requirement in the fabrication of PEMs is that the sign of the surface charge must be reversed after each exposure to a polyelectrolyte, which is called

charge overcompensation. In principle, charge overcompensation has two important consequences: (i) the adsorbed polyelectrolyte repels equally charged molecules from the surface, which allows self-regulation of the adsorption process and restriction to a monolayer, and (ii) an oppositely charged polyelectrolyte can be adsorbed in the subsequent step. Researchers in separation sciences have adopted PEM coating for modifying surfaces of microchannels. Although the formation of PEMs is simple, the internal structure, which has been probed using various techniques such as neutron reflectometry, AFM, and solid state NMR, reveals considerable complexity. The layer deposited consists of stratified structures in which polyanions and polycations of individual layers interpenetrate each other. One layer may be dispersed three or four layers from its original location. In addition to layer interpenetrations, surface roughness and morphology have been shown to be influenced by salt concentrations¹¹⁷.

Figure 2.5. Scheme of typical PEM-coated capillary using a cationic polymer and an anionic polymer (or an anionic polymeric surfactant)¹¹⁷

2.3.4. Biomolecule coating

Cremer's group applied a phospholipids bilayer coated on PDMS microchannels to perform heterogeneous immunoassays. Coating of the microchannels was performed by injecting a solution of small unilamellar vesicles (SUVs) in 10mM phosphate buffer at pH 7.2 into each channel. Coating was performed 3 – 4 minutes after plasma oxidation to ensure that the PDMS was still hydrophilic enough to allow filling of the channels with aqueous solutions by capillary action. The coating efficiency was checked by epifluorescence. Using fluorescence measurements, protein adsorption to the bilayer coated channels was seen to be suppressed by at least two orders of magnitude when compared to the uncoated channels. The bilayer proved to be

stable for several weeks^{85, 118}.

Figure 2.6. Diagram of the sample preparation microsystem used as demonstration model for heparin coating evaluation. The device consists of a lid and a bottom chip, both fabricated in PDMS elastomer. A polymeric membrane filter is incorporated into the lid before bonding. The microchannels underneath the filter collect the filtrate and lead it into a collection funnel⁸⁵.

Modifications of PDMS microchannels with proteins have mainly been used for heterogeneous immunoassays. In the research done by Thormann's group, proteins were immobilized on PDMS/AF45-glass channels, with the aim of reducing adsorption of nonpolar molecules on the channels and carrying out immunoassays. In this work, channels were modified with a three-layer biotin-neutravidin sandwich, which was made by successively depositing biotinylated immunoglobulin G (IgG), neutravidin and biotinylated dextran. Similarly, Eteshola and Leckband developed a heterogeneous sandwich enzyme-linked immunosorbent assay (ELISA) by depositing the proteins A on the PDMS microfluidic channel¹¹⁹.

2.3.5. Others

Radiation induced graft polymerization is a method used for surface modification of polymers for various purposes. If the polymer surface has no chemically reactive functional groups, irradiation would be needed to induce chemical reactions on the surface. The basic aim of irradiation is to generate free radicals; these act as sites for graft polymerization¹²⁰. Hu S demonstrated a one-step procedure to covalently link polymers to the surface of PDMS microchannels by ultraviolet graft polymerization¹²¹. The grafted microchannels generally higher electroosmotic flow (EOF) than native PDMS and lower EOF than oxidized PDMS. The EOF of the grafted channels was stable for only up to 30 hours in air or water, which is not good for long-term quantitative analysis since peak areas vary with migration times, which in turn depend on the EOF magnitude. The limited stability of the grafted coating could still be of concern

in long term applications¹²².

The other trend in polymer surface modification benefits from developments in sol-gel science. Roman, G.T etc. claimed that surfaces modified via sol-gel chemistry could suffer less from hydrophobicity recovery since the silica particles produced are homogeneously distributed throughout the PDMS matrix. Based on the reported data, however, the increase of hydrophilicity through sol-gel modifications is relatively small since the contact angle was reduced by only 17%. Moreover, the resistance to protein adsorption for PDMS-SiO₂ material could still be poor due to the silica-like surface¹²³.

The use of vapor-deposited polymer coatings provides an appealing alternative to the currently employed arsenal of surface modification methods which consist mainly of wet-chemical approaches. Chen, H.Y etc. demonstrated the usefulness of chemical vapor deposition polymerization for surface modification in confined micro geometries with both nonfunctionalized and functionalized poly (p-xylylenes)⁸⁰. For a diverse group of polymer coatings, homogeneous surface coverage of different micro geometries featuring aspect ratios as high as 37 has been demonstrated based on optical microscopy and imaging X-ray photoelectron spectroscopy. The fact that reactive coatings can be deposited within confined microenvironments is an important factor toward the design of new device architectures, with potential relevance to bioanalytical, medical, or “BioMEMS” applications.

Chapter 3 Experimental

3.1. Methodology

Various experimental methodologies were used or developed. The success of a miniaturized lab-on-chip system greatly relies on the correct use of new materials, novel architectures, economical fabrication processes and efficient immobilization of biomolecule probes. These unique methodologies are developed as they are related to the applications.

3.1.1. Polymer substrate preparation

Glass dishes were thoroughly cleaned with ethanol and subsequent DI water washing. A curing agent and PDMS monomer were thoroughly mixed in a 1:10 weight ratio. To remove air bubbles from the precursor mixture, a vacuum was applied for 1 hour. The PDMS precursor was then put in the cleaned glass dishes and cured for 2 hours at 80 °C or overnight at room temperature. After curing, the solid PDMS slab was cut into small pieces, cleaned with ethanol and nitrogen flow dried. The PDMS specimens were used in the following experiments.

A cleaned Polyethylene terephthalate (PET) film was cut into the desired size (75 mm×25 mm). The prepared epoxy/acrylate resin discussed above was dropped on the surface of the PET substrate to form a thin layer of film by spin-coating, followed by UV irradiation under a nitrogen atmosphere with a UV exposure system equipped with an Hg lamp for 5 seconds (UV system, 400W). The cured epoxy film was washed in ethanol and subsequently in DI water for 3 minutes each time to remove any uncured polymer, and was ready to be used for the fabrication of the protein microarray and microfluidic device.

3.1.2. Characterization of polymer and its functionalized surfaces

Polymer (such as PDMS, functionalized PDMS surface, and cured epoxy film) surface characteristics were studied. Surface functional groups were examined with Fourier transform infrared spectroscopy (FTIR), Raman and AFM force measurements. Surface hydrophilicity was studied with contact angle measurements. The morphology of the polymer surface was recorded by AFM. The concentrations of different surface functional groups were measured using different chemical titration methods. The detailed experimental conditions are

also discussed in this text.

3.1.2.1. FTIR

FTIR is specifically used to measure the absorption of various infrared light wavelengths by the material being studied. These infrared absorption bands identify specific molecular components and structures. In this study, FTIR was used to survey the functional groups on polymer surfaces. FTIR spectra were recorded in a range of 400-4000 cm^{-1} (using a Nicolet Magna IR 560 ESP spectrophotometer, Thermo Nicolet, USA). A minimum of 64 scans was averaged using a signal resolution of 0.5 cm^{-1} .

To study PDMS and its functionalized surface, PDMS samples were cut into identical sizes. Figure 3.1 shows the characteristic IR feature of pristine PDMS. Spectra recorded on the functionalized samples were always compared to spectra obtained from pristine PDMS, in which the peaks at 2964, 1259, and 1015 cm^{-1} should be assigned to -methyl CH, -CH₃, and Si-O-Si¹²⁴.

Figure 3.1. FTIR spectra of pristine PDMS

In epoxy resin study, a thin layer of resin was coated on a potassium bromide disc. The FTIR spectrum of the coated resin was recorded before and after UV-polymerization. Attention was focused on the unsaturated double bands of the acrylate group at 1637 cm^{-1} and 810 cm^{-1} ¹²⁵ and the characteristic bands of epoxide groups: the epoxide ring breathing mode at 1258 cm^{-1} , the antisymmetric ring deformation band at 917 cm^{-1} , and the symmetric epoxide ring deformation at 866 cm^{-1} ¹²⁶.

3.1.2.2. Raman spectroscopy

Raman spectroscopy is used in chemistry in order to provide a fingerprint by which the molecule can be identified. In this study, a Raman microscope CRM2000 (Witek, Germany) equipped with a 488nm laser was used to study the PDMS conformation status in a chemical modification process. Figure 3.2 shows the characteristic Raman peaks of pristine PDMS. Spectra recorded on the functionalized samples were always compared to spectra obtained from pristine PDMS. Peaks of interest include the methyl group appearing at 2965 and 2907 cm^{-1} , the methyl bending vibrations appearing at 1412 and 1262 cm^{-1} , the Si-CH₃ rocking vibrations appearing at 862, 787, and 687 cm^{-1} , and the Si-O-Si stretching vibrations appearing at 488 cm^{-1} ¹²⁷.

Figure 3.2. Raman spectra of pristine PDMS¹²⁷

3.1.2.3. Atomic force microscopy

Atomic force microscopy (AFM), a high resolution type of scanning probe microscope, was used to study polymer surface morphology and the protein immobilization process.

Imaging mode

The primary operation mode is tapping AFM, which scans a sharp probe over a surface to image extremely small surface structure. In tapping mode AFM, a cantilever is driven to oscillate up and down at near its resonance frequency by a small piezoelectric element mounted in the AFM tip holder. In this study, AFM images of polymer surfaces were measured in air by AFM (Nanoscopy III,

Digital Instruments, USA). The samples were scanned with a standard Si tip (10-15 nm in diameter) supplied by the microscope manufacturer. Five randomly selected sites on each PDMS sample were imaged to determine the uniformity of the surfaces.

Force-distance measurement

In addition to topographic measurements, AFM force mode was conducted to measure the force-distance curves of polymer surfaces. AFM can record the amount of force felt by the cantilever as the probe tip is brought close to - and even indented into - a sample surface and then pulled away. This can be used to measure the long range attractive or repulsive forces between the probe tip and the sample surface, elucidating local chemical and mechanical properties like adhesion and elasticity, and even thickness of adsorbed molecular layers or bond rupture lengths^{128, 129}. A typical force-distance curve is shown in figure 3.3.

Figure3.3. AFM force curve measurement

In force curve experiments, the AFM tip was made to approach towards and retract from the surface, and the static deflection of the cantilever was monitored as a function of the piezo displacement. The force curve clearly shows the relationship between the set-point and the deflection of the cantilever. Because the set-point defines the value of the deflection signal maintained by the feedback loop, the force curve can be used to calculate the contact force of the tip on the sample if the spring-constant of the cantilever is known. The contact force is defined by the following equation¹³⁰:

$$F = k \times d \quad [3.1]$$

where k is the cantilever spring constant, and d is the deflection measured from the set-point to V_{CSmin} in nanometers.

Image analysis

The obtained AFM images were processed to be analyzed for surface

roughness, section and bearing using the DI software Version 5.12 (Veeco, USA). A third-order polynomial plane fit was used to account for the sample tilt as well as the slight curvature resulting from the coupling between the Z and X-Y piezoelectric positioners.

Generally, section analysis proves most useful for making direct depth measurements of surface features, for example, when measuring vertical distance (depth), horizontal distance and angle between two or more points.

The roughness analysis generates a wide variety of statistics for surfaces. In this study, the roughness analysis gives R_{ms} , the standard deviation of the surface roughness for all Z_{ij} (surface heights) according to the equation:¹²⁸

$$R_{ms} = \sqrt{\sum_{j=1}^N \sum_{i=1}^N \frac{(Z_{ave} - Z_{ij})^2}{N^2}} \quad [3.2]$$

where Z_{ave} and N are the average value and the number (512) of Z_{ij} , respectively. Clearly, R_{ms} can be used to represent the roughness of scanned surfaces.

3.1.2.4. Contact angle measurement

The sessile drop method is a measurement by a contact angle goniometer using an optical subsystem to capture the profile of pure liquids on a solid substrate. The surface hydrophobicity is evaluated from the water contact angle data. In the test, a droplet of 5 μL liquid was automatically dispensed onto the substrate surface, and a CCD camera revealed the profile of the droplet. The angle formed between the liquid/solid interface and liquid/vapor interface is the contact angle. Software calculates the tangent to the droplet shape and the contact angle (FTÅ 200, First Ten Angstroms, USA). Each data point calculated was based on 6 contact angle measurements at different positions on each specimen.

3.1.2.5. Surface functional group quantification

Procedures for evaluating dyes as biological stains were developed to quantify biomolecules of interest, such as nitrogen compounds. In principle, dye methods express the color characteristics of a sample by a simple ratio of color densities at two selected wavelengths; stains are characterized by their complete absorption spectra and by the exact location of their absorption maxima. In this study, surface amino groups, and carboxylic groups were quantified using

fluorescamine assay and toluidine blue O, respectively. The detailed experimental conditions are listed in the following sections.

Fluorescamine assay

A synthetic compound, fluorescamine (4-phenylspiro [furan-2 (3H, 1 phthalan) - 3, 3'dione]) reacts with primary amines, producing fluorophor which can be assayed at 480 nm (395 nm excitation). At room temperature, the reaction of fluorescamine is almost instantaneous, with a half-life of milliseconds. The products are highly fluorescent whereas the reagent and its degradation products are non-fluorescent¹³¹ (figure 3.4). Thus, the intensity of the fluorophor is proportional to the amount of the amine groups in the measured sample.

Figure3.4. Fluorescamine assay

In following chapters, fluorescamine method was employed to quantify the density of NH_2 groups on polymer surfaces¹³². In experiments, a 200 μL fluorescamine/acetone solution (1 mg mL^{-1}) was loaded in triplicate onto testing surfaces for 5 minutes. The reacted solution was loaded into a 96-well micro plate for fluorescence intensity measurement (excitation wavelength 395 nm, maximum emission wavelength 480 nm) on a micro plate reader, Genios plus (Tecan, USA). A calibration curve was obtained with a serials concentration of APTES on PDMS surface reacting with the same fluorescamine/acetone solution (figure 3.5). The surface NH_2 concentration is expressed in mol/cm^2 .

Figure3.5. Calibration curve of Fluorescamine assay of APTES on PDMS surface

Toluidine Blue O assay

Toluidine blue has positively charged amine groups which can adsorb to the negatively charged carboxyl groups. In principle, the density of COO^- groups on the testing surface is estimated under the assumption that every COO^- binds with a single toluidine blue molecule.

Figure3.6. Toluidine blue O structure

The density of carboxylate groups on the functionalized polymer surface was determined using the toluidine blue method¹³³. In experiments, testing samples were immersed in an aqueous solution of toluidine blue (5×10^{-4} M, adjusted to pH 10 with NaOH). The formation of ionic complexes between the COOH group and cationic dye was carried out for 5 hours at room temperature. The excess dye was removed by rinsing with a pH 10 0.01M NaOH solution. The toluidine blue was then desorbed from the surface by incubating the stained surface in 1 mL of 50 wt% acetic acid solution for 5 minutes. The optical adsorption (633 nm) of 1 mL of the desorbed toluidine blue solution was measured using a UV-vis spectrophotometer. The amount of toluidine blue originally bound to the testing sample was then calculated from the optical density. The density of COO^- groups on the testing surface was expressed in mol/cm^2 . Measurements were performed on five separate samples to obtain an average surface COO^- density. A calibration UV adsorption curve was obtained with a serials concentration of toluidine blue O dissolved in 50 wt% acetic acid (figure 3.7).

Figure3.7. Calibration curve of TBO COOH assay

Ninhydrin assay

Solid-phase ninhydrin assay was used to assess the free primary amines on the polymer surface because ninhydrin (triketokhydrindane hydrate) is a chemical widely used to detect ammonia or primary and secondary amines¹³⁴. In order to generate the ninhydrin chromophore (Ruhemann's purple complex), the amine is condensed with a molecule of ninhydrin to give a Schiff base. Thus only ammonia and primary amines can proceed past this step (figure 3.8). This reaction is simple and straightforward. The assay result can be directly observed using the human eye and quantified by UV absorbance. This assay was conducted routinely to check the existence of amine groups in the polymer modification experiments (figure 3.9).

Figure3.9. Images of ninhydrin assay on different solid surfaces
a. pristine PDMS, and b. APTES-PDMS

3.1.3. Protein immobilization on polymer surfaces

Protein passive adsorption and covalent immobilization via chemical groups, e.g., -NH₂, -COOH, -epoxy, were studied with the objective of providing

uniformly protein coated surfaces that exhibit increased assay sensitivity and improved assay precision in immunoassays. In the following sections, protein immobilization experimental procedures are presented. Protein immobilized surfaces were characterized by AFM, direct ELISA or sandwich ELISA.

3.1.3.1. Protein passive adsorption on polymer surface

Adsorption is the simplest way to immobilize biological molecules on the surface. In this study, proteins were diluted in coating buffers (0.05 M pH8.0 Tris-HCl, 0.01 M pH 7.4 PBS, 0.05 M pH 9.6 carbonate–bicarbonate buffer, or 0.05 M citrate- phosphate buffer) of the appropriate concentrations. Protein solutions were directly transferred onto the testing substrate for appropriate time incubation. The un-adsorbed proteins were removed with a washing buffer (TBS plus 0.05% Tween 20, TBST).

3.1.3.2. Covalent immobilization protein on polymer surface

Covalent binding is a superior method for immobilization of biological molecules on the hydrophilic polymer surface. The covalently immobilized antibodies could maintain better immuno-activity than that of adsorption-based attached ones due to minimal denaturation on the surface from elimination of the desorptions during continuous measurements^{135, 136}. In addition, hydrophilic surfaces may have a lower level of non-specific interactions than hydrophobic surfaces¹³⁵. In this study, different covalent bioconjugation approaches were explored according to the polymer surface functional groups, such as amino groups, epoxy groups and carboxylic groups.

Aldehyde-group

Schiff base interactions between aldehyde and amines typically are not stable enough to form irreversible linkages. These bonds may be reduced with sodium cyanoborohydride to form permanent secondary amine bonds (figure 3.10). However, proteins cross-linked by glutaraldehyde, without reduction nevertheless show stabilities unexplained by a simple Schiff based formation. The stability of such unreduced glutaraldehyde conjugates has been postulated to be due to the vinyl addition mechanism, which does not depend on the reaction of

Schiff bases¹³⁷.

Figure3.10. Protein immobilization on aldehyde functionalized surface

In experiment, amino groups on a PDMS surface were activated by 2.5% glutaraldehyde (GA) (pH 9.6) for 1 hour at room temperature. The GA treated surface was carefully rinsed with 0.01 M pH 8.0 Tris-HCl buffer and dried by nitrogen flow. Proteins were dissolved in 0.01 M pH 8.0 Tris-HCl with the desired concentrations and then transferred onto aldehyde-activated surfaces for 1 hour's incubation at room temperature. The surfaces were washed 3 times for 3 minutes each with TBST to remove unbound proteins. The protein immobilized surfaces were used in the following AFM, direct, or sandwich ELISA studies.

EDC-NHS

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) is a zero-length crosslinking agent used to couple carboxyl groups to primary amines. EDC reacts with a carboxyl to form an amine-reactive *O*-acylisourea intermediate. This active species can react with a nucleophile such as a primary amine to form an amide bond. If this intermediate does not encounter an amine, it will hydrolyze and regenerate the carboxyl group. In the presence of N-hydroxysulfosuccinimide (Sulfo-NHS), EDC can be used to convert carboxyl groups to amine-reactive Sulfo-NHS esters. This is accomplished by mixing the EDC with a carboxyl containing molecule and adding Sulfo-NHS¹³⁸ (figure 3.11.). EDC/NHS bridged carboxylic-amine crosslinking was used to covalently immobilize proteins on carboxylic groups functionalized solid surfaces.

Figure 3.11. EDC/NHS-mediated carboxylic-amine conjugation

In the experiment, a mixture of 2 mM EDC and 5 mM NHS (0.05 M pH 5.0 citrate-phosphate buffer) was added on the carboxylic groups- functionalized polymer surface for 1 hour's incubation. The polymer surfaces were then washed with PBS three times for 2 minutes each. Thereafter, proteins dissolved in 0.01 M pH 7.4 PBS with different concentrations were added onto the EDC/NHS-activated surfaces for 1 hour's incubation at room temperature. After protein immobilization, the unbounded proteins were removed by tris buffered saline, 0.01M pH 8.0 containing 0.05% detergent Tween 20 (TBST) washing (3 times for 3 minutes each). The protein immobilized surfaces were used in the following AFM, direct or sandwich ELISA experiments.

Epoxy

An epoxy can react with nucleophiles in a ring-opening process. The reaction can take place with primary amines, sulfhydryls or hydroxyl groups to create secondary amines, thioether, or ether bonds, respectively. During the coupling process, ring opening forms a β -hydroxy group on the epoxy compound¹³⁷ (figure 3.12).

Figure 3.12. Epoxy groups-mediated multiple conjugation

In microarray study, proteins were diluted in 0.01 M PBS, pH 7.4 plus 2.5% glycerol and 0.003% triton X-100 for microarray preparation. Nanoliter volumes of protein per spot were delivered to the epoxy-polymer surface. Following microarray printing, printed proteins were incubated overnight at room temperature. The epoxy-polymer surface was then washed three times for 2 minutes each in a solution of TBS (containing Tris buffer, 0.5% Tween 20) to remove any unbound probes.

3.1.3.3 Characterization of proteins on polymer surface

To evaluate the different protein immobilization methods, the protein immobilized surfaces were characterized. The morphologies of the immobilized protein were recorded using AFM (see *section 3.1.2.3*). The adsorbed protein volumes were analyzed by using the bearing analysis command. The amount of the protein immobilized was indirectly evaluated by direct ELISA, of which the enzyme labeled antibody reacted with the immobilized protein. The reacted enzyme catalyzed the substrate to develop a measurable signal. The higher the signal was, the more the enzyme was immobilized, and the more protein was attached on the solid surface (see *section 3.1.5.1*).

3.1.4. Polymer microfluidic devices fabrication

3.1.4.1. PDMS microfluidic devices

Structure design

Figure 3.13a and b show the top and section views of the fabricated PDMS microfluidic ELISA device, respectively. The device comprises a plurality of

microchannels in the substrate for guiding a fluid flow from the inlet channel into detection well, and then to the outlet channel. Figure.3.13 *b* shows the detail structure of the detection well and its related connections, in which a flow is introduced through the inlet manifold microchannel into the detection well and then drains out to the waste outlet manifold microchannel by overflow. The overflow outlet of the detection well was designed in such a way (Figure.3.13 *b*) that its flow level is lower than the inlet but significantly higher than waste outlet microchannel, thus totally eliminating backflow of the reacted fluid and inhibiting cross-contaminations by the reacted fluid from one detection well to another one due to extremely high fluidic resistances in such a structure. This unique feature can also allow enough contact time for interactions of target and capture proteins in the detection well. The device in more details was previously described in our filed patent¹³⁹. This simple but delicate design can automatically and efficiently conduct sampling, incubation, washing and detection within the plurality of test sites for multiple immunoassay screening. To fabricate the three-dimensional architected microfluidic device, a lithography process involving multiple-step deep reaction ion etch (DRIE) steps was carried out to make the master mold, which could be used to manufacture the device in Figure.3.13*a* and *b* through soft lithography. In the fabrication, the silicon wafer was thermally oxidized to form a 1 μm oxide layer on its surface as the sacrificial layer in the DRIE process and photoresist AZ7220 was applied for the patterning.

Figure 3.13. PDMS microfluidic device: a. top view of the microfluidic system, b. cross-sectional view of the microchannel

PDMS based microfluidic device fabrication

For PDMS casting, the PDMS precursor, made by mixing the PDMS elastomer with a curing agent from the Sylgard 184 kit in a weight ratio of 10:1 (w/w), was poured onto the DRIE fabricated silicon mold and cured at 80°C for 2 hours. After the PDMS replica was peeled from the mold, holes were made using a drill at the inlet and outlet portions of the channel for connection to the sample micropump. A flat PDMS slab, which was used to cover the top of the replicated PDMS microstructure, was obtained by casting and curing the precursor PDMS on a clean glass dish. The replicated PDMS microstructure and flat PDMS cover were washed with ethanol and subsequently, DI water; this was followed by baking at 70°C for 1 hour. They were then treated by oxygen plasma for 17 seconds in a plasma generator (Oxford Instruments, Bristol, UK). After the plasma treatment, the flat PDMS cover was put on top of the replicated PDMS microstructure followed by baking at 70°C for 30 minutes. Silicon tubes were employed to connect the main inlet with a syringe pump to finally make a sealed, flow-through, multi-channel microfluidic device for ELISA applications.

Scheme 3.1. Micro-channel prepared through PDMS replication. a. PDMS precursor is poured on silicon mold and degas for 20 min, b. PDMS is cured at 80°C for 2 h, c. carefully peel off the cured PDMS micro-structure, and d. replicated PDMS microchannels

The character of the fabricated wafer is shown in figure 3.14 (a), (b). The PDMS replica was generated from the silicon mold. The structures of the microfluidic system in the PDMS slab can be seen in figure 3.14 (c) and (d). From the SEM pictures (e) and (f), the channel structure (width× depth× length: 250μm×300μm×2.5mm) can be seen more clearly.

Figure 3.14. PDMS microfluidic system a., b.: microstructures on the silicon master, c., d.: PDMS replica made from the silicon master, e., f.: SEM images of the PDMS microstructures

Coomassie blue dye was injected into the system from the inlet reservoir. Under the pressure driving force, the blue liquid divided into different portions and simultaneously flowed through the multi-channels. The flow through character was videotaped using a CCD camera (figure 3.15). Figure 3.15 shows the liquid filled the inlet main channel before then splitting into the parallel channels with a positive pressure drive applied.

Figure 3.15. The flow-through characters of PDMS microfluidic device

3.1.4.2. UV-embossed microfluidic device with epoxy resin

Fabrication of epoxy microfluidic device

A multi-channel epoxy microfluidic device was made for ELISA experiments. To fabricate the epoxy microfluidic system, a SU-8 photoresist patterned master mold was fabricated via photolithography. The precursor PDMS, prepared by mixing PDMS elastomer with a curing agent in a ratio of 10:1(w/w), was poured onto the master and cured at room temperature for 24 hours to produce a replicated PDMS mold. The prepared liquid UV resin was poured onto the

PDMS mold. After the resin had uniformly filled the pattern of the mold, the prepared polyethylene terephthalate (PET) film with a coated cured-epoxy thin layer was laminated on top of the epoxy resin-filled mold. A glass slide was placed on the PET film and four clamps were used to fix the whole assembly as well as to apply pressure. The assembly was then UV irradiated for 5 seconds followed by demolding; this entailed peeling the soft PDMS mold from the embossed polymeric microstructure including the microchannels (width $\times$ depth $\times$ length: 200 μ m $\times$ 100 μ m $\times$ 10mm) on the PET film. The cured structure was washed successively with ethanol and DI water. After blow-drying, a drill was used to make holes at the inlet and outlet channels to connect to a syringe pump using a silicon tube; the connections were sealed with a HY-9 hydrophilic (adhesive material) coated plastic film.

Scheme 3.2. micro-channel prepared through UV embossing. a. UV curable epoxy resin is poured on PDMS mold, b. polyester film is laminated over the epoxy resin, c. epoxy resin is cured by UV irradiation, and d. carefully peel off the cured micro-structure; e. embossed microchannel

3.1.5. Immunoassay

In this thesis, three types of immunoassay platforms, namely, i) micro-plate based ELISA; ii) microfluidic devices based flow-through ELISA; and iii) antigen/antibody microarray, were studied. A key feature of the flexibility of ELISA is that more than one system can be used to measure the same thing. This allows some scope in adapting assays to suit available reagents as well as to note areas of potential improvement. The following experimental details of the various reaction schemes, direct ELISA, sandwich ELISA, and competitive ELISA, were used in this study.

3.1.5.1. Micro-plate ELISA

Direct ELISA

Direct ELISA can be regarded as the simplest form of the ELISA, and is illustrated in scheme 3.3. In a direct ELISA, 100 μL of different concentration antigens (PBS, pH 7.4) were loaded into micro-plates. The antigen coated plate was covered with paraffin film and incubated for 1 hour at 37°C . After the antigen incubation, any excess antigen was removed by flooding and emptying the wells thrice with 150 μL TBST for 2 minutes each time. Unoccupied surfaces were blocked with 150 μL blocker (1% BSA or BlockerTM) for 2 hours at 37°C . The plate was then emptied and washed again (3 minutes, thrice) with TBST. The enzyme-labeled antibody was diluted in TBST with the appropriate concentrations and added to each well (100 μL /well) for a 30 minute incubation at 37°C . The plate was emptied and washed again (3 minutes, 4 times). A 100 μL TMB solution was then added into the wells for the appropriate incubation. The absorbance of the peroxidase catalyzed substrate was quantified by ELISA reader (GENios plus, Tecan, USA).

Scheme3.3. Micro-plate direct ELISA process: a. Protein coated solid surface. b. After washing, blocker protein was use to block the unoccupied surface. Enzyme labeled antibody was added in and reacted with the immobilized protein. c. Enzyme labeled antibody reacted with the immobilized protein; free protein and labeled antibody were washed off. d. Immobilized enzyme catalyzed substrate developed color. The higher the absorbance was, the more the enzyme was immobilized, and the more protein was immobilized on the solid surface.

Sandwich ELISA

In sandwich ELISA (scheme 3.4), a 100 μL probe antibody, diluted in 0.01 M pH 7.4 PBS with the desired concentrations, was added to the wells for a 1 hour incubation at 37°C . The wells were then emptied and the unoccupied surface of the wells was blocked with a 150 μL of blocking buffer for 2 hours at 37°C . The wells were emptied and washed three times with TBST. The antigens diluted in TBST with different concentrations were added into the wells (100 μL antigen per well) for an appropriate time incubation at 37°C . The wells were then

emptied again and washed three times with a wash buffer. The procedure of the reactions of enzyme-labeled antibodies and TMB were the same as those for direct ELISA.

Scheme 3.4. Micro-plate sandwich ELISA process. a. Antibody coated solid surface. b. After washing, blocker protein was used to block the unoccupied surface. Sample (antigen) was added in c. Enzyme labeled antibodies reacted with the captured antigen; free labeled antibody was washed off. d. Immobilized enzyme catalyzed substrate developed color. The higher the absorbance was, the more the enzyme was immobilized, and the more protein in the sample on the solid surface.

Competitive inhibition ELISA

In competitive ELISAs (scheme 3.5), probe proteins on the pre-coated micro-plate were used to capture a specific protein in a complex, which consisted of target proteins which were the same as the coated protein, and enzyme conjugated antibodies. The immobilized protein and the target protein in the mixture is a competitive pair for the antigen binding sites of the enzyme-linked antibody. Therefore, as the concentration of the competitive protein increases, the amount of antibodies which could be captured by the immobilized protein decreases. With the addition of the enzyme substrate reagent solution, the amount of captured enzyme conjugated antibodies is detected, resulting in an inverse relationship between absorbance and target protein concentration: the higher the absorbance, the lesser the amount of target protein in the sample.

Scheme 3.5. Competitive inhibition ELISA a. Protein coated solid surface, b. The reaction mixture of enzyme labeled antibody and protein. The protein here competes with the immobilized protein for the antigen-binding site, c. Enzyme labeled antibody reacted with the immobilized protein; free protein and labeled antibody were washed off, d. Immobilized enzyme catalyzed substrate developed color. The high the absorbance was, the more the enzyme was immobilized, the lesser the protein in the reaction mixture.

Micro-plate ELISA data analysis

For a given assay and set of reagents, the optimization of the assay sensitivity is a compromise (or balance) between the ratios of the reagent concentrations and the errors associated with signal measurement. In this study, all immunoassays were characterized in terms of their detection range and their lower limits of detection.

Absorbance curves were obtained by plotting absorbance against the logarithm of analytes concentrations using Origin 7.0. In an ELISA, sensitivity may be defined as the limit of detection or quantification. The lower limit of detection (LOD) is defined as the smallest concentration of an analyte that is able to produce a signal that can be distinguished from the signal obtained in the absence of that analyte¹.

Statistically, since all the ELISAs in this study were performed in at least triplicate, the LOD can be defined as the concentration that gave a signal (S_{LOD}), eq.3.4:

$$S_{LOD} = S_0 + 3s \quad [3.4]$$

where S_0 is the signal obtained in absence of analytes (negative control), and s is the standard deviation of the signal S_0 .

The working range is defined as the concentrations from the LOD to the concentration at which a plateau is reached on the dose-response curve.

In competitive ELISAs, the inhibition degrees were measured by the inhibition percentage, which was derived from the absorbance against the logarithm of analytes concentration. The inhibition rate can be expressed as

$$\text{Inhibition percentage (\%)} = [(S_0 - S_x) / S_0] \times 100\% \quad [3.6]$$

where S_0 is the absorbance of the negative control, in which there is no competitive protein in the reaction system and S_x is the absorbance value of the tested spots which correspond to the different competitive protein concentration.

3.1.5.2. Flow-through ELISA

Flow-testing experimental setup

Silicon tubes were employed to connect the main inlet with a syringe pump to make a flow-through, multi-channel microfluidic device for ELISA experiments. During the flow-through ELISA, reagents were pumped step-by-step into the

microfluidic device. Scheme 3.6 illustrates the experimental setup which was used to detect and capture the enzymatic converted signal from the microfluidic devices. Oblique Coherent Contrast (OCC) illumination system and charge-coupled device (CCD) camera were used as the detection equipment for the signal. The captured images were analyzed by image software, Nikon ACT-2U.

Scheme3.6. Flow-testing experimental setup

Direct ELISA on microfluidic device

In flow-through direct ELISA, antigens were diluted in 0.01 M pH 7.4 PBS with the desired concentrations and pumped into the microfluidic device for the appropriate time incubation. After protein immobilization, the excess antigen was removed with a 3 minute flow washing process. A casein solution was then pumped into the microchannels for the desired incubation time followed by flow-through washing with TBST for 3 minutes. The peroxidase conjugated antibody was pumped into the fluidic devices and incubated for the appropriate time followed by flow-through washing for 5 minutes. Finally, the enzyme substrate, TMB, was pumped into the microfluidic devices to react with the captured enzyme. The oxidized substrates were pumped out and collected in the 384-well ELISA-plates. The absorbance of the substrate was quantified using the ELISA reader. The images of the enzyme-catalyzed colored TMB filled microchannels were captured with CCD camera. The images were quantified based on their RGB (red-green-blue) brightness intensity (Nikon ACT-2U version 1.3)¹⁴⁰.

Scheme 3.7. Flow-through direct ELISA process. a. Protein was coated on the surface of the ELISA reaction portion. b. After washing, BSA was used to block the unoccupied surface. c. enzyme-labeled protein was added. d. the free enzyme labeled protein was washed off. The enzyme reacted with the substrate and chromatics enzyme converted substrate was developed.

Sandwich ELISA on functionalized microfluidic device

In flow-through sandwich ELISA (scheme 3.8.), antibodies (probes) dissolved in a 0.01 M pH 7.4 PBS in the desired concentration was pumped into the channels and incubated for the proper time period. After protein immobilization, the unbounded antibodies were removed by a flow washing process for 3 minutes. The same blocking and flow-through washing were performed similar to that done in the studies for flow-through direct ELISA. The analytes, antigens diluted in human serum or tris buffered saline, 0.01M pH 8.0 containing 0.05% detergent Tween 20 (TBST), were transported into the fluidic devices and incubated for the desired time, followed by flow-through washing with TBST for 4 minutes. The enzyme- labeled antibodies were then pumped into the channels for the appropriate time incubation followed by 5 minutes of flow through washing. Finally, the enzyme substrate, sodium metaperiodate and 3, 3', 5, 5'-tetramethyl benzidine, TMB, (1.25 mM TMB, 2.21 mM H_2O_2 , pH 3.1), was pumped into the channels. The peroxidase converted color-substrate TMB was quantified similarly to that done in the flow-through direct ELISA study.

Scheme 3.8. Flow-through sandwich ELISA at functionalized microfluidic device. a. section view of the intact microchannel, b. polymer functionalized microchannel, c. probe immobilized microchannel, d. analytes were injected into the microchannel, e. enzyme labeled antibodies were pump into the microchannel, f. substrates are enzymatically converted into colorimetric product

Flow-through ELISA data analysis

Absorbance data

The enzymatic converted substrates can be recorded as absorbance for quantitative analysis. In this case, the enzymatic converted substrates were pumped out and collected in the ELISA-plates. The absorbance of the substrate was quantified using the ELISA reader. The absorbance data was plotted against the assayed protein concentrations to obtain the dose-response curve. The working range and lower limit of detection are defined similarly to that in the microplate ELISA study (section 3.1.5.1).

RGB data

One of the advantages of the ELISA is that the colored end-product can be read by eye to assess whether tests have worked (avoiding waiting for results where machine reading is essential, such as in radioimmunoassay)^{141, 142}. For this purpose, the channels filled with enzymatic converted substrates were imaged using CCD camera. The color changes in each channel can be quantified via an imaging process. An image analysis software, Nikon ACT-2U, was used to quantify the RGB brightness. The brightness is based on the RGB colors of red, green, and blue, where brightness based on S_{RGB} color = $0.229 \times \text{red} + 0.587 \times \text{green} + 0.114 \times \text{blue}$ ¹⁴⁰. The RGB brightness was plotted against the assayed protein concentrations to obtain the dose-response curve (figure 3.16).

Figure3.16. Characteristic CCD image of the micro-channels and RGB brightness

3.2. Materials and equipments

3.2.1. Reagents and chemicals

Proteins

Protein A, goat immunoglobulin G (Cy3 labeled), goat IgG, rabbit IgG, rat IgG, human IgA, Anti-rabbit IgG peroxidase conjugate, monoclonal anti-goat IgG alkaline phosphatase conjugate, monoclonal anti-human IgA (α -Chain specific), anti-Rat IgG peroxidase conjugate, anti-human HLA-I antibody, anti-Human HLA-A2 antibody, anti-HLA-DR antibody and anti- β_2 -microglobulin antibody, anti-mouse IgG peroxidase conjugate, bovine serum albumin (BSA), human serum albumin, chicken serum albumin, were purchased from Sigma, USA BlockerTM Casein in PBS was purchased from Pierce, Germany. IL-5 and monoclonal anti-IL-5 antibodies were kindly provide by BD Bioscience, USA.

Chemicals

Poly (demethylsiloxane) elastomer kits (Sylgard 184) were purchase from Dow Corning, USA Poly (vinyl alcohol) (PVA, MW 89 000-98 000, 99% hydrolyzed), dextran, alkaline phosphatase yellow (pNPP) liquid substrate were purchased from Sigma-Aldrich, USA. 96% ethanol, sodium metaperiodate and 3, 3', 5, 5'- tetramethyl benzidine (TMB), hydrochloric acid and hydrogen peroxide were purchased from Merck KGaA, Germany). 3-amino-propyltriethoxysilane (APTES), succinic acid anhydride (SAA), glutaraldehyde (GA), BlockerTM Casein in PBS and 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide hydrochloride (EDC) were purchased from Pierce, Germany. All other used chemicals were of analytical grades and obtained from common commercial supplies.

Trimethylolpropane tricarylate (TMPTA, SR-351), dipropylene glycol diacrylate (DPGD, SR-508) are supplied by Satomer Company Exton, USA. Ebecryl 3605 (Trade Mark) is a partially-acrylated bisphenol A epoxy resin available from UCB SA Company, Belgium. The UV curing for patterning the structure was initiated with the photoinitiator 2,2-dimethoxy-2-phenylacetophenone supplied as Irgacure 651 by Ciba Speciality Chemicals, Switzerland.

Photoresist AZ7220 and their developer PGMEA (propylene glycol methyl ether acetate) were purchased from MicroChem Corp, USA. The PMMA array was made by mechanical processing (Bace Engineering, Singapore).

Buffers

Citrate-phosphate buffer (0.05 M, pH 5.0), Tris-HCl, phosphate buffered saline (PBS, 0.01 M pH 7.4), Carbonate-bicarbonate buffer capsules (CCB, 0.05 M, pH 9.6), Tris buffered saline with 0.5% tween 20 (pH 8.0, TBS washing buffer) were obtained from Sigma, USA. The deionized water used in all experiments was produced by a water purification system, Q-Grad ®1, from Millipore Corporation, USA.

3.2.2. Equipment

Field emission scanning electron microscopy (FESEM) (JSM-6700F JOEL, Japan) provided the SEM photos.

Atomic force microscopy (AFM) (SPM 3100, Veeco Instruments Inc., USA) was used to characterize the morphology of our samples.

Fourier transform infrared (ATR-FT-IR) spectroscopy was performed by using the Nicolet Magna IR 560 ESP spectrophotometer (Thermo Nicolet, USA) incorporated with a “Golden Gate” ATR module (Graseby Specac, USA).

X-ray photoelectron spectroscopy (XPS, Kratos AXIS,) fitted with an aluminium anode (1486.6 eV) and a 4 keV Ar⁺ ion sputter gun was used to characterize the sample formation.

ELISA absorbance measurement was carried out by using Genios plus (Tecan,

USA) at 450 nm.

A Raman microscope CRM2000 (Witek, Germany) equipped with a 488nm laser was used for Raman spectrum measurements.

UV-VIS was measured on HITACHI U-2800 double beam system.

Contact angle measurement was carried out on FTÅ 200, First Ten Angstroms, USA

VersArray chip writerTM contact system (Bio-Rad, USA) was used to array probes onto the substrate for fabrication microarray. Microarrays were scanned at 530 nm using a scanning laser confocal fluorescence microscopy (ScanArray system, PerkinElmer, USA) at a 5- μ m resolution. The images were analyzed by quantifying the pixel density (intensity) of each spot by Scan Array Express software (PerkinElmer, USA).

Chapter 4 Conformative and quantitative studies of adsorption of protein A on PDMS surface by AFM

4.1. Introduction

This chapter studies the protein adsorption on PDMS by AFM not only to examine the conformation of the absorbed molecule at nanoscale, but also to quantitatively characterize the adsorption on the solid surface at a molecular level.

Proteins play critical roles in biological processes. Protein analysis is essential in molecular biology and many biomedical applications. It is known that ELISA, a mainstream assay used in clinical diagnostics for infectious diseases, and protein microarray/microfluidic devices, an advanced technology for high-throughput analysis, greatly rely on surface chemistry for efficiently immobilizing proteins on a solid support while retaining their native conformation and bioactivity^{15, 143-147}. Thus, the study of protein surface modification can provide not only deep fundamental insights into surface chemistry, but also optimal approaches for the enhancement of protein assays. Currently the advanced analytical technologies are related to the miniaturization and integration of an assay system such as microarrays and microfluidic chips for economical and high-throughput analysis. This is mainly enabled by microfabrication and has been rooted in polymer science to reduce the complexity and the manufacturing cost by usage of plastic substrates³³, of which PDMS is of particular importance⁸⁶ due to their excellent mold characteristics with high fidelity. PDMS strongly interacts with apolar and partly apolar analytes through its hydrophobicity¹⁴⁸. The PDMS/aqueous interface behavior is described like the octane/water system, in which small molecules such as toluene can diffuse into PDMS by adsorption mechanisms, while larger molecules containing apolar domains adsorb onto the surface. Spontaneous protein adsorption on surfaces is often caused by hydrophobic or hydrophilic interactions of their functional groups. Intrinsic hydrophobicity of a protein is determined by the relative number of hydrophobic and hydrophilic amino acids in its molecule¹⁴⁹, and therefore the property of the solid substrate would probably dominate the protein adsorption process. Recently, it is reported that the surface functional groups such as $-\text{CH}_3$, $-\text{POH}$, and $-\text{CF}_3$ on a solid surface determines

the surface hydrophobicity and this affects the protein adsorption on the surface⁹⁵. As a surface rich in methyl group, PDMS has a strong tendency to entrap proteins from the PDMS/aqueous interface. It is interesting and significant to further investigate the surface chemistry of protein adsorption on PDMS in a solid phase immunoassay (SPI) for improvement of PDMS based protein assays and lab-on-chips. Although various synthetic solid phases are used to immobilize the probe protein in modern immunoassays¹⁵⁰⁻¹⁵², the designs are mainly dependant on empirical data, without clear understanding of the protein/surface interaction at a molecular level.

Currently, investigations on protein adsorptions on a solid surface are conducted with surface tensiometry¹⁵³, ellipsometry¹⁵⁴, infrared spectroscopy¹⁵⁵, immunoassay⁹⁷ and surface plasmon resonance^{53, 156}. These methods may be sensitive to the amount of adsorption, but they are very difficult to provide the conformation state of the absorbed proteins on the surface. As one of the recognized label-less methods, the SPR method is almost completely insensitive to a refractive index variation of a film greater than 300 nm on silver or gold surfaces¹⁵⁷ since the evanescent field created on the surface decays exponentially and falls to one third of its maximum intensity at approximately 300 nm from the surface. However, many polymeric films used for fabricating microdevices may be too thick for SPR analysis. In fluorescence-based methods, the labeled dye could affect the affinity of the protein and may alter the conformation of the protein. Besides, different dye-to-protein ratios after labeling bring complexity to the quantitative interpretation of the fluorescent intensity. As a probe method suitable for “soft” biological samples, AFM has been used to study protein-surface and protein-protein interaction, particularly to depict conformation and spreading of the surface-bound proteins.^{158, 159}. More interestingly, the tensiometric and depletion measurements normally only detect ten¹⁵ or more molecules, whereas AFM can examine surface changes induced by a few proteins¹⁶⁰. The accuracy and sensitivity of the adsorption information provided by AFM is over several orders of magnitude higher than that often used, and thus can potentially be used to study surface adsorption kinetics and dynamics at a molecular level. Currently, AFM is reported to provide a valid estimation of adsorption density of the absorbed proteins on a solid surface but there is a large margin of error due to the assumptions involved¹¹, and not at a

molecular scale.

Protein A, a cell wall constituent of *staphylococcus aureus* from most mammalian species, is highly stable in the presence of denaturing agents over a wide range of temperatures and pH values¹⁶¹ and is a well recognized function protein used in IgG (Immunoglobulin G) associated assays¹⁶²⁻¹⁶⁴. The N-terminal part of protein A exhibits five highly homogenous IgG binding domains, namely E, D, A, B and C, of which domain B and E possesses 19 hydrophobic amino acid residues with a cylinder-like structure having a diameter of 3nm and a length of ~ 4.5 nm along the β -sheet, resulting in a molecule length of about 20-30 nm¹⁶⁵. Because of its unique structure and properties, protein A can be an excellent template molecule for the study of protein adsorptions on a solid surface. To date, most of the research work on protein A adsorption are focused on substrates like silicon wafer, mica and glass¹⁶⁵⁻¹⁶⁷.

There is a possibility to directly examine how the conformation state of the adsorbed protein A is affected by different adsorption conditions while quantitatively determining the amount of adsorption at the molecular scale by using the AFM approach.

4.2. Protein A adsorption and characterization

The AFM images of the surfaces with and without adsorbed proteins were measured in open air by AFM. The samples were scanned with a standard Si tip supplied by the microscope manufacturer. During scanning, the applied force was always minimized to avoid deforming the soft proteins layer. Five randomly selected sites on each PDMS sample were imaged to determine the uniformity of the protein A adsorption and its reproducibility. The measured AFM images were analyzed for surface roughness, section and bearing. The section analysis can be used to depict the sectional profile of the adsorbed protein molecules. The roughness analysis gives Rms , the standard deviation of the surface roughness for all Z_{ij} (surface heights) (eq. [4.1]) as follows:¹²⁸

$$Rms = \sqrt{\sum_{j=1}^N \sum_{i=1}^N \frac{(Z_{ave} - Z_{ij})^2}{N^2}} \quad [4.1]$$

where Z_{ave} and N are the average values and the number (512) of Z_{ij} , respectively. Clearly, Rms can represent the roughness introduced by the protein adsorption. The theoretical molecular volume ($V_{M.V.}$) was calculated from the

protein molecular weight using eq.4.2¹⁶⁸

$$V_{MV} = (M_0 N_0)(V_1 + dV_2) \quad [4.2]$$

where M_0 is the molecular weight, N_0 is Avogadro's number, V_1 and V_2 are the partial specific volumes of the individual protein ($0.74\text{cm}^3.\text{g}^{-1}$) and water ($1\text{cm}^3.\text{g}^{-1}$), respectively, and d is the extent of protein hydration (0.4 M of H_2O per mole of protein). The bearing absorbed volume V_0 and V_b , before and after the protein molecule adsorption were measured by using the bearing analysis command. Thus the adsorbed protein A molecular number can be calculated by

$$\Gamma_{M.N} = \frac{V_b - V_0}{V_{MV} * A_s} \quad [4.3]$$

where A_s is the surface area measured by AFM, which is $25\text{ }\mu\text{m}^2$ in this study.

Protein A was diluted with different pH citrate-phosphate buffers. In the adsorption experiment, 400 μL of the protein solution was loaded into a small beaker, and a 5 mm $\times$ 5mm PDMS slab was mounted on the protein solution. The beaker was hermetically sealed with parafilm which allows a negligible evaporation of the solution during the adsorption process. The PDMS surface was then thoroughly rinsed with TBS and deionized water respectively, followed by nitrogen flow dry and kept in a dry box before AFM measurement.

4.3. Results and Discussion

4.3.1. pH Effect on adsorption

The effects of solution pH on protein A adsorption were examined using 1 $\mu\text{g mL}^{-1}$ protein A and the adsorption time was 30 min. Figure 4.1 shows the AFM images (5 $\times$ 5 μm , 15 nm gray scale; inset: 1 $\times$ 1 μm , 15 nm gray scale) before and after the protein adsorption on the PDMS. The PDMS surface exhibits a smooth surface (figure 4.1a) before adsorption while distinguished conformations of the adsorbed protein A can be seen after adsorptions at different pH conditions. The adsorbed protein A displays a tortuose line-like conformation at pH 2.0 (figure 4.1b), but has two architectures at pH 3.0: tortuose line and dendrites with 2 or 3 tails (figure 4.1c). At pH 5.0, it mainly forms dendritic structures with 3 or 4 tails (figure 4.1d). The section analysis of each AFM top view numerically shows that the expanding areas of the adsorbed protein A at pH 2.0 and 3.0 are larger than

those at pH 5.0. Likewise, the space between the protein clusters is larger at pH 2.0 and pH 3.0 than at pH 5.0. This indicates that protein A adsorbed at pH 5.0 has a denser distribution on the PDMS surface. Since the isoelectric point of protein A is 5.0, the adsorption in pH 5.0 solution has an environment close to the *IP* of the protein, resulting in an almost neutral form of the protein in the solution. Theoretical modeling based on the adsorption quantity and solution viscometry reveals that the protein conformation is very compact at the zero-point of charge (ZPC)¹⁶⁹. Thus, the dendritic structure formed at pH 5.0 is due to the compactly folded domains of protein A, resulting in a higher distribution density to increase the amount of the adsorbed proteins. The linearly stretching structure of protein A at pH 2.0 and 3.0 is most likely the result of charged protein molecules that could induce the intramolecular lateral electrical repulsion to reduce the amount of the protein adsorption. The adsorption of protein A at pH 6.0 and 8.0 were also studied (figure 4.1 e, f), indicating the conformation of the dendritic structure formed in pH 5 solution changes back to the tortuose line structure as observed in pH 2.0 and 3.0 solutions. These results reveal that the conformation of the absorbed protein is simply dependant on the amount of charges, irrespective of whether they are positive or negative charges.

Figure 4.1. AFM images for $1 \mu\text{g mL}^{-1}$ protein A adsorption under different pH solution. a. bare PDMS, b. pH 2.0, c. pH 3.0, d. pH 5.0, e. pH 6.0, and f. pH 8.0. The section analysis shows the protein A layer thickness and density.

4.3.2. Effect of protein concentration on the adsorption

The effect of protein concentration on the adsorption on the PDMS surface was investigated at pH 5.0 over a range of 10 to 1000 ng mL^{-1} with adsorption time of 30 minutes. The results are shown in figure 4.2a-c ($5 \times 5 \mu\text{m}$, 15 nm gray scale; inset figures: $1 \times 1 \mu\text{m}$, 15 nm gray scale). Visible protein clusters were observed after adsorption in a 10 ng mL^{-1} protein A solution (figure 4.2a), while the characteristic dendritic structure occurred with adsorption in the 100 ng mL^{-1} solution (figure 4.2b) and became denser with adsorption in the 1000 ng mL^{-1} solution (figure 4.2c). *Rms* changes introduced by protein adsorptions are plotted in figure 4.2d, showing 0.7 and 1.29 of *Rms* after adsorptions in 10 and 100 ng mL^{-1} solutions, respectively, before achieving a plateau value of ~ 1.45 of *Rms* for adsorptions in the protein solutions with a concentration of equal to or higher than 1000 ng mL^{-1} . The thickness of the adsorbed protein layers produced by different protein concentrations is depicted in the section profile below for every top view image. Interestingly, the thickness of the adsorbed protein layer via

adsorption in the 10 ng mL^{-1} protein solution is $\sim 1 \text{ nm}$, which is smaller than the mean size of 3 nm for protein A in solution, whereas the thickness of the same protein layer via adsorption in protein solutions equal to or higher than 100 ng mL^{-1} increases to $2.5\text{-}3.5 \text{ nm}$, which is the same size as that of the protein A in solution. The result may indicate that the adsorption in a dilute protein solution for a short time only has partial coverage of protein molecules on the PDMS surface and could stretch the protein molecule to deform its native conformation due to its strong interaction with the solid surface. This is supported by the AFM images in figure 4.2, which shows that the adsorbed proteins from the 10 ng mL^{-1} (figure.4.2a) solution has less tails and are more stretched than proteins from the 100 and 1000 ng mL^{-1} solutions. However, the adsorption in higher protein concentrations had strong protein-protein interactions to inhibit the stretching effect caused by the interaction with the solid surface, thus tending to keep the native conformation of the proteins and resulting in the sizes of the adsorbed status to be the same as their sizes in the solution. This may indicate that the native conformation of the adsorbed protein could be obtained by an adsorption in a relatively full coverage of adsorbed protein molecules. In addition, the fact that the thickness of the adsorbed layer is equal to the protein size in the solution at the adsorption plateau region may strongly suggest that the adsorbed protein A could only form a monolayer.

Figure 4.2. AFM images of the deposition solution concentration at pH 5 a. 10 ng mL⁻¹, b. 100 ng mL⁻¹, c. 1 μg mL⁻¹ and d. *Rms* plots protein A solution concentration

In this work, a method is employed to quantitatively analyze the adsorption of protein A on a PDMS surface. The bearing volume of a bare PDMS (V_0) and a protein adsorbed PDMS (V_i) in eq. 4.3 can be obtained from the bearing analysis³⁵ to represent the protein adsorption by their difference ($\Delta V = V_i - V_0$). To calculate the adsorbed molecule number on the PDMS from eq. 4.3, the volume of a single molecule (V_{MV}) is needed. It has been proved that V_{MV} of a protein measured by AFM is in good agreement with that calculated from its molecular weight based on eq. 4.2^{168, 170}. The V_{MV} of protein A as calculated from its molecular weight of 45,000 is 85 nm³. The relationship of the adsorbed molecule number on the PDMS surface ($\Gamma_{M,N}$), calculated in terms of eq. 4.3, versus protein concentration is shown in figure 4.3, demonstrating that $\Gamma_{M,N}$ increases with the increase of the protein concentration until a saturation value of $\sim 1.20 \times 10^4$ molecule.μm⁻² forms a plateau at the protein concentrations equal to or larger than 1000 ng mL⁻¹, which is very similar to the relationship of *Rms* vs. protein concentrations in the adsorption process, and this indicates that the maximum adsorption is $\sim 1.20 \times 10^4$ molecule.μm⁻². The effects of the protein

concentration on the adsorptions were also examined at pH 3.0 and 7.0, and similar trends were observed. The characterization of the protein adsorptions using other methods such as attenuated total reflection¹⁷¹, solution-depletion¹⁶⁰, and resonant elastomeric surface-tension¹⁷² were conducted in very high protein concentrations of 0.57-3.04 mg mL⁻¹¹⁷¹, 0.1-0.5 mg mL⁻¹¹⁶⁰, and 0.05-0.2 mg mL⁻¹¹⁷², significantly higher than the range of 1 ng mL⁻¹ to 100 µg.mL⁻¹ used in this work. It is difficult to use these methods to study the adsorption process in relatively dilute protein solutions, particularly in very fast adsorption cases. Thus, the main advantage of AFM measurement for quantitative study of protein adsorption introduced in this work is its ability to study the adsorption process in low protein concentrations of the solution and can be used directly to estimate the absorbed molecule number on a solid surface, obviously due to the high sensitivity of AFM for the surface characterization.

Figure4.3. Surface molecular concentration vs. protein A bulk solution concentration.

4.3.3. Effect of adsorption time

The effects of protein adsorption times were studied in a 10 µg mL⁻¹ protein A solution at pH 5.0 for 0.08, 0.25, 0.50, 0.75, 1, 3, 5, 8, 10, 15, 20, 25, 30 and 60 minutes, respectively, and the measured AFM images (5×2.5 µm, 15 nm gray scale) and AFM section analysis (y-axis: 4.5 nm, x-axis: 800 nm) are shown in figure.4.4, illustrating a relatively stretched and non-molecule networked conformation structure (figure.4.4a) for 0.08 minute adsorption. However, as adsorption times increased, more protein molecules were absorbed, and stronger molecule-molecule interactions made more networked protein molecules

(Figure.4. 4b-h). In the measured section profiles, as shown in the insets below each topography view image in figure.4.4, the peak separation indicates the distance between adsorbed single or networked molecules for different adsorption times while the width of the peak (x-dimension) represents the size of the adsorbed species. The large peak separation in figure.4.4a and b indicates less adsorbed protein molecules on the PDMS surface, while it decreases in figure.4.4c-e due to more adsorbed molecules. Very interestingly, the peak separation in figure 4.4g, h becomes large again, which is likely due to the protein-protein interactions at a high adsorbed surface concentration to form a network. In fact, the AFM images in figure 4.4g and h clearly show the networked protein molecules like a large “molecule” and then strongly support the proposed mechanism. The widths of the peaks in the section profiles of figure 4.4 g and h significantly increase in comparison to the others, further demonstrating that highly concentrated adsorbed protein molecules can interact with each other for a networked, large “molecule”.

Figure 4.4. AFM top view of $10 \mu\text{g mL}^{-1}$ at different time a. 5 second, b. 30second, c.1 minute, d. 5 minute, e.10 minute, f.20 minute, g. 30 minute, and h. 60minute

$\Gamma_{\text{M.N.}}$ calculated from eq.4.3 vs. adsorption times is shown in figure.4.5, in which $\Gamma_{\text{M.N.}}$ increased with the adsorption time until a maximum adsorption of

1.22×10^4 molecule. μm^{-2} is reached at 10 minutes, which is in agreement with that obtained by measuring the relationship of adsorption vs. protein concentration. Dubrovsky et al. developed a Langmuir film of recombinant protein A which was obtained on a poly-organosiloxane-activated silicon and quartz substrate surface¹⁷³, in which the thickness of the adsorbed protein A monolayer was 4 nm, a value very well in agreement with that measured by AFM in this work. The protein surface density of the monolayer measured by microgravimetric measurement⁴¹ is 3.67×10^4 molecule. μm^{-2} , which is larger than that on the PDMS surface measured by the AFM approach developed in our work. The difference between the two methods is understandable. Dubrovsky et al. uses a carboxyl terminus truncated protein A (M.W.32,000) and a thin film is formed by covalent immobilization rather than physical adsorption, which should be larger than that of the adsorbed amount on the PDMS in this work with a larger protein A (M.W.45,000) and using a physical adsorption process. In addition, the use of different substrate surfaces could introduce the different adsorbed amount of the molecules.

Figure4.5. Surface molecular concentration vs. adsorption time

4.4. Conclusion

This work developed a new approach by using AFM to study the conformation and kinetics of the adsorption of protein A on the PDMS surface at a molecular level. Through investigations of the effects of pH, protein concentrations, and contact times on the adsorption, the results show that the conformation of protein A is significantly affected by the pH and adsorbed surface concentrations. Protein

A shows tortuous line shape with adsorption at pH away from isoelectric point (pI) point of protein A (5.0), while displaying dendrite conformation at pH equal to pI of the protein (5.0), which is ascribed to the electrical repulsion effect of charged protein molecules in the solution with pH away from pI . High adsorbed surface concentration of protein A is found to form networks like a large “molecule” from a number of single molecules. The results also reveal that the protein A produces a monolayer film with a maximum adsorption of 1.22×10^4 molecules. μm^{-2} . This work demonstrates that AFM is able to study the kinetics of an adsorption process in relatively low concentrations of proteins due to its high sensitivity.

Chapter 5 Efficient probe immobilization on PDMS for sensitive detection of protein

5.1. Introduction

Surface modification by a silanization reaction has been established in numerous fields of bionanotechnology, especially in the field of biomaterials, where cellular adhesion and proliferation often need to be promoted for better biocompatibility^{107, 174}. To date, the silanization studies have been focused on glass and silicon^{73, 175}. There are a wide range of reaction conditions used to deposit APTES films. Usually, devices fabricated in either glass, fused silica, or silicon (Si/SiO₂) can be conveniently used to explore silane-based surface modification chemistries. In the case of APTES modified glass, the reaction is self-catalyzed by the amine group of APTES. For this case, rapid hydrolysis of APTES produces ethanol and trisilanols. The Si-C bond is hydrolytically stable and the aminopropyl group will not be cleaved, while the transient silanol groups will condense with other silanols to yield aminopropyl-functional resins. Following the trend of APTES deposition on glass or silicon, special treatment could be done on PDMS, such as oxygen plasma, or UV irradiation to generate silanol groups on the PDMS¹⁰⁰. However, energy sources such as plasma⁹⁹, ultraviolet light¹⁰¹, and corona discharges tend to roughen the surface and leads to physical damage to the surface of the PDMS, producing “cracks” or other microstructures on the surface. Therefore, ambient methods are highly desirable. In this study, a simple surface functionalization approach was developed. The surface of the PDMS was functionalized with amine groups by APTES adsorption.

The covalent immobilization of proteins on PDMS has not been carefully investigated for ELISA applications. In this work, the amino-groups grafted on PDMS can be activated by succinic anhydride to import carboxylic groups on the PDMS. The activated PDMS surfaces were explored as solid support for the inhibition competitive ELISA method and the sandwich ELISA method. The topographies of the solid PDMS surfaces with different immobilization approaches were studied by AFM to further characterize the bioconjugation effects. Finally, an APTES-PDMS surface was employed for HLA molecule

detection in two groups, namely, healthy peripheral blood mononuclear cell (PBMC) and malignant dermatoid carcinoma cell.

5.2. Preparation and measurement

5.2.1. PDMS functionalization

Amino-group functionalized PDMS

APTES was used to modify the PDMS in order to introduce amino-groups onto the PDMS. Experimentally, the PDMS surface was exposed to APTES solutions of 75%, 50%, 25%, 10%, and 5% (V/V, APTES/ethanol). The reaction times tested were 1, 5, 10, 15, 30, 45, 60, and 120 minutes. After reaction, the APTES solutions were emptied and the free APTES was washed off by ethanol twice, for 3 minutes each time. After ethanol washing, the APTES treated PDMS was washed by DI water twice, for 2 minutes each time. The PDMS sample was then dried under a nitrogen flow and baked at 100°C in a vacuum oven for 2-4 hours. The APTES treated PDMS was characterized using FTIR (*chapter3, section 3.1.2.1*) and AFM force measurement. Ninhydrin test and fluorescamine assay (*chapter3, section 3.1.2.5*) were conducted to quantify the amino groups on the APTES treated PDMS. In the following sections of this text, the APTES functionalized PDMS is named as NH₂-PDMS.

Carboxylic groups functionalized PDMS

To implant carboxylic groups on the PDMS surface, the APTES modified PDMS was further treated using succinic acid anhydride (SAA). On nucleophilic attack, the anhydride yields one carboxylic acid for every acrylated product. For SAA, a dicarboxylic acid, on reaction with a nucleophile, the ring structure of the anhydride opens, forming the acrylated product modified to contain a newly formed carboxylate group. In the experiment, the PDMS/APTES patches were placed into a 50 mg mL⁻¹ SAA solution, while using 3 M NaOH to keep the solution stable at pH 6.0. The reaction was allowed to take place for 2 hours at room temperature. The patches were then rinsed with a 0.05 M pH 5.0 phosphate-citrate buffer and dried under a nitrogen flow. The SAA treated PDMS was characterized using FTIR and AFM force measurement (*chapter3, section 3.1.2.1*). The surface carboxylic groups were quantified using the toluidine blue

O test (*chapter3, section 3.1.2.5*). In the following sections of this text, the SAA activated APTES-PDMS is named as COOH-PDMS.

5.2.2. Competitive ELISA on PDMS

Carboxylic groups functionalized PDMS were used to prepare the immune-solid phase for competitive ELISA application. To demonstrate competitive ELISA on the COOH functionalized PDMS surface, a covalent probe immobilization approach was used. The experimental conditions are summarized in table 5.1. The competitive ELISA experimental procedure is described in *chapter3, section 3.1.5.1*. The reaction conditions were optimized in terms of probe and enzyme-linked antibody solution concentration, blocking reagent, and competitive reaction time.

Table 5.1. Parameters of competitive ELISAs on PDMS surfaces

solid surfaces	probe immobilization	probe solution concentration	competitive analytes	enzyme-linked antibody
pristine PDMS	passive adsorption	11.2 $\mu\text{g mL}^{-1}$	5.6 $\mu\text{g mL}^{-1}$ to 5.6 pg mL^{-1} in 10 fold dilution	1:20,000
NH ₂ -PDMS		11.2 $\mu\text{g mL}^{-1}$		
COOH-PDMS	covalent immobilization EDC/NHS	1.1 $\mu\text{g mL}^{-1}$		

5.2.3. Sandwich ELISA for gp120 detection

Sandwich ELISA (*chapter3, scheme 3.5*) was conducted to measure gp120 in human serum. To construct the sandwich ELISA system as illustrated in *scheme 3.5*, the immobilized probe was rabbit anti-gp120 antibody, the analyte was the gp120 diluted in human serum, and the enzyme labeled antibody was biotin conjugated goat anti-gp120 antibody. In immune-sorbent phase preparation, pristine PDMS, NH₂-PDMS and COOH-PDMS were exposed to probe solutions. The probe immobilization conditions are listed in table 5.2. Probes were passively adsorbed on pristine PDMS and NH₂-PDMS surfaces. EDC/NHS mediated protein immobilization approach was used to covalently immobilize probes on COOH-PDMS. The analytes, gp120 diluted in human serum ranging

from 1 pg mL^{-1} to $100 \text{ }\mu\text{g mL}^{-1}$, were added on the probe-immobilized surfaces to incubate for an hour. To detect the probe captured gp120, biotin conjugated anti-gp120 antibody was added with the concentration of $2 \text{ }\mu\text{g mL}^{-1}$. Finally, HRP labeled streptavidin ($5 \text{ }\mu\text{g mL}^{-1}$ in TBST) was used to detect biotin conjugated anti-gp120 antibody. As described in *chapter 3, section 3.1.5.1*, the HRP substrate TMB was introduced into the reaction system and the TMB signal was quantified as A_{450} by the ELISA reader.

Table 5.2. Parameters of sandwich ELISAs for gp120 detection

solid surfaces	probe immobilization	anti-gp120 antibody	gp120	biotin-anti gp120
pristine PDMS	passive adsorption	$10 \text{ }\mu\text{g mL}^{-1}$	from $100 \text{ }\mu\text{g mL}^{-1}$ to 1 pg mL^{-1}	$2 \text{ }\mu\text{g mL}^{-1}$
NH ₂ -PDMS		$10 \text{ }\mu\text{g mL}^{-1}$		
COOH-PDMS	covalent immobilization EDC/NHS	$10 \text{ }\mu\text{g mL}^{-1}$	10 fold dilution	

5.2.4. HLA-molecules detection on COOH-PDMS

Dermatoid carcinoma cell

Dermatoid carcinoma cells were cultured in Eagles' medium. After harvesting and washing in sterile PBS, the cell density was adjusted to $1 \times 10^7 \text{ mL}^{-1}$. Sulfo-NHS-S-S Biotin was added to the cell suspension at a concentration of 1 mg mL^{-1} . Cells were incubated at 4°C for 60 minutes for surface protein biotinylation. The cells were then washed three times with cold Tris-HCl, pH 7.4 to quench the reaction and remove the excess NHS. The biotin-labeling cells were lysed at 4°C in PBS with 1% Triton X-100. Lysed cell suspensions were incubated on ice for 15 minutes and then centrifuged at 1000 rpm for 5 minutes at 4°C to remove the intact nuclei. The supernatant was collected for direct ELISA test.

Peripheral blood mononuclear cell isolation

The blood samples from healthy people were centrifuged at 20°C in a horizontal rotor for 25 minutes at 2000 rpm. After centrifugation, mononuclear cells and plates were formed in a whitish layer just under the plasma layer. The cell layer was collected with a pipette and transferred into a 15 mL size centrifuge tube. PBS was used to bring the final volume to 10 mL. After centrifugation for

15 minutes at 1000 rpm, the supernatant was collected by careful aspiration. The cell pellet was then re-suspended by PBS and the wash steps were repeated. The harvested PBMC was put through the biotinylation process to labeling the surface protein, as described in the sections above. The harvested supernatant was collected for ELISA experiments.

ELISA on COOH-PDMS

First, the COOH-PDMS was activated by EDC/NHS mixture as described in chapter 3. Four kinds of protein probes, namely anti-human HLA-I antibody, anti-Human HLA-A2 antibody, anti-HLA-DR antibody and anti- β_2 -microglobulin antibody were then added on the EDC/NHS activated COOH-PDMS. The concentrations of the probes are listed in table 5.3. The cross-linking process was kept for 1 hour at room temperature, and stopped by Tris-HCl buffer washing. The probes coated surfaces were then immersed in a 1% BSA solution to incubate for 2 hours at 37°C to block unoccupied surfaces. The prepared probes immobilized PDMS solid phases were exposed to the collected biotinylated PBMC and dermatoid carcinoma cell supernatants. The captured biotinylation HLA-I molecules reacted with the HRP-labeled streptavidin. Finally, the HRP enzymatically catalyzed the TMB to develop a blue color. The absorbance of the enzymatic substrate retrospectively evaluates the quantity of the HLA-I associated molecules.

Table5.3. Parameters for HLA-molecular detection

HLA-molecular	HLA-I	HLA-A2	HLA-DR	β_{2m}
probe concentrations	anti-HLA-I: 100 μ g mL ⁻¹	anti-HLA-A ₂ 100 μ g mL ⁻¹	anti-HLA-DR 100 μ g mL ⁻¹	anti- β_{2m} 100 μ g mL ⁻¹
Supernatant of PBMC			1:100	
Supernatant of DCC			1:100	

5.3. Results and discussion

5.3.1. Surface characterization of modified PDMS

The PDMS surfaces were characterized using FTIR, contact angle and AFM force curve measurement. The surface density of the functional groups was quantified by fluorescamine assay and a toluidine blue O test.

First, the FTIR spectra of the APTES adsorbed PDMS was recorded (figure

5.2). The spectra recorded on the modified solid surface were always compared to spectra obtained from pristine PMDS (figure 5.2a), in which the peaks at 2964, 1259, 1015, and 851 cm^{-1} should be assigned to -methyl CH, $-\text{CH}_3$, Si-O-Si and Si-C^{124} respectively. The APTES treatment resulted in the presence of a peak at 1650-1560 cm^{-1} which is assigned to -NH. The presence of nitrogen on the surface of APTES-treated PDMS suggests successful chemical grafting of aminosilane to the PDMS surface. Figure 5.2c represents the IR spectra of the SAA activated surface where a significant change appears at 1640-1580 cm^{-1} which indicates the $-\text{C}=\text{O}$ group. IR spectra also show that SAA activation is characterized by a broad band at 3650-3300 cm^{-1} which is assigned to the carboxyl group.

Figure5.2. FTIR spectra from different solid surfaces

Table5.4. Comparison of FTIR spectral in each of the modification steps			
Peak assignment	pristine PDMS	APTES-PDMS	SAA-activated APTES-PDMS
NH_2	—	1563,	-
carboxylic acid -OH	—	—	3450
methyl CH	2970	2973	2973
carboxylic acid $-\text{C}=\text{O}$	—	—	1690-1580
CH_3	1265	1259	1259
Si-O-Si	1015	1015	1015

AFM force measurement was conducted to probe the surfaces chemical groups by measuring the AFM tip-surface interaction in different pH buffers. In the experiments, triangular silicon nitride cantilevers were used with a force constant

of 0.05 N/m. Force measurements were obtained in an open liquid AFM cell (Veeco Instruments, USA).

First, force measurements were conducted on pristine PDMS in aqueous solutions at pH 2.2, 5.0, 7.0, and 9.6 (0.01 M NaCl). For each solution, force measurements were conducted at five randomly selected spots, with 16 curves for each spot. The characteristic retrace curves for each pH condition are presented in figure 5.3a. The force curves in figure 5.3a have similar shapes; they differ in the factor of deflection distance, d , which decreases along with the increase of the solutions' pH value. In the case of the NH_2 -PDMS surface, the deflection distance at pH 5.0 is the largest one (figure 5.3b). For the COOH -PDMS surface, the maximum d is at pH 2.2 (figure 5.3c).

Figure 5.3. Force curves for different PDMS surfaces, a. pristine PDMS, b. NH_2 -PDMS, and c. COOH -PDMS

The adhesive forces between AFM tips and different PDMS surfaces were calculated based on eq. 3.1 (*chapter 3. section 3.1.2.3*). Figure 5.4 shows the adhesive force between the tip and surfaces under different pH conditions. The adhesive force decreases continuously along increasing pH values for the pristine PDMS surface. The decreasing trend can also be observed for the COOH -PDMS and the change tendency is much more significant. However, the force-pH plot shows a volcano-like curve and peaks at pH 5.0. Considering the isoelectrical point of Si_3N_4 tip is pH 5-6, thus at different pH the tip keeps a different charge; it is positively charged when pH is lower than 5.0 and negatively charged when pH is higher than 6.0. However, the NH_2 -PDMS is characterized with IP of NH_2 which is positive at all the measurement pH conditions. Meanwhile, COOH -PDMS is negatively charged at pH values higher than 2.2. The charge

status of the tip and the surfaces affect the electrostatic force between them. It was noticed that the change of adhesive forces for the pristine PDMS is similar to that for the COOH-PDMS. Therefore, groups other than the neutral CH_3 group may exist that display characteristics of the carboxylic group on the pristine PDMS surface. It has been proved, by MS study, that there are Si-OH groups on the pristine PDMS which are helpful in explaining the decrease of adhesive forces in figure 5.4. Therefore, the AFM force measurement provides supplementary chemical information for functionalized surfaces.

Figure5.4. Force curve on APTES-PDMS surface: ($\square$) pristine PDMS, ($\circ$) COOH-PDMS, and (Δ) NH_2 -PDMS

To optimize the APTES adsorption conditions, the APTES concentration and APTES/PDMS reaction time were studied. In the experiments, different concentrations of the APTES solution were prepared by diluting the APTES with ethanol (APTES/ethanol v/v: 75%, 50%, 25%, 10%, and 5%). The APTES/PDMS reaction times were for 120, 60, 50, 40, 30, 20, 10, and 5 minutes. Based on the calibration curve of fluorescamine assay which was presented in *chapter3, figure3.5*, the surface NH_2 are expressed as the surface NH_2 concentration (Γ_{NH_2} , mol cm^{-2}). The Γ_{NH_2} of the PDMS, modified with different reaction conditions, is shown in figure 5.5. It was observed that Γ_{NH_2} increases sharply during the first 30 minutes of the reaction for all APTES concentrations. The Γ_{NH_2} keeps increasing at APTES concentrations of 5% and 10%, and does not reach a maximum value after 2 hours of reaction. The Γ_{NH_2} increases slowly for 30-60 minutes of the reaction time and no increase are observed after 60 minutes of reaction at APTES concentrations of 25% and 50%. For APTES

concentration of 75% APTES, Γ_{NH_2} reaches a maximum value after a 60 minute reaction and decreases slightly for reaction times longer than 60 minutes. In the following experiments to prepare the NH_2 -PDMS, 50% APTES was used to treat the PDMS for 30 minutes.

Figure 5.5. NH_2 surface concentration of PDMS with different APTES adsorption conditions

Based on the results shown in figure 5.5, 5%, 10% and 25% APTES were chosen to investigate APTES adsorption kinetics, since these represent different stages of immobilization versus concentration; further results are shown in figure 5.6. For APTES adsorption on the PDMS surface, a simple and reversible process is proposed:

where A is the APTES in solution, B is the PDMS surface, AB is the adsorbed APTES, k_a is the association rate constant, and k_d is the dissociation rate constant, The AB formation rate can be expressed as follows^{53, 156}:

$$d[AB]/dt = k_a[A][B]_t - k_d[AB]$$

because

$$[B]_t = [B]_0 - [AB]$$

thus,

$$d[AB]/dt = k_a[A]([B]_0 - [AB]) - k_d[AB] \quad [5.1]$$

where $[AB]$ and $[A]$ are the concentrations of AB and A, respectively, and $[B]_0$ represents the concentration of B at time zero. Let fluorescence intensity represents the concentration of complex AB on the film surface and denote as R; therefore,

$$E^* = k_a[A]R_{\max} / (k_a[A] + k_d)$$

and

$$k_s = k_a[A] + k_d \quad [5.2]$$

Equation 5.1 can be further transformed into

$$R_t = E^* (1 - e^{-k_s t}) + R_0 \quad [5.3]$$

where R_0 is the response at $t=0$, E^* is activation energy.

On the basis of eq.5.3, the values of k_s , E^* , and R_0 were calculated by fitting the binding curves of a set of concentrations of the APTES (figure 5.6). The values of k_a and k_d calculated from the fitting equation are $4.45 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ and $2.19 \times 10^{-5} \text{ s}^{-1}$, respectively.

Figure 5.6. APTES adsorption curves: (□) 25% APTES, (○) 10% APTES, and (Δ) 5% APTES

For SAA activation of the NH_2 -PDMS, the SAA concentration and reaction time were optimized. In the experiments, different concentrations of SAA were tested while the activation times were varied. The density of COOH on the functionalized PDMS surface was determined using the toluidine blue method. Based on the calibration curve of toluidine blue method which was presented in chapter 3, figure 3.7, the COOH are expressed as surface COOH concentration (Γ_{COOH} , mol cm^{-2}). The Γ_{NH_2} of SAA activated APTES-PDMS surface was quantified by fluorescamine assay. Figure 5.7a shows the Γ_{NH_2} and Γ_{COOH} of SAA activated 50 % APTES adsorbed PDMS. The figure shows that Γ_{COOH} increases from $\sim 2.5 \times 10^{-8} \text{ mol cm}^{-2}$ to $\sim 5.0 \times 10^{-8} \text{ mol cm}^{-2}$ when the SAA concentration increases from 10 mg mL^{-1} to 50 mg mL^{-1} . There is no significant Γ_{COOH} variation for SAA concentrations higher than 50 mg mL^{-1} (from 50 mg mL^{-1} to 100 mg mL^{-1}). Interestingly, the Γ_{NH_2} on SAA activated PDMS shows a decreasing trend:

the Γ_{NH_2} is $\sim 1.35 \times 10^{-7} \text{ mol cm}^{-2}$ for the 10 mg mL^{-1} SAA treated surface, and $\sim 1.10 \times 10^{-7} \text{ mol cm}^{-2}$ for the 50 mg mL^{-1} SAA treated surface. In the following experiments to prepare the COOH-PDMS, 50 mg mL^{-1} SAA was used to treat APTES-PDMS for 2 hours.

Figure 5.7. Γ_{NH_2} and Γ_{COOH} of SAA activated APTES-PDMS: ($\square$) surface COOH, ($\circ$) surface NH₂

5.3.2. Competitive ELISA on PDMS surfaces

The functionalized PDMS was applied to immunoassays. First, the reaction conditions of the ELISA, such as the blocking reagent conditions, reaction time, probe concentration and enzyme tracer concentration, were optimized. Based on the optimized experiment, a competitive ELISA was successfully conducted for rabbit IgG detection.

Blocking reagent effect

Blocking is one of the important strategies in ELISA and is employed to avoid false positive results by reducing non-specific binding on the un-related proteins. To find out the best blocking effect experimentally, six blocking reagents, 1% BSA, 1% human serum albumin, 1% chicken serum albumin, 0.5% BSA + 0.5 % casein, 3% BSA, and 1% casein were screened by direct ELISA. Experimental results demonstrate that the best blocking capability on a PDMS solid-surface is given by 1% BSA in TBS. It is known that high temperatures accelerate biological interactions, and is often used to reduce the reaction time of biological or chemical reactions. Incubation times for all blocking reagents were tested for 2

hours at 37 °C or overnight at 4 °C respectively. Experimental results demonstrate that both conditions have similar blocking performance. Thus, for reduction of the assay time, the blocking conditions used in all other immunoassay experiments were 1% BSA at 37 °C for 2 hours.

Table5.5. A_{450} at different blocker prepared direct ELISA

blocker T	1%BSA	1%HSA	1%CSA	1%casein	3%BSA	0.5% BSA/casein
37°C, 2h	0.10±0.01	0.16±0.05	0.21±0.03	0.13±0.04	0.10±0.02	0.14±0.03
4°Covernight	0.09±0.01	0.15±0.03	0.20±0.04	0.12±0.02	0.09±0.03	0.13±0.01

Antibody and enzyme tracer concentration

In competitive assays, the lower the concentrations of the antibody and the tracer, the better the sensitivity since the analyte ‘competes’ more effectively when reagent concentrations are low. However, low reagent concentrations give low signals with high variability. Thus the concentrations of the antibody and the tracer should be optimized. The saturating conditions were first determined via a non-competitive chessboard titration method over a wide range of protein concentrations. From the results of the chessboard titration (table 5.4), it can be read that the signal is decreasing along with the decrease in the immobilized antigen concentration. The enzyme labeled antibody concentration is also related to the signal. The optimal antigen immobilization concentration is 11.2 $\mu\text{g mL}^{-1}$, while the enzyme labeled antibody titration is 1:5000.

Table5.6. A_{450} of chessboard titration

Ag \ Ab*	1:2500	1:5000	1:10,000	1:20,000	1:40,000
112 $\mu\text{g mL}^{-1}$	1.89±0.54	1.94±0.82	1.34±0.21	0.82±0.35	0.52±0.21
11.2 $\mu\text{g mL}^{-1}$	1.63±0.86	1.74±0.25	1.37±0.45	1.03±0.85	0.73±0.34
1.12 $\mu\text{g mL}^{-1}$	1.50±0.63	1.30±0.15	0.95±0.85	0.75±0.18	0.56±0.18
112ng mL^{-1}	0.37±0.41	0.33±0.10	0.26±0.58	0.24±0.11	0.23±0.09
11.2ng mL^{-1}	0.11±0.53	0.11±0.05	0.10±0.07	0.10±0.05	0.09±0.05
1.12ng mL^{-1}	0.09±0.06	0.09±0.05	0.08±0.05	0.10±0.03	0.08±0.02
112pg mL^{-1}	0.08±0.08	0.08±0.04	0.08±0.01	0.08±0.02	0.08±0.03
0	0.07±0.02	0.07±0.01	0.07±0.01	0.07±0.02	0.08±0.02

Optimized concentrations in a narrow range were then combined and examined by competitive assays. In all cases, the minimum absorbance (background signal) obtained with excess of analytes was approximately zero. The parameters of the inhibition competitive ELISA are shown in table5.1.

Reaction time

In the competitive ELISA, the influence of the off-spot incubation time of the competitive mixture was first investigated for incubation time effects. The competitive mixtures were incubated for different off-spot times of 0, 10, and 30 minutes, before being added onto the PDMS surfaces. The experimental results show that off-spot incubation times had insignificant effects on the assay results (table 5.7). This indicates that the competition degree is mainly determined by the amount of the competitive protein, and not by the off-spot incubation time.

Table5.7. A_{450} of different off-spot incubation time

$\text{Ag}^c \backslash \text{T}$	0in	10min	30min
56 $\mu\text{g mL}^{-1}$	0.51 $\pm$ 0.04	0.47 $\pm$ 0.04	0.40 $\pm$ 0.02
5.6 $\mu\text{g mL}^{-1}$	0.57 $\pm$ 0.03	0.51 $\pm$ 0.02	0.45 $\pm$ 0.02
560ng mL^{-1}	0.69 $\pm$ 0.05	0.56 $\pm$ 0.03	0.56 $\pm$ 0.03
56ng mL^{-1}	0.77 $\pm$ 0.04	0.71 $\pm$ 0.03	0.67 $\pm$ 0.02
5.6ng mL^{-1}	0.80 $\pm$ 0.05	0.81 $\pm$ 0.05	0.76 $\pm$ 0.03
560pg mL^{-1}	0.85 $\pm$ 0.03	0.86 $\pm$ 0.03	0.86 $\pm$ 0.04
56pg mL^{-1}	0.87 $\pm$ 0.03	0.90 $\pm$ 0.04	0.92 $\pm$ 0.04
0	0.94 $\pm$ 0.03	1.11 $\pm$ 0.03	1.00 $\pm$ 0.03

The second time factor investigated was the time the competitive mixture reacted with the immobilized probes. Reaction times of 15, 30, 45, 60, and 120 minutes were tested. Experimental results show that a 45 minute reaction time had the best performance (table 5.8.).

Table5.8. A_{450} of different Ag-Ab incubation time

$\text{Ag}^c \backslash \text{T}$	15min	30min	45min	60min	120min
56 $\mu\text{g mL}^{-1}$	0.51 $\pm$ 0.04	0.47 $\pm$ 0.04	0.40 $\pm$ 0.02	0.34 $\pm$ 0.03	0.37 $\pm$ 0.02
5.6 $\mu\text{g mL}^{-1}$	0.57 $\pm$ 0.03	0.51 $\pm$ 0.02	0.45 $\pm$ 0.02	0.44 $\pm$ 0.03	0.39 $\pm$ 0.03
560ng mL^{-1}	0.69 $\pm$ 0.05	0.56 $\pm$ 0.03	0.56 $\pm$ 0.03	0.55 $\pm$ 0.02	0.50 $\pm$ 0.02
56ng mL^{-1}	0.77 $\pm$ 0.04	0.71 $\pm$ 0.03	0.67 $\pm$ 0.02	0.69 $\pm$ 0.03	0.65 $\pm$ 0.02
5.6ng mL^{-1}	0.80 $\pm$ 0.05	0.81 $\pm$ 0.05	0.76 $\pm$ 0.03	0.81 $\pm$ 0.02	0.70 $\pm$ 0.02
560pg mL^{-1}	0.85 $\pm$ 0.03	0.86 $\pm$ 0.03	0.86 $\pm$ 0.04	0.85 $\pm$ 0.03	0.87 $\pm$ 0.03
56pg mL^{-1}	0.87 $\pm$ 0.03	0.90 $\pm$ 0.04	0.92 $\pm$ 0.04	0.94 $\pm$ 0.03	0.89 $\pm$ 0.03
0	0.94 $\pm$ 0.03	1.11 $\pm$ 0.03	1.00 $\pm$ 0.03	1.10 $\pm$ 0.02	0.93 $\pm$ 0.02

Competitive ELISA results

Towards construction of the ELISA, covalent methods were used to immobilize the probes on the functionalized PDMS surfaces. In experiments, the EDC/NHS approach was conducted to immobilized probes on

COOH-PDMS; the immobilization reaction is illustrated in chapter 3. Using the optimized immunoassay reaction conditions discussed above, competitive ELISA were carried out to detect rabbit IgG, rat IgG, goat IgG, and human IgA. Figure 5.8 shows the typical dose-response curve obtained from the competitive immunoassay for rabbit IgG detection. In figure 5.8, the curve (a) represents the absorbance changes measured with the rabbit IgG immobilized on pristine PDMS, and curve (b) shows the absorbance changes on NH₂-PDMS. They show that the absorbance values obtained from the immobilization via passive adsorption methods (a, b) are lower than those obtained from bioconjugation via the covalent immobilization method (c), where the immobilization protein concentration employed in the experiments was 1.12 $\mu\text{g mL}^{-1}$, which was 10 fold lower than the concentration used in the passive adsorption method immobilization. The lowest protein that can be distinguished from the test is 5.6 pg mL^{-1} with 27% inhibition.

Figure 5.8. Inhibition competitive ELISA results obtained from protein immobilized (□) pristine PDMS, (○) NH₂-PDMS, and (△) COOH-PDMS

As an indicator of the quantity of the immobilized protein on the solid-surface and the enzyme-linked antibody, the absorbance can be used as the factor to evaluate the efficiency of the immobilization product. Covalent immobilization method, EDC/NHS, surpasses the passive adsorption in inhibition percentage and absorbance aspects. The results obtained with the immunoassay are well in agreement with that observed from AFM topographies for protein immobilization on different PDMS solid surfaces. In the AFM experiment, the samples for characterization were prepared according to the method described in chapter 3.5. 11.2 $\mu\text{g mL}^{-1}$ rabbit IgG solution was used for protein

immobilization on solid surfaces. Figures 5.9 (a) and (b) are the AFM images of protein passively adsorbed onto the pristine PDMS and NH_2 -PDMS surfaces, respectively, while figure 5.9 (c) is the AFM image of protein covalently immobilized onto COOH-PDMS. Figure 5.9 (a) shows the surface after incubation in $11.2 \mu\text{g mL}^{-1}$ rabbit IgG solution for 1 hour at room temperature. Although protein can be observed on the solid surface, significant conglomeration dominates the whole surface morphology. This is mainly due to the highly hydrophobic PDMS surface that was ready to interact with the hydrophobic groups of the protein. The agglutination of the proteins could either denature or impair the function of the PDMS based structure. Figure 5.9 (b) is the result of protein passive adsorption onto a NH_2 -PDMS surface. Apparently, there is no significant protein immobilization observed, indicating poor protein immobilization capability in the direct passive protein adsorption on the APTES-PDMS substrate. In figure 5.9 (c), the morphology characteristics are obviously different from (a) and (b). More protein immobilization can be observed on the solid surfaces in (c), in which case the protein was covalently immobilized on the SAA activated APTES-PDMS surface. Figure 5.9 (c) shows that the immobilized protein is uniformly distributed on the PDMS solid surface. The AFM morphology demonstrates that SAA generated sufficient carboxyl groups to react with the heterobifunctional cross-linker EDC, which could bridge the amino groups of the protein and carboxyl groups on the solid surface for higher and more uniform probe immobilization. AFM based experimental results demonstrate that AFM is a powerful tool for directly investigating biomolecular interactions at the molecular scale.

Figure 5.9. AFM images ($5 \times 5 \mu\text{m}$, 15 nm gray scale) of PDMS (a) protein passively adsorbed on to pristine PDMS surface, (b) protein passively adsorbed onto PDMS-APTES surface, and (c) SAA/EDC immobilized protein (rabbit IgG).

5.3.3. gp120 ELSIA detection

The gp120 is a glycoprotein exposed on the surface of an HIV envelope. The human immunodeficiency virus (HIV) can mutate frequently to stay ahead of the human immune system. Fortunately, there is a highly conserved region in the virus genome near its receptor binding site. The glycoprotein gp120 is anchored to the viral membrane through non-covalent bonds along with gp41, both coming from a cleaved protein, gp160¹⁷⁶. It infects any target cell with a CD4 receptor, particularly the T_H cell, by binding to that receptor. Gp120 analysis is important because immune escape mechanisms have been assigned to the extensive glycosylation of HIV-1 envelope gp120¹⁷⁷. For detection and quantification of gp120, any specific anti-gp120 antibody can be used, provided that it is able to recognize the particular gp120 protein under evaluation and has a high enough affinity. The bound antibody is then detected using an enzyme-labeled anti-antibody of appropriate specificity^{178, 179}.

Towards the construction of a sandwich ELISA for gp120 detection, a covalent method (EDC/NHS) was used to immobilize the probes (anti-gp120 antibody) on a COOH-PDMS surface. For comparison purposes, probes were passively adsorbed on pristine PDMS and NH₂-PDMS (table 5.2). A chessboard titration procedure, similar to that described for competitive ELISA in the previous section, was conducted to find the optimal probe and enzyme-linked antibody solution concentrations. The typical dose-response curve obtained from the sandwich immunoassay for gp120 detection is shown in figure 5.10, where the curve (a) represents the absorbance changes measured with anti-gp120 antibody immobilized on a pristine PDMS, and curve (b) is the absorbance changes on the NH₂-PDMS. They show that the absorbance values obtained from the immobilization by the passive adsorption methods (a, b) are lower than those obtained from bioconjugation via the covalent immobilization method (c). More importantly, the slope of the dose-response curve (c) is significantly larger than the slopes of curves (a) and (b), which means that the sensitivity of the ELISA on the COOH-PDMS is higher than that of passive adsorption based assays on either a pristine PDMS or on the NH₂-PDMS. The lowest detection limit of the assay based on the EDC mediated probe immobilization is 100 pg mL⁻¹; the detection range is over 4 orders of magnitude,

from 100 pg mL^{-1} to 100 ng mL^{-1} .

Figure 5.10. Sandwich ELISA results for gp120 detection Linear fit: ($\square$) pristine PDMS: $y = -0.1 + 0.162x$, $R = 0.986$ ($\circ$) NH_2 -PDMS: $y = -0.623 + 0.208x$, $R = 0.999$ (Δ) COOH -PDMS: $y = 0.019 + 0.0109x$, $R = 0.993$

5.3.4. HLAs detection on COOH -PDMS

The major histocompatibility complex (MHC) is a large genomic region or gene family found in most vertebrates. It plays an important role in the immunosystem, autoimmunity, and reproductive success^{180, 181}. The human leukocyte antigen (HLA) system is the name of the human MHC¹⁸². The immune system uses the HLAs to differentiate self cells and non-self cells^{183, 184}. Studies with a broad panel of monoclonal antibodies (mAbs) in cryopreserved tissue sections indicate that a high frequency of HLA down regulation occurs in human cancer¹⁸⁵. Cervical carcinomas show a 70% reduction in HLA molecules and the loss of HLA-B₄₄ in premalignant lesions is associated with tumor progression^{186, 187}. These data seem to indicate that HLA changes occur early in tumor development, and the selection of HLA-defective tumor clones confers an advantage that is preserved throughout the life of the tumor cells. The assessment of the frequency of HLA class I antigen loss or down regulation in various types of malignancies has biomedical significances for immunological events in their pathogenesis and in their clinical course. In this experiment, four antibodies (anti-human HLA-I antibody, anti-Human HLA-A2 antibody, anti-HLA-DR antibody and anti- β_2 -microglobulin antibody) were immobilized on SAA-activated APTES-PDMS. The absorbance data in figure 5.16 clearly shows that healthy PBMC has a stronger signal compared to malignant dermatoid carcinoma cells.

Figure 5.11. HLA protein detection on SAA-activated PDMS: the left column of each group represents the data from the healthy PBMC, the right column of each group represents the data from dermatoid carcinoma cell.

5.4. Conclusion

A simple adsorption approach was developed to graft APTES onto the PDMS surface. FTIR and AFM force curves proved that APTES adsorption can immobilize amino groups onto PDMS surfaces. SAA was employed to import COOH groups on the APTES-PDMS. The COOH-PDMS surfaces were explored as solid support for inhibition competitive ELISA method and sandwich ELISA. The optimization of immobilized probe concentration and incubation time for sensitive immunoassay was conducted. The detection limit using a simple and efficient protein immobilization method was reached at 5.6 pg mL⁻¹. This detection limit is sensitive and could have wide applications in clinical diagnostics. The topographies of the solid PDMS surfaces under different immobilization approaches were studied by AFM to further characterize the bioconjugation effects. Finally, the APTES-PDMS surface was successfully employed as the solid substrate for HLA molecule detection in two groups, namely healthy PBMC and malignant dermatoid carcinoma cell. The surface immobilization methods reported here could provide a great potential approach to building a PDMS based ELISA lab-on-chip system for clinical diagnostic applications.

However, the APTES functionalized PDMS surface is characterized by a contact angle of 84.7°, which means that the surface is not hydrophilic. The research work should aim to improve the hydrophilicity of the PDMS.

Chapter 6 Poly (vinyl alcohol) functionalized poly (dimethylsiloxane) solid surface for immunoassay

6.1. Introduction

This chapter developed an approach to covalently immobilize poly (vinyl alcohol) (PVA) on PDMS surface to suppress non-specific adsorption. The PVA functionalized PDMS was used towards the development of a solid phase material for sandwich ELISA. In addition, flow-through immunoassays were successfully demonstrated with the detection of HBsAg, HBeAg and α -FP in the PVA-functionalized PDMS microfluidic devices.

Surface modification is generally considered as an applicable method to improve the biocompatibility of a substrate surface⁹⁶. Tethering of polymers to solid surfaces via chemical bonding provides an interesting way to modify the surface properties and is therefore of significant interest in applications such as wettability, nonfouling surface technology, and biomedical science⁹⁸. Polymer coatings designed to reduce protein adsorption have been used to modify a variety of polymeric substrates. Among them, long flexible hydrophilic polymers can provide a beneficial microenvironment for the immobilization of various biological macromolecules. Hydrophilic matrices have been put in use to improve the performance of batch immunoassays like ELISA¹⁸⁸. Poly (vinyl alcohol) or PVA is one of the most hydrophilic polymers with stable chemical properties, good dissolution and strong adhesion power. PVA surface coatings have attracted much interest in recent years. For example, although numerous hydrophilic capillary coatings have been developed for microchip capillary electrophoresis, those based on PVA have proven to exhibit particularly good performance¹⁸⁹⁻¹⁹¹. Although some research have been successfully demonstrated on various hydrophobic polymer surfaces, such as poly- (styrene) and hydrophobic self-assembled monolayer surfaces prepared on silicon, it has been reported that PVA has no obvious adsorption on an original PDMS surface. In addition, it is very difficult to produce homogeneous PVA films on a hydrophobic surface via adsorption. Nevertheless, PVA can be adsorbed on oxygen plasma treated PDMS since the resulting surface possesses a highly hydrophilic layer with hydroxyls, silanols, and carboxylic acids. Hydrogen bonding, instead of hydrophobic interaction, is probably the dominant driving

force for PVA adsorption on such treated PDMS surfaces¹⁹¹.

Hepatitis B virus (HBV) infection is a serious global health problem, with a billion people infected worldwide, and 350 million people suffering from chronic HBV infection. In Asia and most of Africa, chronic HBV infection is common and usually acquired perinatally or in childhood¹⁹². Thus, making a correct diagnosis of HBV infection will be important for hepatitis B therapy and hepatocellular carcinoma prevention. HBV markers which can be used diagnostically to monitor an HBV infection include HBs Ag, HBeAg, IgM anti-HBc, total anti-HBc, anti-HBe and anti HBs. In general, it costs 30-50 Singapore dollars for a set of blood tests. The patients have to wait 6-8 hours to obtain the test results. Thus, it is desirable for microchip-based immunoassays to do the screening test with the faster analysis times, smaller amounts of required samples, and good portability.

The PVA-functionalized PDMS surface was prepared and characterized by AFM and contact angle. Effects of the PVA in ELISA were characterized in terms of protein specific immobilization, non specific bonding and ELISA sensitivity. PVA-functionalized PDMS microfluidic devices were fabricated and characterized for the detection of HBsAg, HBeAg and α -FP in human serum.

6.2. PVA-PDMS preparation and applications

6.2.1. Preparation of PDMS-PVA Solid Substrate

Amino groups on APTES immobilized PDMS solid surface may be reacted with the bis-aldehyde compound glutaraldehyde to form activated derivatives that are able to cross-link with PVA. The reaction process for this modification is shown at figure 6.1.

Figure6.1. PDMS functionalized by aldehyde groups

The PVA conjugation process is illustrated in scheme 6.1. The APTES-PDMS surface were immersed in a 2.5% v/v GA in sodium carbonate buffer (CB) for 1 hour at room temperature to introduce aldehyde groups on the surface, followed by rinsing with DI water. A 10 mg mL⁻¹ aqueous solution of PVA was prepared according to Yakovleva et al⁷³. In brief, 0.3 g PVA was suspended in Milli-Q water and allowed to wet sufficiently. The suspension was heated in a water bath until it became transparent and then the pH was adjusted to 1.0. Finally, the PVA concentration was adjusted to 10 mg mL⁻¹. The 2.5 % GA activated APTES-PDMS was immersed in the PVA solution for 30 minutes at room temperature. The PDMS was rinsed with PBS and dried under a nitrogen flow. In the following sections, the PVA modified PDMS is named as PVA-PDMS.

Scheme 6.1. PVA modify PDMS

6.2.2. Protein passive adsorption

In protein passive adsorption experiments, pristine PDMS and PVA-PDMS were exposed to a 10 mg mL⁻¹ BSA protein solution. After 1 hour passive adsorption at room temperature, the free proteins were removed by careful rinsing. The surface morphology of the protein adsorbed pristine PDMS and PVA-PDMS were imaged by AFM.

In direct ELISA, rabbit IgG in Tris-HCl was directly added to the surface, followed by 1 hour incubation at room temperature. After careful rinsing with Tris-HCl, the solid surface was then incubated with a blocking solution consisting of 1% BSA, 0.5% Tween 20 for 1 hour at room temperature. HRP conjugated anti-rabbit IgG antibody was added for 30 minutes incubation (table 6.1). The antibody-antigen reaction intensity can be quantified by adding in TMB.

The same procedure was conducted on the unmodified PDMS surface as a control experiment.

Table 6.1. direct ELISA on PDMS and PVA-PDMS

solid surfaces	probe immobilization	proteins	antibody*
pristine PDMS PVA-PDMS	passive adsorption	rabbit IgG 11.8 $\mu\text{g mL}^{-1}$	HRP-anti-rabbit IgG: 5 $\mu\text{g mL}^{-1}$

6.2.3. Protein covalent immobilization

PVA-PDMS (described in *section 6.2.1*) was treated with 5% GA (pH 1.0) for 30 minutes to cross-link the PVA polymer matrix layer and introduce aldehyde-groups on the surface. This will be called aldehyde-PVA-PDMS in the following sections of this text. After rinsing with a Tris-HCl buffer, a pH 8, 10 mg mL^{-1} BSA protein solution was added onto the aldehyde-PVA-PDMS surface. The protein immobilization took place at room temperature for 1 hour. Unbound protein was removed from the surface upon tris buffered saline, 0.01M pH 8.0, containing 0.05% detergent Tween 20 (TBST) washing. The protein coated surfaces were dried under nitrogen flow and keep in a dry box before AFM measurement.

6.2.4. Sandwich ELISA Assay

Sandwich ELISA for rabbit IgG detection was conducted on pristine PDMS and aldehyde-PVA-PDMS. To construct the sandwich ELISA system as described in *chapter 3, section 3.1.5.1*, the immobilized probe was goat anti-rabbit IgG antibody, the analyte was a serials concentration of rabbit IgG diluted in TBST, and the enzyme labeled antibody was HRP conjugated anti-goat IgG antibody. Pristine PDMS and aldehyde-PVA-PDMS were exposed to probe solutions. The probe immobilization methods are listed in table 6.2. During the experiment, the target protein, rabbit IgG was gradually diluted ten-fold to produce different concentrations ranging from 0.112 pg mL^{-1} to 11.2 $\mu\text{g mL}^{-1}$. The antigen-antibody reaction time was 1 hour at room temperature. To detect the probe captured rabbit IgG, HRP conjugated anti-rabbit IgG antibody was added with a dilution of 1:5000. As described in *chapter 3, section 3.1.5.1*, the HRP substrate TMB was introduced into the reaction system and the TMB signal was quantified as A_{450} by the ELISA reader.

Table 6.2. Parameters of sandwich ELISAs on PDMS and PVA-PDMS

solid surfaces	probe immobilization	probes	analyte	antibody*
pristine PDMS	passive adsorption	1:2000 diluted	11.2 $\mu\text{g mL}^{-1}$ to 0.112 pg mL^{-1}	1:5000
PVA-PDMS	covalent immobilization	in Tris-HCl	10 fold dilution	

Probes: anti-rabbit IgG, analyte: rabbit IgG, antibody*: HRP-linked anti-rabbit IgG

6.2.5. PVA functionalized channel preparation

A silicon master with parallel microchannels was fabricated (*chapter 3*). PDMS microchannels were replicated from the silicon master and packaged as described in *chapter 3, section 3.1.4.1*. The modification process of the PDMS is summarized in table 6.3. The functional microfluidic device preparation process starts from the APTES treatment. Initially, APTES (50%) was pumped into the micro-channels for 30 minutes incubation before being washed by ethanol, DI water and dry air. The APTES treated microfluidic device was then baked in an oven for 2 hours at 100°C. After rinsing with DI water followed by drying under an air flow, the amino groups of APTES-activated PDMS were reacted in 2.5% v/v GA in a sodium carbonate buffer (CB, pH 9.6) for 1 hour at room temperature to introduce aldehyde groups on the surface. This was followed by rinsing with CB. A 10 mg mL^{-1} aqueous solution of PVA was injected into the 2.5% GA treated microchannels at room temperature for 30 minutes. The microchannels were then washed by flushing with DI water and dried under an air flow. The PVA modified PDMS surface was treated with 5% GA as described previously (*section 6.2.1*).

Table 6.3. Parameters of the PVA functionalized PDMS microfluidic device

	50% APTES	2.5% GA	PVA	5.0% GA	probes
PDMS microchannel	30 min, bake 2h.	60 min	10 mg mL^{-1} 30 min	30 min	antibodies in PBS

6.2.6. Flow-through ELISA: HBV-biomarkers detection

The flow-through ELISA was conducted on the PVA-PDMS microfluidic devices which were treated with 5% GA as described at *section 6.2.3*. The effect of incubation times on probe immobilization and antigen-antibody interaction on the PVA-functionalized microfluidic device was examined by pumping a 10 μg

mL^{-1} HRP-labeled IgG into the channels and then incubating for different time periods. After probe immobilization, the unbounded protein was removed by a flow washing process for 3 minutes. The enzyme substrate, TMB was then pumped into the channels. The peroxidase converted color-substrate TMB was quantified as described in *chapter3, section 3.1.5.1*. To determine the optimal antibody-antigen interaction time, the microchannels were immobilized by IgG using a $10 \mu\text{g mL}^{-1}$ IgG / 0.01 M PBS, followed by blocking with casein for 15 minutes, and the device was then used to detect the absorbance responses after flowing-in of $5 \mu\text{g mL}^{-1}$ anti-IgG into different micro-channels for different incubations times.

The flow-through sandwich ELISA procedure is illustrated in *scheme 3.5*. The optimal incubation time identified in the probe-immobilization studies was applied to attach the antibodies. First, the immobilized probes were diluted with a PBS (pH 7.4, 0.01 M) to produce different concentrations (table 6.4.) The probes were pumped into the microfluidic device to incubate for 20 minutes. After probe immobilization, the unbounded probes were removed by a flow washing process for 3 minutes, while the blocking was performed in the microchannels with casein for a 15 minute incubation period. The analytes (table 6.4) diluted in human serum were pumped into the PVA-PDMS microfluidic device and incubated for 15 minutes followed by a flow-through washing with TBS for 4 minutes. The biotin-labeled antibodies were pumped into the channel for another 15 minutes incubation period followed by a 5 minute flow-through washing process. Then $2 \mu\text{g mL}^{-1}$ HRP-avidin/TBST was pumped into the channels for a 15 minutes reaction followed by a 5 minute flow washing. Finally, TMB was pumped into the microfluidic devices. The peroxidase converted substrates were pumped out into the ELISA plates for absorbance measurement. The data collection and processing procedures are described in *chapter3, section 3.1.5.2*.

Table 6.4. Parameters of sandwich ELISAs on PVA functionalized PDMS microfluidic device

	probes	analytes	antibody*
channels for HBsAg	anti-HBsAg IgG 2 $\mu\text{g mL}^{-1}$	HBsAg 10 $\mu\text{g mL}^{-1}$ to 10 pg mL^{-1}	goat anti-HBsAg 2 $\mu\text{g mL}^{-1}$
channels for HBeAg	anti-HBeAg IgG 5 $\mu\text{g mL}^{-1}$	HBeAg 10 $\mu\text{g mL}^{-1}$ to 10 pg mL^{-1}	mouse anti-HBeAg 2 $\mu\text{g mL}^{-1}$
channels for α -fetal protein	anti- α -FP IgG 12 $\mu\text{g mL}^{-1}$	α -FP 10 $\mu\text{g mL}^{-1}$ to 10 pg mL^{-1}	mouse anti-AFP 2.5 $\mu\text{g mL}^{-1}$
channels for control	—	+	+

6.3. Results and discussion

6.3.1 PVA-functionalized PDMS

In this study, a covalent approach was proposed to immobilize PVA on the PDMS surface to make a hydrophilic surface. FTIR spectrophotometer was employed to survey the surface groups on the PDMS specimens at each of the modification steps. For clarity, spectra of the modified PDMS are always compared to spectra obtained from pristine PMDS (figure 6.2a), of which the peaks at 2964, 1259, 1015, and 851 cm^{-1} are assigned to -methyl CH, -CH₃, Si-O-Si and Si-C¹²⁴. From the spectra obtained from the APTES treated PDMS (figure 6.2b), a new broad band at 3400 to 3000 cm^{-1} , assigned to -NH₂ stretch vibration¹⁹³, can be observed. The presence of primary nitrogen on the surface of the APTES-treated PDMS suggests that aminosilane was successful grafted onto the PDMS surface. New peak at 3550-3300 cm^{-1} is assigned to primary alcohols, -OH^{194,195}, indicating that PVA is grafted onto the APTES-treated PDMS (figure 6.2c). The peaks appearing in the fingerprint region (1410 and 1310 cm^{-1}) are indicative of PVA coating (figure 6.2c). Moreover, the new peak in the region of 1720–1760 cm^{-1} is assigned to the aldehyde group¹⁹⁶, which is the functional group expected to contribute to efficient covalent immobilization of proteins for highly sensitive immunoassays.

Figure 6.2. FTIR spectra a. Pristine PDMS, b. APTES silanized PDMS, and c. PVA-conjugated PDMS

Contact angle measurement was performed on PDMS specimens. For comparison purposes, the pristine PDMS substrates were measured as control data. The average contact angle value was 107.8° . The PVA layer was characterized by a decreased contact angle value, 37.5° (figure 6.3). This result indicates that the hydrophilicity of PDMS can be enhanced by PVA immobilization.

Figure 6.3. Contact angle measurement on solid surface a. pristine PDMS and b. PVA-functionalized PDMS

AFM measurement was used to image the PVA coated surface after different reaction times. The measurements were carried out after 10, 20, 30, 40, 50 and 60 minutes of reaction time. There are some bright spots can be observed at a 10 minute reaction (figure 6.4a). At the image of 20 minutes reaction time (figure 6.4b), some circles can be observed and the bright spots disappear. More circles can be observed at the image of 30 minutes reaction time and the diameter of the circle decrease (figure 6.4c). When comparing the images from the 30 minute and the 40 minute reactions, No significant differences can be observed between the images of the 30 minute (figure 6.4 d) and the 40 minute reactions (figure 6.4.e), but as reaction time reaches the 50 minute mark, the distribution of circles

becomes much denser (figure 6.4e). The smaller pictures inserted in figure 6.4 are $1 \times 1 \mu\text{m}$ AFM phase images at different PVA immobilization times. The phase images can be observed to gradually change along with an increase in the immobilization times. The surface roughness (R_{ms}) of each image was calculated based on eq. 3.2 (Chapter3. section 3.1.2.3). R_{ms} changes introduced by PVA immobilization are plotted in figure 6.5, showing that the R_{ms} first has a decreasing trend as conjugation time increases. The R_{ms} value starts to increase slightly after 30 minutes immobilization. It is likely that initial orientation of PVA polymer immediately following immobilization ($t = 10$ minutes) induces a rougher surface. Rearrangement of the immobilized PVA polymer ($t = 20$ -40 minutes) will results in flatter and denser layer of the PVA on the glutaraldehyde activated PDMS surface. In the following experiments, the PVA covalent immobilization time was 30min.

Figure6.4. AFM images ($5 \times 5 \mu\text{m}$, 25 nm gray scale; inset: phase images, $1 \times 1 \mu\text{m}$, 20° gray scale) of PVA modified PDMS surface at different PVA conjugation times a. 10 minutes, b. 20 minutes, c. 30 minutes, d. 40 minutes, e. 50 minutes, and f. 60 minutes

Figure6.5. Surface roughness of PVA modified PDMS surface morphology at different PVA conjugation times

6.3.2. Protein repelling capability

The protein repelling capabilities of PVA functionalized PDMSs were demonstrated using different techniques. First, AFM was used to characterize the surface morphology changes after protein adsorption. Since AFM images can provide direct information about the morphological changes on the solid surface, it was able to deduce the protein adsorption intensities from the comparisons between the surface morphology before and after protein adsorption. As discussed in the previous section, the morphology of the PDMS changed from a flat feature to a surface dominated by circles after the treatment of PVA (figure 6.6a, b). Regarding the protein passive adsorption, only an insignificant amount of bovine serum albumin (BSA) was adsorbed by the PVA modified PDMS surface (figure 6.6c). In contrast, the unmodified PDMS exhibited a drastic change in its morphology after its exposure to BSA solution, indicative of non-specific adsorption via hydrophobic-hydrophobic interaction (figure 6.6d). Such data demonstrate that the modification of PDMS with PVA suppresses protein adsorption and reduces non-specific bonding. Rafati et al. studied the interactions of a homologous series of cationic surfactants with BSA and reported that hydrophobic interactions played an important role in the BSA-surfactant binding, whereas electrostatic interactions played only a minor one.

Figure 6.6. AFM images ($5 \times 5 \mu\text{m}$, 15 nm gray scale): a. Pristine PDMS, b. after modification with PVA, c. 10 mg mL^{-1} BSA passive adsorption on PVA modified surface, and d. 10 mg mL^{-1} BSA passive adsorption on pristine PDMS

To illustrate the passive adsorption process of IgG onto the PVA-PDMS, AFM images were taken at 30, 60 and 120 minutes of the protein adsorption process. The presented 3-D AFM images (figure 6.7) show that there are slightly protein on the PVA-PDMS surface which confirms that the PVA modification can indeed improve the PDMS protein repelling capability (figure 6.7).

Figure 6.7. 3-D topographical changes of PVA modified PDMS solid-surface at different protein passive adsorption times: a. 10 minutes, b. 30 minutes, and c. 120 minutes

The second assay used to demonstrate the protein repelling capability was direct immunoassay. During the experiment, probes were allowed to passively adsorb onto pristine PDMS and PVA-PDMS. In principle, the more the probes immobilized, the more the enzyme labeled analytes could be captured, consequently resulting in a high signal in the end. The ELISA results clearly show a high signal from the protein passive adsorption onto pristine PDMS (figure 6.8). A low ELISA signal means that fewer probes were attached onto the solid-phase. The ELISA data further proves that PVA-coating can improve the protein repelling performance.

Figure 6.8. direct ELISA on pristine PDMS and PVA-modified PDMS

6.3.3. Protein covalent immobilization

6.3.3.1. Protein covalent immobilization

To study the protein covalent immobilization on PVA- PDMS, the PVA modified PDMS surfaces were reacted with one aldehyde group of glutaraldehyde whereas a second aldehyde became available for coupling with immunoglobulin or BSA. It should be noted that glutaraldehyde might cross-link

two adjacent PVA chains or even react with two hydroxyls of the same PVA molecule. Therefore, the reaction was performed with excess glutaraldehyde to ensure the availability of abundant aldehydes for the subsequent bimolecular attachment. As shown in figure 6.9, BSA (67 kDa), represented by small dots with an average diameter of 100 nm, is distributed rather evenly on the surface. Compare this to the covalent bonding of IgM (900 kDa), a kind of protein having a higher molecular weight than BSA, which results in much larger circles; presumably the proteins were linked to the PVA backbone (figure 6.9b).

Figure 6.9. Different molecular weight protein covalently immobilized on aldehyde-PVA-PDMS solid-phase (2 μm $\times$ 2 μm , 15 nm gray scale) a. BSA, and b. IgM

6.3.3.2. Protein immobilization kinetics

AFM morphological changes were then used to monitor the kinetics of protein immobilization. Two solid surfaces for protein (rabbit IgG, 112 $\mu\text{g mL}^{-1}$) immobilization were studied. They were aldehyde-PVA-PDMS and pristine PDMS surface. The surface morphologies were recorded at 3 time intervals: 0, 10, and 30 minutes. The washing steps helped to distinguish passive agglutination and solid immobilization. Figure 6.10 shows that the protein adsorption intensities of pristine PDMS surfaces (figure 6.10 a-c) are much lower than the covalent immobilization on aldehyde-PVA-PDMS (figure 6.10 d-e), where the surface protein coverage is significantly increased at the 30 minute interval. The more significant difference was that the distribution of the immobilized protein on the aldehyde activated PVA-PDMS surface is much more uniform than that on the pristine PDMS. From this comparison (figure 6.10), it is safe to say that the PVA-PDMS surface has a high efficiency to sit specific immobilized proteins. This is good for preparation of efficient ELISA solid phases.

Figure 6.10. 3-D AFM images of proteins on pristine PDMS and PVA-PDMS: a. pristine PDMS, b. after 10 min of incubation with rabbit IgG, c. after 30 min of incubation with rabbit IgG, d. PVA modified PDMS, e. 10 min after covalent bonding with rabbit IgG, and f. 30 min after covalent bonding with rabbit IgG. In all experiments, the amount of IgG was $112 \mu\text{g mL}^{-1}$.

6.3.4. Sandwich ELISA

A sandwich immunoassay was then conducted using the PVA-PDMS to anchor anti-rabbit IgG via the glutaraldehyde activated surface for the detection of rabbit IgG. HRP conjugated anti-rabbit IgG formed a tertiary complex with the anti IgG-IgG pair and was detected spectrophotometrically. The detection range of the PVA modified PDMS substrate was 1.12 pg mL^{-1} to 11.2 ng mL^{-1} (figure 6.11), which was significantly wider than the detection range of the unmodified PDMS surface (11.2 pg mL^{-1} to 11.2 ng mL^{-1}). The detection limit was also much lower than the reported value of 15 ng mL^{-1} immunoglobulin for non-PVA treated PDMS¹⁹⁷. Together with AFM imaging, our results revealed that the PVA modified substrate provided uniformly distributed aldehyde groups on the backbone of the PVA. High densities of such functional groups, coupled with the hydrophilic property of PVA, led to the immobilization of a high amount of antibodies, i.e., improved detection sensitivity as well as reduced false positive responses. The tendency to adsorb the protein non-specifically by unmodified PDMS resulted in a higher detection limit. Such results were not completely unexpected since the binding sites of the protein/antibody are mostly located in the hydrophobic domain and are no longer available for target binding because of their preferential association with the PDMS.

Figure 6.11. Sandwich ELISA results on PVA modified PDMS and pristine PDMS

Linear fit: (□) pristine PDMS: $y = 8.021E - 4 + 0.271x$, $R = 0.976$

(○) PVA-PDMS: $y = 0.027 + 0.257x$, $R = 0.989$

6.3.5. Flow-through ELISA for HBV-biomarker detection

PVA-functionalized PDMS ELISA microfluidic devices were fabricated to study the protein immobilization and antibody-antigen interaction in its microchannels and further explore its application in multiple flow-through detection of Hepatitis B related biomarkers. The device fabrication and different protein immobilizations in the microchannels were conducted according to the procedure presented in *section 6.2.6*. The immobilization of proteins, including incubation and the subsequent washing step, in the microchannels of the device was conducted in a flow-through mode as well.

The effects of incubation time on the protein immobilization in the PVA-functionalized PDMS microfluidic device were investigated. The absorbance results in figure 6.12 show that the highest signal is obtained from protein with a 20 minute immobilization and the absorbance signal produced in the microfluidic device reaches a plateau with 15 minutes of antibody-antigen interaction.

Figure 6.12. Absorbance signal at different antibody immobilization times. Insert figure: absorbance signal at different antigen-antibody interaction times

Compare with sandwich ELISA conducting at 96-well microtiter plate, the flow-through sandwich ELISA is more cost-effective. The reagent consumption and assay times of each step were compared in Table 6.5. It can be seen that the consumption of reagents (the most expensive ones are antigen and antibodies) on the microfluidic device was 30 μ L, which is one tenth that of the 96-well microtiter plate (300 μ L). The assay time on the microfluidic device was a little more than an hour, which is also about one tenth of that required for the microtiter plate. It is expected that with further improvement the reagent consumption can be reduced to few microliter for each step and each assay can be finished within half an hour.

Table 6.5. ELISA comparison between a 96-well microtiter plate and a microfluidic device

	reagent volume (μ L)		incubation time (min)	
	96-well	*microfluidic	96-well	*microfluidic
probe immobilization	100	10	60	20
blocking protein	150	10	60-120	20
analytes	100	10	60	15
the second antibody	100	10	45-60	15
substrate	100	10	10-30	1-5
total	550	50	235-330	75

Flow-through sandwich ELISAs were carried out in the PVA-functionalized microfluidic devices for detection of HBsAg, HBeAg and α -FP, biomarkers related to Hepatitis B.

First, the assay was performed to test alpha-fetoprotein (α -FP), a main tumor marker used to monitor testicular cancer and teratoma in any location. α -FP is a glycoprotein that is normally produced by the fetal yolk sac, the fetal gastrointestinal tract, and eventually by the fetal liver. For α -FP detection, antibodies were immobilized onto the PVA-functionalized PDMS channels. The collected enzyme converted substrates were measured for absorbance value and the results are listed in figure 6.13a. The adsorbance calibration curve shows a linear signal/dose relationship with a detection limit of 10 pg mL^{-1} , a dynamic range of 5 orders of magnitude and a saturation value of 100 ng mL^{-1} . To record the colorimetric results, the channels filled with TMB were captured by CCD camera at 5 minutes reaction time. From the resumed CCD images (figure 6.13b, insert), no color is observed in the channels with detection of negative control (background). However, a blue color can be distinguished clearly in the channels with detection of a concentration equal to or larger than 10 pg mL^{-1} . Furthermore, the CCD images were quantified by means of RGB brightness based on the color red, green, and blue (Nikon ACT-2U version 1.3), where $S_{RGB} = 0.229 \times \text{red} + 0.587 \times \text{green} + 0.114 \times \text{blue}^{140}$. The S_{RGB} calibration curves of IL-5 detection shows a detection limit of 10 pg mL^{-1} , and a dynamic range of 5 orders of magnitude (figure 6.13b), which are in good agreement with that measured with the ELISA plate reader.

Figure 6.13. Dose response curve for α -FP flow-through ELISA

a. Absorbance data, linear fit $y = 0.156 + 0.154x$, $R = 0.992$

b. S_{RGB} data, inserted images: α -FP detection: Enzymatic converted substrate in different channels (the channel width is $250 \mu\text{m}$). From left to right, the reaction analytes concentrations were: 0 , 10 pg mL^{-1} , 100 pg mL^{-1} , 1 ng mL^{-1} , 10 ng mL^{-1} , 100 ng mL^{-1} , $1 \mu\text{g mL}^{-1}$, and $10 \mu\text{g mL}^{-1}$; anti- α -FP immobilization time (t_{immob}): 20 min .

The presence of HBeAg indicates that an individual is of higher infectivity, and seroconversion to anti-HBe correlates with reduced infectivity. In an acute infection, it suggests that the infected person is progressing towards resolution of their infection. Therefore, HBeAg detection has medical significance in disease diagnoses, therapy and prognosis¹⁹⁸. For HBeAg flow-through ELISA detection, a calibration curve as shown in figure 6.14a reveals a dynamic range of 4 orders of magnitude (100 pg mL^{-1} to 100 ng mL^{-1}), a detection limit of 100 pg mL^{-1} for HBeAg, and a saturation detected concentration of 100 ng mL^{-1} . The same colorimetric analysis process used in the α -FP study was repeated to analyze the CCD images. The S_{RGB} calibration curve shows a dynamic range of a broad 6 orders of magnitude (100 pg mL^{-1} to $10 \text{ } \mu\text{g mL}^{-1}$), and a detection limit of 100 pg mL^{-1} .

Figure 6.14. Dose response curve for HBeAg flow-through ELISA

a. Absorbance data linear fit: $y = 0.344 + 0.149x$, $R = 0.997$

b. S_{RGB} data, insert images: enzymatic converted substrate in different channel (the channel width is $250 \mu\text{m}$) From left to right, the reaction analytes concentrations were: 0 , 10 pg mL^{-1} , 100 pg mL^{-1} , 1 ng mL^{-1} , 10 ng mL^{-1} , 100 ng mL^{-1} , $1 \text{ } \mu\text{g mL}^{-1}$, and $10 \text{ } \mu\text{g mL}^{-1}$; anti-HBeAg immobilization time (t_{immob}): 20 min .

The HBsAg is typically a qualitative serological marker for diagnosing an ongoing HBV infection¹⁹⁹. PVA-functionalized PDMS microfluidic devices were fabricated for use in HBsAg detection. The absorbance calibration curve obtained for detection of HBsAg is presented in figure 6.15a, showing a detection limit of 100 pg mL^{-1} , a dynamic range of 4 orders of magnitude, and a saturation value of 100 ng mL^{-1} . The S_{RGB} calibration curves of HBsAg detection shows a detection limit of 100 pg mL^{-1} , and a dynamic range of 5 orders of

magnitude (figure 6.15b).

Figure 6.15. Dose response curve for HBsAg flow-through ELISA:

a. Absorbance data linear fit. $y = -0.062 + 0.233x$, $R = 0.909$

b. S_{GRB} data, insert images: enzymatic converted substrate in different channels (the channel width is 250 μm). From left to right, the reaction analytes concentrations were: 0, 10 pg mL⁻¹, 100 pg mL⁻¹, 1 ng mL⁻¹, 10 ng mL⁻¹, 100 ng mL⁻¹, 1 μg mL⁻¹, and 10 μg mL⁻¹; anti-HBsAg immobilization time (t_{immob}): 20 min.

The limit of detection (LOD) of HBsAg, HBeAg and α-FP on PVA-functionalized PDMS microfluidic devices was compared to some commercial ELISA kits. The flow-through ELISA microdevices possess a detection limit of 10 pg mL⁻¹ (α-FP) and 100 pg mL⁻¹ (HBsAg, HBeAg), which are better or comparable to the LOD of the commercial ELISA kits as listed in their product data sheet (Table 6.6.). The analysis of these biomarkers demonstrates that the microfluidic ELISA system has the comparable performance as the conventional ELISA method, and has advantages such as decreased reagent consumption, shorter assay time and point-of-care detection over the conventional method.

Table 6.6. Limit of detection (LOD) of some commercial 96-well plate sandwich ELISA kit

sandwich ELISA	analytical LOD	company
α -fetal protein	348 pg mL ⁻¹	IBL immuno Biological Laboratories
	348 pg mL ⁻¹	Calbiotech, Inc
	700 pg mL ⁻¹	Diagnostic System; Laboratories Inc
	312 pg mL ⁻¹	R&D System
HBsAg	500 pg mL ⁻¹	Atlaslink Biotech Co.Ltd
	200 pg mL ⁻¹	Shantest TM
	39 pg mL ⁻¹	Biocare Diagnostic Ltd
	200 pg mL ⁻¹	DiaColon Tech.
HBeAg	78 pg mL ⁻¹	Usclife TM
	200 pg mL ⁻¹	DiaColon Tech.

It is promising to find that the results of S_{RGB} are in good agreement with that measured with the ELISA plate reader, demonstrating that the colorimetric detection can be used for qualitative screening of positive and negative assays, then providing potential to fabricate portable immunoassay devices for health point of care and infectious diseases screening.

6.4. Conclusion

In brief, this chapter describes a simple but effective technique to suppress non-specific adsorption; this can be used towards the development of a solid phase material for immunoassays. Biomolecules can be covalently immobilized to the PVA-functionalized PDMS surface in an even distribution. The application of the PVA functionalized surface in sandwich ELISAs results in excellent detection sensitivity (1 pg mL⁻¹) and linearity of up to 10 ng mL⁻¹ with negligible non-specific protein adsorption. Flow-through immunoassays were successfully demonstrated with the detection of HBsAg, HBeAg and α -FP in the PVA-functionalized PDMS microfluidic devices. The flow-through ELISA microdevices possess a detection limit of 10 pg mL⁻¹, a dynamic range of 4 orders of magnitude, and a saturation value of 100 ng mL⁻¹ for detection of α -FP, HBsAg and HBeAg in human serum.

Chapter 7 Sensitive Dextran flow-through functionalized poly-(dimethylsiloxane) microfluidic ELISA devices

7.1. Introduction

In this chapter, a dextran modified PDMS microfluidic ELISA device was fabricated. The dextran functionalization was conducted with a simple, economic and fast flow-through process in a fabricated PDMS microfluidic device, and the device was used to simultaneously detect multiple important biomarker IL-5, HBsAg, and rabbit IgG.

Infectious diseases can be caused by various pathogenic microorganisms, such as bacteria, viruses, parasites or fungi. The clinical diagnoses of infectious diseases often requires the use of different and tedious, labor-intensive tests for the screening of various possibilities via immunoassays such as ELISA¹⁷ (Enzyme-linked immuno-sorbent assay), immunofluorescence¹⁸, western blot¹⁹ and immunodiffusion²⁰, of which ELISA is the most important method. Advances in microfluidics and MEMS technologies could provide an economical, high throughput and real-time diagnostic ELISA tool²¹. In general, the successful construction of a microfluidic immunosensor involves a number of basic aspects, which includes the selection of substrate materials, microfabrication methods, probe immobilization and detection.

Dextran has been investigated as a biocompatible polymer for cell repelling biomaterial coatings. Immobilization of dextran is a less toxic and milder process, involving conversion of glucose residues to hemiacetal structures via periodate oxidation^{200,119}. In this work, PDMS microchannels were functionalized with dextran solution via a flow-through process to enhance the hydrophilicity and further achieve higher immobilization efficiency of proteins through its abundant hydroxyl groups. IL-5 is predominantly produced by CD4⁺ T cells²² and regulates growth, differentiation, activation, and survival of eosinophils²³; it is a marker of disease activity and treatment efficacy in bronchial asthma²⁴. Hepatitis B virus (HBV) infection is a major cause of chronic hepatic damage and of hepatocellular carcinomas worldwide²⁰¹; it can be diagnosed by HBsAg, a qualitative serological biomarker for an ongoing HBV infection¹⁹⁹. IgG can serve as a specific marker for neuromyelitis optica, an inflammatory demyelinating

disease²⁵. Herein, a novel portable dextran-functionalized PDMS ELISA microfluidic device was designed and fabricated to simultaneously and colorimetrically detect IL-5, HBsAg and IgG in human serum using the naked eye. To confirm the accuracy, the device was also characterized by absorbance for its sensitivity and specificity.

7.2. Preparation and measurement

7.2.1. Dextran functionalized PDMS

The dextran coating process is illustrated in scheme 7.1. In general, the polymer coating experiment consists of three major steps. First, 0.475 g dextran was dissolved in 10 mL deionized water to obtain the solution used in the functionalization. Sodium periodate (NaIO_4 , 0.232g) was added into the dextran solution. The NaIO_4 /dextran mixture was stirred overnight for complete oxidation of the dextran by the periodate to produce aldehyde groups. The dextran solution after the oxidation process was immediately used for PDMS modification.

The PDMS surface was first oxidized in a 5 minute reaction by a mixture consisting of an oxidative solution, hydrochloric acid and hydrogen peroxide ($\text{H}_2\text{O}/37\% \text{HCl}/30\% \text{H}_2\text{O}_2$ in a volume ratio of 5:1:1), followed by rinsing with DI water and absolute ethanol washing. The acid treated PDMS surface was then treated with APTES in ethanol (50%, v/v) for 30 minutes. The excess APTES was removed by ethanol and DI water washing. The freshly prepared oxidized dextran solution was added onto the APTES-PDMS surface for an hour to allow a conjugation reaction to occur between aldehydes of the oxidized dextran and amines of the APTES on the PDMS surface. The un-reacted dextran was removed by DI water washing. The grafted dextran was further oxidized by a NaIO_4 solution (0.1 M) for 2 hours. The excess NaIO_4 was then washed away with DI water. The PDMS surface was finally functionalized by the oxidized dextran for probe protein immobilization.

Scheme 7.1. Dextran modification process of PDMS surface PDMS oxidized by $\text{HCl}/\text{H}_2\text{O}_2$; the silanization reaction was carried out to modify the PDMS Dextran oxidation: dextran was oxidized by NaIO_4

7.2.2. Sandwich ELISA on dextran-PDMS

Sandwich ELISA for rabbit IgG detection was conducted on pristine PDMS and dextran-PDMS. To construct the sandwich ELISA system illustrated in *chapter 3*, the immobilized probe was goat anti-rabbit IgG antibody, the analyte was a serials concentration of rabbit IgG diluted in TBST, and the enzyme labeled antibody was HRP conjugated anti-goat IgG antibody. In immuno-sorbent phase preparation, both the pristine PDMS and dextran-PDMS were exposed to probe solutions. The probe immobilization methods are listed in table 7.1. During the experiment, the target protein, rabbit IgG was diluted 10-fold into different concentrations ranging from 0.112 pg mL^{-1} to $11.2 \text{ } \mu\text{g mL}^{-1}$. To detect the probe captured rabbit IgG, HRP conjugated anti-rabbit IgG antibody was added with a dilution of 1:5000. As described in *chapter 3*, the HRP substrate TMB was introduced into the reaction system and the TMB signal was quantified as A_{450} by ELISA reader.

Table 7.1. Parameters of sandwich ELISAs on PDMS and dextran-PDMS

solid surfaces	probe immobilization	probes	analyte	antibody*
pristine PDMS	passive adsorption	1:2000 diluted	11.2 $\mu\text{g mL}^{-1}$ to 0.112 pg mL^{-1} 10 fold dilution	1:5000
dextran-PDMS	covalent immobilization	in Tris-HCl		

probes: anti-rabbit IgG; analyte: rabbit IgG; antibody*: HRP-linked anti-rabbit IgG

7.2.3. Dextran-flow-through functionalization of PDMS microchannels

A silicon master with parallel microchannels was fabricated (*chapter 3*). PDMS microchannels were replicated from the silicon master and packaged as described in *chapter 3, section 3.1.4.1*. The modification process of the PDMS is summarized in table 7.2. The PDMS microchannels were first oxidized by pumping a mixture of an oxidative solution, hydrochloric acid and hydrogen peroxide ($\text{H}_2\text{O}/\text{HCl}/\text{H}_2\text{O}_2$ in a volume ratio of 5:1:1) into the channels for 5 minutes, followed by rinsing with DI water flow washing for 10 minutes and by absolute ethanol flow washing for 5 minutes. APTES in ethanol (50%, v/v) was then pumped into the microchannels for 30 minutes pretreatment. The excess APTES was removed by ethanol flow washing for 3 minutes followed by DI water flow-through washing for 6 minutes. The freshly prepared oxidized dextran solution was pumped into the APTES treated PDMS channels for an hour to allow a conjugation reaction between aldehydes of the oxidized dextran and amines of APTES on the PDMS surface to occur. The un-reacted dextran was removed by DI water flow washing for 5 minutes. The grafted dextran was further oxidized by injecting NaIO_4 (0.1 M) for a 2 hour treatment. The excess NaIO_4 was then washed away by DI water flow for 5 minutes. The channels were finally functionalized by oxidized dextran for probe protein immobilization.

Table 7.2. Parameters of dextran functionalize PDMS microchannel

	HCl/H ₂ O/H ₂ O ₂	APTES	Oxidized dextran	NaIO ₄	probes
PDMS Micro-channels	5min	50% 30 minute	5mM 1 hour	0.1M 2 hour	antibodies in PBS

7.2.4 Enzyme labeled anti-IL-5 antibody preparation

HRP-conjugated antibody was prepared by modified sodium periodate method. Monoclonal anti-IL-5 antibody (McAb Clone TRFK 5) was labeled with HRP. The 5 mg high purity ($R_z \geq 3.0$) HRP was dissolved in 1 ml. DI water. 0.2 ml. 0.1 M sodium periodate (NaIO_4) was added into the HRP solution. The mixture was stirred for 20 minutes at room temperature. After being dialyzed against acetate acid buffer (pH 4.4, 1 mM) overnight at 4°C, the oxidized HRP solution was used to label Mc Ab (Clone TRFK5). Before conjugation, a 20 μL 0.2 M pH 9.5 carbonate-bicarbonate buffer was used, dropwise, to raise the pH of the HRP solution to 9.0. Consequently, 1.6 mg antibody (Clone TRFK5) in 0.2 ml. CB buffer was mixed with the treated HRP solution and gently stirred at room temperature for 2 hours. To stop the conjugation process, freshly prepared 0.05 mL 4 mg mL^{-1} sodium tetraborate (NaBH_4) was added into the HRP-IgG mixture, and left for 2 hours at 4°C. To purify the HRP conjugated antibody, the HRP-IgG mixture was dialyzed against PBS overnight at 4°C. Finally, the HRP conjugated antibody was harvested and characterized by UV spectrophotometer. Sandwich ELISA was used to determine the valency of the labeled antibody.

7.2.5 Flow-through ELISA of the dextran-functionalized PDMS device

The effect of incubation times on probe immobilization and antigen-antibody interaction on the dextran-functionalized microfluidic device was examined by pumping a 10 $\mu\text{g mL}^{-1}$ HRP-labeled IgG into the channels and then incubating for different time periods. After probe immobilization, the unbounded protein was removed by a flow washing process for 3 minutes. The enzyme substrate, TMB was then pumped into the channels. The peroxidase converted color-substrate TMB was quantified as described in *chapter3, section 3.1.5.1*. To determine the optimal antibody-antigen interaction time, the microchannels were immobilized by IgG using a 10 $\mu\text{g mL}^{-1}$ IgG / 0.01 M PBS, followed by blocking with casein for 15 minutes, and the device was then used to detect the absorbance responses after flowing-in of 5 $\mu\text{g mL}^{-1}$ anti-IgG into different micro-channels for different incubations times.

Micro-channels modified by dextran were used in flow-through sandwich ELISA (*scheme 3.5*). The reagent used in each step was summarized in table 7.3.

The calibration of IL-5 was measured. A $10 \mu\text{g mL}^{-1}$ anti-IL-5 monoclonal antibody (clone BCA4) was pumped into different channels for a 15 minute incubation to attach the probes; this was followed by flow washing with TBST for 3 minutes to remove the unbounded proteins. Casein was then pumped into the device for a 20 minute incubation to block the possible non-specific bindings. The analytes, IL-5 diluted in human serum ranging from 100 pg mL^{-1} to $10 \mu\text{g mL}^{-1}$, was pumped into the device and incubated for 15 minutes followed by flow-through washing with TBST for 4 minutes. The HRP labeled monoclonal anti-IL-5 antibodies were then pumped into the channels for a 15 minute incubation followed by a 5 minute flow-through washing. Finally, the enzyme substrate, TMB was pumped into the microfluidic channels to react with the captured HRP for colorimetric detection (read by the naked eye). The peroxidase catalyzes the conversion of the substrate TMB into a chromophore. The signal collection and processing procedure was described in *chapter 3*.

Table 7.3. Parameters of sandwich ELISAs on dextran-PDMS microfluidic device

	probes	analytes	antibody*
dextran-PDMS microchannels	anti-IL-5 IgG $10 \mu\text{g mL}^{-1}$	IL-5 $100 \mu\text{g mL}^{-1}$ to 1 pg mL^{-1}	anti-IL-5 (HRP) $5 \mu\text{g mL}^{-1}$
negative controls	—	+	+

The diagnoses of multiple proteins with the flow-through ELISA device was demonstrated by detection of important biomarkers IL-5, hepatitis B virus surface antigen (HBsAg), and IgG in different microchannels. The immobilization of the probe proteins, anti-IL-5 monoclonal antibody, anti-HBsAg, and anti-IgG onto different detection wells and the subsequent multiple detection used the same method as that used for the determination of the calibration curve of IL-5 detection as discussed above. The probe coated in each channel is listed in table 7.4.

Table 7. 4. Multiply biomarker detection of flow-through ELISA

channel		a	b	c	d
Immobilized probes		—	anti-IL-5 antibody	anti-HBsAg antibody	anti- IgG antibody
Test samples	A	IL-5(100ng mL ⁻¹), HBsAg(100ng mL ⁻¹), IgG(100ng mL ⁻¹)			
	B	IL-5(100ng mL ⁻¹), IgG(100ng mL ⁻¹)			
	C	IL-5(100ng mL ⁻¹), HBsAg(100ng mL ⁻¹);			
labeled antibody		HRP-labeled anti-IL-5 antibody(2μg mL ⁻¹), HRP-labeled anti-HBsAg antibody(2μg mL ⁻¹), HRP-labeled anti-IgG antibody(2 μg mL ⁻¹)			

7.3. Results and discussion

7.3.1. Surface characterization: Dextran functionalized PDMS

The FTIR spectra of the modified PDMS at each modification step are always compared to that of the pristine PMDS (figure 7.1.a), where the peaks at 2964, 1259, 1015, and 851cm⁻¹ are assigned to -CH₂CH, -CH₃, Si-O-Si and Si-C¹²⁴. The spectra of the APTES-treated PDMS (figure 7.1.b) show a new broad band from 3400 to 3000 cm⁻¹, which can be assigned to -NH₂ stretch vibration¹⁹³. The existence of nitrogen on the surface of APTES-treated PDMS suggests successful chemical grafting of aminosilane. New peaks at 3550-3300, 1717 and 1560 cm⁻¹ observed after dextran immobilization are assigned to the stretching of the primary functional groups of alcohols, carboxylics and carboxylates^{194,195}, indicating that it is covalently attached on the APTES-thread PDMS (figure 7.1 c) surface. Another new peak in the region of 1720–1760 cm⁻¹ displayed after dextran immobilization can be assigned to the aldehyde group¹⁹⁶, which is the key functional group for efficiently and covalently immobilizing proteins for the highly sensitive flow-through ELISA.

Figure 7.1. FTIR spectra (a) Pristine PDMS, (b) APTES modified PDMS, and (c) oxidized-dextran grafted APTES-PDMS

The Raman results (figure 7.2.) can be used to further confirm the dextran immobilization of PDMS. The new peak observed after reaction with dextran at 3345cm^{-1} is assigned to OH stretching¹⁹⁴ (figure 7.2.c), one of the characteristic peaks of dextran. The imported hydroxyl groups can improve the hydrophilicity of the PDMS surface. This result supports the conclusion that dextran immobilization can enhance not only the covalent binding through its aldehyde group, but also the hydrophilicity through its OH groups.

Figure 7.2. Raman spectra (a) Pristine PDMS, (b) APTES modified PDMS, and (c) oxidized-dextran grafted APTES-PDMS

The water contact angle with the pristine PDMS was measured at 109.28° (figure 7.3 a). After the hydrochloric acid/ H_2O_2 treatment, the water contact angle showed a significant reduction to 22.82° . However, the contact angle after APTES-grafting was raised to 66.97° due to the increased amine groups on the surface. After the subsequent oxidized dextran-modification, the contact angle was reduced to 48.63° , since the attachment of the oxidized dextran introduced OH groups on the surface to improve the hydrophilicity.

Figure 7.3. Contact angle (a) Pristine PDMS, (b) $\text{HCl}/\text{H}_2\text{O}_2$ oxidized PDMS, (c) APTES

modified PDMS, and (d) dextran grafted APTES-PDMS

7.3.2. Sandwich ELISA on dextran-PDMS

A sandwich immunoassay was conducted on dextran-PDMS to detect rabbit IgG. The detection range of the dextran-PDMS substrate is 1.12 pg mL^{-1} to 11.2 ng mL^{-1} (figure 7.4), which is significantly wider compared to that of the pristine PDMS surface. High densities of such functional groups coupled with the hydrophilic property of dextran led to the immobilization of a higher amount of antibodies, i.e. improving detection sensitivity as well as reducing false positive responses. The protein non-specifically adsorption on unmodified PDMS resulted in a higher detection limit. Such results were not completely unexpected since the binding sites of the protein/antibody are mostly located in the hydrophobic domain and are no longer available for target binding because of their preoccupied association with PDMS.

Figure 7.4. Sandwich ELISA signal/dose response on dextran-PDMS and pristine PDMS

Linear fit: ($\square$) pristine PDMS: $y = 0.441 + 0.057x$, $R = 0.952$

($\circ$) dextran-PDMS: $y = 0.566 + 0.234x$, $R = 0.997$

7.3.3. Flow-through ELISA with the dextran-functionalized PDMS device

Dextran-functionalized PDMS ELISA microfluidic devices were fabricated to study the protein immobilization and antibody-antigen interaction in its microchannels. The absorbance results in figure 7.5 show that the highest signal is obtained from protein with a 15 minute immobilization and the absorbance signal produced in the microfluidic device reaches a plateau with 15 minutes of antibody-antigen interaction.

Figure 7.5. Absorbance signal at different antibody immobilization times. Insert figure: absorbance signal at different antigen-antibody interaction times

Figure 7.6 shows the calibration curve of IL-5 over a concentration range of 1 pg mL^{-1} to $100 \text{ } \mu\text{g mL}^{-1}$. The experiments were repeated 5 times to create the error bars and the standard deviations were calculated by Origin 7.0. A sigmoid can be seen over the whole detected concentration range and the linearity lies between 1×10^2 to $1 \times 10^5 \text{ pg mL}^{-1}$, illustrating a typical immunoassay characteristics. The limit of detection (LOD) is determined as three standard deviations above the background and is *ca.* 100 pg mL^{-1} . The dextran-functionalized PDMS microfluidic device demonstrates much better performance than that of the hydrophobic PDMS based ELISA device (LOD: $15\text{--}35 \text{ ng mL}^{-1}$ and dynamic range: 1-3 orders of magnitude¹¹⁹). The high sensitivity of the device fabricated in this work is likely ascribed to the dextran-modified PDMS surface with hydrophilic nature, then resulting in efficient covalent probe immobilization. The device performance is also much better than the plasma-treated PDMS based microfluidic immunoassay device (LOD: 100 ng mL^{-1} and dynamic range: 3 orders of magnitude²⁰²), which has covalently immobilized proteins as probes, indicating its significant improvement over the existing flow-through devices.

Figure 7.6. Absorbance curve for the flow-through ELISA. Linear fit: $y = -0.07 + 0.261x$, $R = 0.998$

Interestingly, the conjugated HRP oxidized product of TMB has a blue color and the colorimetric dose responses shown in the detection channels for IL-5 detection can be assessed by the human eye, as shown in the inset of figure 7.7, demonstrating a great potential for the fabrication of a portable microfluidic ELISA device with multiple detections that are visible to the naked human eye. In fact, quantitative analysis of the recorded colorimetric images, captured by CCD camera by means of RGB brightness based on the colors red, green, and blue (Nikon ACT-2U version 1.3, where $S_{RGB} = 0.229 \times \text{red} + 0.587 \times \text{green} + 0.114 \times \text{blue}^{140}$), shows a result (figure 7.7) identical to that measured by absorbance (figure 7.6), strongly supporting the suggestion that the colorimetric detection is accurate and could be used to assess the positive or negative nature of the detected biomarker simply by observing with the naked eye.

Figure 7.7. IL-5 detection: RGB brightness in different channels. Inset image: enzymatic converted substrate in different channels (the channel width is 250 μm); from left to right, the

reaction analytes concentrations were: 0, 1 pg mL⁻¹, 10 pg mL⁻¹, 100 pg mL⁻¹, 1 ng mL⁻¹, 10 ng mL⁻¹, 100 ng mL⁻¹, 1 µg mL⁻¹, 10 µg mL⁻¹, and 100 µg mL⁻¹

Three 4-channel PDMS microfluidic ELISA devices fabricated in this work were used to colorimetrically detect IL-5, HBsAg, and IgG in a flow-through scheme. The results are shown in figure 7.8 (1) and (2), where *A*, *B* and *C* represents the three devices, while *a*, *b*, *c*, and *d* denote the different channels with immobilized probes (for control, anti-IL-5, anti-HBsAg, and anti-IgG, respectively). In order to demonstrate the multiple detection, device *A* was used to detect a sample with mixed IL-5 (100 ng mL⁻¹), HBsAg (100 ng mL⁻¹), and IgG (100 ng mL⁻¹), while devices *B* and *C* were employed to detect samples with a mixture of IL-5 (100 ng mL⁻¹) and HBsAg (100 ng mL⁻¹), and a mixture of IL-5 (100 ng mL⁻¹) and IgG (100 ng mL⁻¹), respectively. Results shown in figure 7.8 (1) illustrate that no blue color is observed in the background channel (channel *a*). Channel *d* of device *B* and channel *c* of device *C* also has no observable blue color; these are correct negative responses that we designed. Other channels in all the devices show a significantly identical blue color indicating that the target biomarkers in the different channels have the same concentration. Figure 7.8 (2) displays the bar plot of RGB brightness vs. target biomarker for all three devices, which clearly demonstrate that all the measured background and negative responses are significantly lower than the positive responses; these are the results expected from our design. According to the colorimetric calibration curve in figure 7.7, the measured concentrations of the three biomarkers in devices *A*, *B*, and *C* are 95.94, 103.27 and 99.54 ng mL⁻¹, respectively, which are in good agreement with the target concentration in the samples (100 ng mL⁻¹), showing a good assay accuracy and high specificity in multiple detections. Since the color changes for multiple protein detection can be observed directly by human eye, the microfluidic device provides a great potential to fabricate a portable, flow-through lab-on-chip system for high-throughput screening of infectious diseases in health point-of-care applications.

Figure 7.8. Multi-detection test of the flow-through ELISA in human serum solution: (1). CCD image of the enzyme catalyzed substrate (the channel width is 250 μm); (2) Bar plot of RGB signal at each microchannel. Immobilized probe of each microchannels: a. negative probe; b. anti-IL-5 antibody; c. anti-HBsAg antibody; d. anti-rabbit IgG antibody, Test A. injected testing sample contains IL-5 (100 ng mL⁻¹), HBsAg (100 ng mL⁻¹) and rabbit IgG (100 ng mL⁻¹), Test B. injected testing sample contains IL-5 (100 ng mL⁻¹) and HBsAg (100 ng mL⁻¹); Test C. injected testing sample contains IL-5 (100 ng mL⁻¹) and rabbit IgG (100 ng mL⁻¹).

7.4. Conclusion

A dextran modified PDMS microfluidic ELISA device with a unique architecture for eliminating crossover interference was fabricated. The dextran functionalization of the PDMS was conducted in a simple, economical and fast flow-through process, and demonstrated great enhancement of the hydrophilicity and efficient covalent immobilization of proteins on the PDMS microchannel surface. The microfluidic device was used to simultaneously detect multiple important biomarkers, which included IL-5, HBsAg, and IgG, showing high sensitivity of 100 pg mL⁻¹ and a dynamic range of 4 orders of magnitude; this is much better than similar results reported for both hydrophobic PDMS based microfluidic ELISA devices and plasma-treated PDMS microfluidic immunoassay device with covalently attached protein probes. Moreover, the fabricated PDMS device demonstrated its capability for colorimetric detection of proteins and this could be observed clearly by the human eye. Thus, the dextran-modified PDMS microfluidic device not only provides a great potential for fabrication of an economical and sensitive lab-on-chip system for high throughput screening of various infectious diseases, but also provides an excellent opportunity for developing a portable microfluidic ELISA device via human eye examination for health point-of-care services.

Chapter 8 One-step fabrication of sensitive UV-curable epoxy-based immunoassay microarray and microfluidic device

8.1. Introduction

In this chapter, immunoassay devices including microarrays and microfluidic systems were fabricated with an UV-curable resin by a new economic approach, which can not only simply produce a 3-D patterned structure, but also simultaneously introduce functional epoxide groups for efficient protein immobilization.

Efficient immobilization of capture proteins on a solid surface is critical for a sensitive immunoassay. Protein microarrays have been fabricated on various substrates such as glass²⁰³, organic polymer^{48, 204}, silicon²⁰⁵ and metal plate⁴⁶. To attach capture proteins on these substrates, epoxide-immobilized substrates have been proposed to efficiently attach various biomolecules through its direct reaction with amine or carboxylic group of the biomolecules without additional preactivation or crosslinking process^{126, 206}. Among these works, introduction of epoxide groups onto a substrate surface can be achieved by self-assembly of epoxysilanes²⁰⁷, but it is limited to only a few substrates. In Liu et al. reported work, glycidyl methacrylate (GMA) is used to react with aminolyzed polyethylene terephthalate (PET) surface for immobilization of the epoxide groups, which can introduce high density of epoxy groups for further effectively attach capture proteins. This approach involves time-consuming multiple steps. In all these reported works, surface modification of the epoxide group is conducted by physical adsorption and/or chemical reactions. Most importantly, the reported methods so far for epoxy-activation are all completely surface modifications and are not amenable to fabricate 3-dimensional patterned microfluidic structure at the same time.

Most of the early microfluidic immunosensors devices were made using silicon and glass as the substrates, because their microfabrication can directly borrow the matured technology from the semiconductor industry. As an economic alternative, polymer substrate was employed to fabricate the microfluidic device by simple molding instead of the expensive patterning process. Poly (dimethylsiloxane) (PDMS) is one of the polymer substrates mostly used due to

its high cast fidelity in a mold²⁰⁸. However, its resin takes a long time (a number of hours) to cure, and the immunoassay device has strong, non-specific interference caused by its hydrophobic nature. Therefore, surface modification for improvement of PDMS hydrophilicity is usually a must^{79,97}. Additionally, the polymer microfluidic device so far needs several manufacture processes to fabricate its 3-D patterned structure including master microfabrication, polymer structure molding and protein surface modification. This is still a costly manufacture process although relatively less expensive than silicon and glass based microfluidic devices.

To overcome the difficulties with PDMS, UV-curable epoxy was come out as valuable alternative approach to fabricate an immunoassay microarray and microfluidic device with low cost and performance improvement. In this chapter, we investigate a new and simple approach using an epoxy resin to directly fabricate a 3-D microfluidic structure while simultaneously introducing the epoxide group to efficiently immobilize capture proteins for high performance immunoassay devices. A light-induced polymerization has advantages of short curing time (tenths of a second)^{209,210} and room temperature operation, and is employed to cure the epoxy resin in this work. PET as a plastic substrate has been widely used in tissue engineering and biomedical devices because of its low cost and desirable physicochemical properties, and is used as the substrate for the immunoassay devices in this work. The current approach to immobilize the capture proteins onto PET surface is conducted with introduction amino groups by silanization through 3-aminopropyltriethoxysilane and aminolysis, but requires additional crosslinkers such as glutaraldehyde and sebacoyl chloride for the protein attachment²¹¹. In this work, the formulation and polymerization of the UV-curable resin were investigated to produce functional epoxy groups on PET surfaces for efficient protein immobilization. The printing buffer optimization and protein immobilization kinetics were studied to improve the microarray performance. The fabricated immunoassay microfluidic device was explored to simultaneously and colorimetrically detect IL-5 and rabbit IgG for portable, economical and multiple immunoassays. The protein microarray and the flow-through immunoassay device were characterized to demonstrate their performance and application potential.

8.2. Epoxy-based microarray and microfluidic device preparation and applications

8.2.1. Preparation of UV-curable epoxy resin

TMPTA and DPGD were used as the solvents to adjust the viscosity of the resin and also to effectively dissolve the photoinitiator. For 3-D microstructure fabrication, the UV curing was initiated with the photoinitiator Irgacure 651. The epoxy resin (Ebecryl 3605) was put in a brown glass beaker, weighted, and then heated to 60 °C for about half an hour until it was melted. SR508, SR-351 and 0.5% (w/w) photoinitiator were added into the beaker to mix with the melted epoxy by stirring for overnight. The chemical structures of Ebecryl 3605, SR-508, SR-351 and Irgacure 651 are shown in figure.8.1.

Figure 8.1. Chemical structure of (a) SR-508, dipropylene glycol diacrylate (b) SR-351, trimethylolpropane triacrylate (c) Ebecryl 3605 (d) Irgacure 651

Eight resin formulations were prepared with different epoxy acrylate to triacrylate group ratios (table 8.1). The E/A ratios were calculated, taking into account the ratio of the functional groups of the epoxy resin and the TMPTA. The concentration of the photoinitiator in each mixture was 0.5% (w/w).

Table 8.1. Resin formulation with different E/A

resin sample	1	2	3	4	5	6	7
epoxy acrylat: triacrylate (mole ratio)	0	0.02	0.04	0.08	0.12	0.24	0.36

8.2.2. Fabrication of Epoxy film on PET substrate

The procedure to prepare epoxy film on PET substrate was described in *chapter 3, section 3.1.1*. The cured epoxy film was washed in ethanol and subsequently in DI water for 3 minutes each time to remove any uncured polymer, and was ready to be used for the fabrication of the protein microarray and

microfluidic device.

8.2.3. Fabrication of epoxy microfluidic device

The procedure to prepare epoxy microfluidic system was described in *chapter 3, section 3.1.4.2*. The fabricated structure was washed with ethanol and DI water successively. After blow-drying, holes at the inlet and outlet channels were made by drilling to connect a syringe pump with a silicon tube. The connections were sealed by a HY-9 hydrophilic (adhesive material) coated plastic film. Then, a multi-channel microfluidic device for ELISA experiments was finally made for use.

8.2.4. Fabrication of epoxy film based protein microarray

The protein probe was dissolved in the printing buffer for the desired concentration and was transferred to the 384-well micro-titer plate before printing. To optimize the printing buffer, 0.01 M PBS containing different concentrations of glycerol ranging from 0 to 40% and different amounts of triton X-100 ranging from 0.001% to 0.012% were examined. In other experiments, the optimal printing buffer was employed. In the study of the protein immobilization kinetics, 25, 100, 500 $\mu\text{g mL}^{-1}$ of Cy3-conjugated anti-goat IgG were used while a range from 5 to 1000 $\mu\text{g mL}^{-1}$ of the protein solution was employed for characterization of protein loading capacity. VersArray chipwriterTM (Bio Rad, USA) was used to fabricate microarray by contact printing mode at 60% humidity.

The probe immobilization for the kinetic study was carried out in a humid chamber with incubation times of 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 12 hours, respectively, and for other studies was conducted with an overnight incubation time. The protein-immobilized slides were washed 3 times for each 2 minutes with TBST (0.01 M TBS with 0.05% Tween 20) to remove unbound probes. Then it was immersed in BlockerTM casein/TBS solution for 1 hour to not only quench the unreacted epoxy functional groups on the substrate surface, but also to produce a layer of casein that could reduce the nonspecific bindings in subsequent steps. For the immunoassay, 20 μL of Cy3-labeled anti-goat IgG/TBS buffer was applied to the fabricated microarray in such a way that different concentrations of Cy3-labeled anti-goat IgG from 1 pg mL^{-1} to 10 $\mu\text{g mL}^{-1}$ were

used for various groups of spots (subarrays) for 1 hour reaction at 37°C. The microarray was washed again (2 minutes $\times$ 4 times) with TBST, followed by washing (twice) with DI water for 2 minutes to remove the detergent and salt. After drying, the microarray was scanned at 530 nm using a scanning laser confocal fluorescence microscopy (ScanArray GX, PerkinElmer, USA) at a 5- μ m resolution. The acquired images were quantitatively analyzed with ScanArray® Express analysis software. The fluorescent intensities were local background-subtracted means for downstream statistical analysis. The acquired data were input to Origin 7.0 for further analysis and plots.

8.2.5. Flow-through ELISA with UV-embossed device

The effect of incubation time on probe immobilization and the following antigen-antibody interaction on the embossed microchannel surface was studied by pumping a 10 μ g mL⁻¹ HRP-labeled IgG into the channels and then incubating for different times. After probe immobilization, the unbounded protein was removed by a flow washing process for 3 minutes. TMB was then pumped in for the enzymatic reaction to yield colors, which were captured by CCD camera. In addition, the peroxidase converted substrates were pumped out into the 384-well ELISA-plates for absorbance measurement (Genios plus, Tecan, USA).

To fabricate a microfluidic device for the flow-through sandwich ELISA, a 10 μ g mL⁻¹ anti-IL-5 monoclonal antibody solution was pumped into the microchannels for a 5 minutes incubation, the optimal condition identified above in the probe attachment. The same flow-through washing procedure as that used in the study of probe immobilization was performed, while the blocking was performed in the microchannels using the same as that used in the protein microarray fabrication. The analyte, IL-5 diluted in human serum ranging from 10 pg mL⁻¹ to 10 μ g mL⁻¹, was pumped into the fluidic device and incubated for 10 minutes, followed by flow-through washing with TBS for 4 minutes. The HRP labeled monoclonal anti-IL-5 was then pumped into the channel for a 10 minutes incubation period followed by a 5 minutes of flow-through washing. Then, the enzyme substrate, TMB was pumped into the microfluidic channels to react with the captured HRP for colorimetric detection. Additionally, the peroxidase converted substrates were pumped out into the 384-well ELISA-plates for absorbance measurement.

Table 8.2. Parameters of flow-through IL-5 detection on epoxy microchannel

	probes	analytes	antibody*
epoxy microchannels	anti-IL-5 IgG 10 $\mu\text{g mL}^{-1}$ 5 min	IL-5 10 $\mu\text{g mL}^{-1}$ to 10 pg mL^{-1} 10 min	anti-IL-5 (HRP) 5 $\mu\text{g mL}^{-1}$ 10 min
negative controls	—	+	+

8.3. Results and discussion

8.3.1. UV-curable epoxy resin and cured film

The prepared epoxy resin and its UV-cured film were characterized by ATR-FTIR spectrophotometer. In figure 8.2, it can be observed that the characteristic unsaturated double bands of the acrylate group at 1637 cm^{-1} and 810 cm^{-1} ¹²⁵ disappears, apparently after UV irradiation, indicating the polymerization via acrylate groups occurs. However, the characteristic bands of epoxide groups including the antisymmetric ring deformation at 917 cm^{-1} , and the symmetric epoxide ring deformation at 866 cm^{-1} ¹²⁶, can be seen before and after the UV irradiation, showing that the epoxy groups are still intact for covalently immobilizing proteins after UV polymerization.

Figure 8.2. FTIR spectra of the resin before: a. and after b. UV exposure

The epoxy concentration in the resin was controlled by adjusting the epoxy and acrylate molar (E/A) ratio. The water contact angles measured from different cured films were around 73° with no significant difference for different E/A ratios. After argon-plasma treatment, the water contact angle significantly decreased to 32° (figure 8.3a). Protein immobilization was conducted with different E/A ratios to obtain an optimal condition. The protein loading capacity was determined by

the method described in section 8.2.4. The results shown in figure 8.3b illustrate that the samples with an E/A ratio of less than 0.03 have no detectable fluorescent signal, indicating that the immobilized protein is negligible. The detected fluorescent intensity increases steadily with an increase in the E/A ratio over the range of 0.03 to 0.08, until the plateau fluorescence intensity is reached at E/A ratios higher than 0.08 (figure 8.3b). To compromise the maximum protein immobilization capability with a low viscosity for non-fragile epoxy films, the optimal E/A molar ratio of the resin is selected as 0.08. Interestingly, after treatment by argon plasma, the fluorescent intensity of the epoxy film did not show significant change (figure 8.3b), although its hydrophilicity had remarkable improvement (figure 8.3a). This might clearly indicate that the protein nonspecific adsorption introduced by the hydrophobic interaction was insignificant, and the covalent binding of the epoxy group with amino group of the protein was the dominant factor in the protein immobilization. This can eliminate or reduce the non-specific adsorption interference from the hydrophobic interaction.

Figure 8.3. Hydrophilicity of epoxy films and effects of epoxy ratio and hydrophilicity on protein immobilization. a. water contact angle vs. epoxy ratio and b. fluorescence intensity vs. epoxy ratio

8.3.2. Protein microarray and its immobilization kinetics

The fabrication of the UV-cured epoxy protein microarrays was optimized by the probe concentration, printing buffer, and incubation time. The properties of the printing buffer can significantly affect the probe binding capacity and the spots quality. The effect of the printing buffer pH was evaluated by directly

spotting Cy3-IgG on the cured epoxy films. As shown in figure 8.4, among various pH in PBS buffers, the pH 7.4 solution gives the highest fluorescence signal for the best protein immobilization. Thus, a pH 7.4 PBS was used to prepare the printing buffer for other optimization experiments.

Figure 8.4. Signal/ printing pH plot

Glycerol is an additive in the printing buffer to prevent the spotted probe from dehydration and subsequent denaturation, since the nanoliter-droplets could quickly evaporate even at room temperature¹⁶. Additionally, adding triton X-100, a non-ionic surfactant, to the printing buffer can increase the homogeneity and diameter of the spots^{212, 213}. In our previous work, both additives were used to improve antibody microarray performance; however, they were only effective at a certain threshold concentration, and deviation from this threshold could cause negative effects¹⁶. A delicately tuned optimization is necessary when using them in the printing buffer. In this work experiments, glycerol concentrations of 2.5%, 5%, 10%, 20%, and 40%, and triton X-100 concentrations of 0.001%, 0.003%, 0.006%, and 0.012% in the printing solution were examined. The scanned image of the fabricated microarray and the fluorescence intensity vs. the glycerol and triton concentrations are shown in figure.8.5 (a) and (b), respectively. The results reveal that the buffer containing 2.5% glycerol, 0.003% triton X-100 has the highest signal intensity. Thus, the optimal composition of the printing buffer is 0.01M PBS (pH 7.4) containing 2.5% glycerol and 0.003% triton X-100.

Figure8.5. Signal/ surfactant concentration plot

The protein immobilization kinetics was studied by spotting different concentrations of Cy3-labeled IgG. The dependency of fluorescence intensity on the printed protein concentrations is shown in figure 8.6, illustrating that the intensity sharply increases with increasing the protein concentration, until a plateau intensity is reached at a concentration equal or larger than 250 $\mu\text{g mL}^{-1}$. However, the 500 $\mu\text{g mL}^{-1}$ deposited spots have both the best uniformity and the highest fluorescence intensity.

Figure8.6. Signal/ concentration curve

Based on the results shown in figure 8.6, concentrations of 25, 100 and 500 $\mu\text{g mL}^{-1}$ were chosen to investigate protein immobilization kinetics, since they can represent different stages of the immobilization. The results are shown in figure 8.7; of which the inset is the images of the tested microarrays. The results clearly illustrate that the fluorescent intensity for the immobilization solution of 500 μg

mL^{-1} increases with the immobilization time much faster than that of concentrations of 25 and $100 \mu\text{g mL}^{-1}$, very fast reaching its maximum value in less than 4 hours. However, the immobilization with 25 and $100 \mu\text{g mL}^{-1}$ could not reach its maximum value even after 12 hours. The protein immobilization kinetics on the cured epoxy surface was studied by using the following equation [8.1]^{53, 156}:

$$R_t = E^* (1 - e^{-k_s t}) + R_0 \quad [8.1]$$

$$\text{and} \quad k_s = k_a [A] + k_d \quad [8.2]$$

Where E^* , k_a , k_d , R_0 , and A are the activation energy, the association rate constant, the dissociation rate constant, the response at $t=0$ and the adsorption protein concentration in the aqueous solution, respectively. The values of k_a , k_d , E^* , and R_0 are determined by fitting the binding curves of fluorescent intensity vs. times for different immobilization concentrations. The fitted k_a and k_d are $1.38 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ and $9.82 \times 10^{-6} \text{ s}^{-1}$, respectively.

Figure 8.7. Signal/ time curve of different probe concentration

8.3.3. Analytical characterization of antibody microarray

The dynamic range and limit of detection (LOD) of protein microarrays fabricated on the epoxy resin film was characterized by using direct immunoassay with goat IgG and anti-goat IgG as model proteins. $500 \mu\text{g mL}^{-1}$ goat IgG in the optimal printing buffer discussed above was contact-printed on the epoxy film for immunoassay microarrays. Various concentrations of anti-goat IgG (Cy3 label) from 10 pg mL^{-1} to $10 \mu\text{g mL}^{-1}$ with 10-fold dilution were used to incubate each array. Figure 8.8.A illustrates that the fluorescence intensity rises with the increase of analyte concentration. The fluorescence intensity of each

spots was quantitatively analyzed with ScanArray® Express analysis software, and a plot of fluorescence intensity versus concentrations of the analyte (anti-goat IgG) was obtained by using Origin 7.0. Figure 8.8.B shows the calibration curve of anti-goat IgG over a concentration range of 10 pg mL^{-1} to $10 \text{ } \mu\text{g mL}^{-1}$. A sigmoid curve ($R^2=0.989$) can be seen over the whole detected concentration range, showing a linear range (the concentration range that gave the best fit to the linear equation) from 10 to $1 \times 10^6 \text{ pg mL}^{-1}$ on the cured epoxy film. It indicates that the dynamic range is from 10 to $1 \times 10^6 \text{ pg mL}^{-1}$. LOD is determined as three standard deviations above the background and is *ca.* 10 pg mL^{-1} . The dynamic range and LOD are two important parameters to characterize the performance of a protein microarray. In detection of IgG, the LOD of 150 pg mL^{-1} and detection dynamic range of 4 orders of magnitude on carboxyl-terminated dendrimer-coated surface²¹⁴, and the detection dynamic range of 50 to 200 ng mL^{-1} on 3D micro/nano porous silicon structures have been reported²¹⁵. In comparison to these works, the epoxy resin-based protein array exhibits very comparable LOD and larger dynamic range. It is also much simpler and less expensive, since the inherent functional epoxide group in the epoxy resin can directly bind proteins without need of the additional multiple surface reactions for functionalization and preactivation.

Figure 8.8. A. Fluorescent image of microarray; B. Dose dependent curve of UV-curable epoxy resin-based protein microarray for detection of anti-goat IgG.

8.3.4. Epoxy-based ELISA microfluidic device

UV-cured epoxy-based ELISA microfluidic devices were fabricated to explore its application in simultaneous multiple flow-through detection of proteins. The

protein immobilization and detection including incubation and washing step were conducted with a flow-through operation in the device.

The effects of incubation time and probe concentration on the protein immobilization in the epoxy-microchannel were investigated. The absorbance results in figure 8.9a show that the highest signal is obtained from protein with a 5 minutes-immobilization. To study the optimal antibody-antigen interaction time, the microchannels were immobilized by IgG with $10 \mu\text{g mL}^{-1}$ IgG/0.01M PBS, followed by blocking with casein for 10 minutes. The device was then used to detect the absorbance responses after flowing-in of $5 \mu\text{g mL}^{-1}$ anti-IgG into the different micro-channels for different incubation times. The results (figure 8.9b) show that the absorbance produced in the microchannel reaches a plateau after 10 minutes of antibody-antigen interaction, and the optimal protein immobilization time and the antibody-antigen incubation time are 5 and 10 minutes, respectively. The total assay time, including immobilization, blocking and detection, for the microfluidic device is ~ 35 minutes, which is significantly reduced than the typical assay time (normally 3-4 h) in the commercially available micro-plates.

Figure 8.9. Absorbance signal at different antibody immobilization times. Insert figure: absorbance signal at different antigen-antibody interaction time

Simultaneous detection of IL-5 and IgG was conducted in the epoxy-based microfluidic device, which had 14 microchannels for detecting 6 concentrations of each target protein and background. IL-5 and IgG are of great importance in biomedical science and clinic diagnoses, since they are involved in a wide range of allergic reactions and mediate immune reactions against parasites^{216 217}. IL-5 is a marker of disease activity and treatment efficacy in bronchial asthma²⁴, while

IgG can serve as a specific marker for neuromyelitis optica, an inflammatory demyelination disease. The absorbance calibration curves obtained for the multiple detection of IL-5 and IgG plus background are shown in figure 8.10, displaying a detection limit of 100 pg mL^{-1} and a dynamic range of 4 orders of magnitude. The performance of this one-step fabricated epoxy microfluidic device was demonstrated in analytes containing serum, and it is still very comparable with that detected in PBS with the microfluidic devices fabricated using a more complicated multiple step process^{11, 21, 57}.

The fabricated epoxy microfluidic device can be used for simple colorimetric detection as shown in the inset of figure 8.10, in which no color is observed from the channels with detection of background and 10 pg mL^{-1} IL-5. However, blue colors can be observed clearly from the channels with detections of a concentration equal to or higher than 100 pg mL^{-1} . The results are in agreement with that measured using the ELISA plate reader. Since the color changes for the protein detection can be directly observed by the naked human eye, the microfluidic device demonstrates a great potential for fabricating a portable, flow-through, detection equipment-free device for screening diseases using the immunoassay method. The microdevice could also be fabricated using a web to web embossing manufacturing process developed in our previous work for mass production²¹⁸.

Figure 8.10. Calibration curve of IL-5 at epoxy-microchannel by using anti-IL-5 antibody as probe (□), Calibration curve of IgG at epoxy microchannel (△). Insert image: channels (the channel width is $200 \mu\text{m}$), from left to right, the IL-5 concentration are: 0, 10 pg mL^{-1} , 100 pg mL^{-1} , 1 ng mL^{-1} , 10 ng mL^{-1} , 100 ng mL^{-1} , and $1 \mu\text{g mL}^{-1}$; probes immobilization time: 10 min.

8.4. Conclusion

In brief, a new and economic approach to fabricate protein microarray and microfluidic immunoassay device with a UV-curable epoxy resin was investigated. The microarray showed an extremely low standard deviation error, a broad dynamic range of 5 orders of magnitude, and a LOD of 10 pg mL^{-1} . More promisingly, the ELISA microfluidic device also possesses a LOD of 100 pg mL^{-1} and a dynamic range of 4 orders of magnitude in serum sample, which is better or at least comparable to that measured in PBS with the microfluidic devices made by more complicated fabrication processes. The work also demonstrates the feasibility of a microfluidic device for colorimetric immunoassays that can be detected with the naked human eye, then providing potential to fabricate portable immunoassay devices for health point of care and infectious diseases screening.

Chapter 9 General conclusion and future work

9.1. General conclusion

In this dissertation, the advancements in miniaturized immunoassays in recent years were thoroughly reviewed. The details of the detection schemes, miniaturized immunoassay construction and signal processing were presented and several comparative studies were made. Drawbacks such as the hydrophobic nature of pristine PDMS still restrain its performance and application in microfluidic immunoassays. To conquer these challenges, bioconjugated methods were investigated thoroughly and used to enhance the flow-through ELISA system for protein detection.

A new approach used AFM to study the conformation and kinetics of the adsorption of protein A on the PDMS surface at a molecular level. Through investigations of the effects of pH values, protein concentration, and contact time on the adsorption, the results indicate that the conformation of protein A is significantly affected by pH and adsorbed surface concentration. Protein A shows tortuous line shapes with adsorption at pH values far from the *pI* point of protein A (5.0), while displaying dendrite conformation at pH values equal to *pI* of the protein (5.0). This phenomenon is ascribed to the electrical repulsion effect of charged protein molecules in solutions with pH values far from *pI*. Networks, formed like a large “molecule” from a number of single molecules, were found in higher adsorbed surface concentrations of protein A. The results also reveal that the protein A produces a monolayer film with a maximum adsorption of 1.22×10^4 molecule μm^{-2} . This work demonstrates that AFM can be used to study the kinetics of an adsorption process in relatively low concentrations of proteins due to its high sensitivity.

A mild physiosorption assembled APTES was investigated to graft amino-groups onto the PDMS surface. The method presented does not increase the surface roughness by mechanically damaging the PDMS; this is a common side effect of other surface treatments such as irradiation with UV light, corona discharges and oxygen plasma. Moreover, fewer chemicals are used and fewer experimental steps are required compared to existing PDMS modification methods. SAA/EDC based protein immobilization is identified as a simple and

effective approach to attach probe proteins on an aminated-PDMS solid surface for sensitive protein detection. The detection limit using this simple and efficient protein immobilization method was reached at pg mL^{-1} level.

A simple but effective technique to suppress non-specific adsorption for the development of a solid phase material for immunoassays was studied. Hydrophobicity of PDMSs can be silanized with APTES, followed by PVA coating and glutaraldehyde activation. Biomolecules can be covalently attached to the modified surface in an even distribution. The application of the PVA functionalized surface in sandwich ELISAs results in excellent detection sensitivity (1 pg mL^{-1}) and linearity up to 10 ng mL^{-1} . A multiple PVA-functionalized PDMS microfluidic device was demonstrated for the detection of HBsAg, HBeAg and α -FP. The microfluidic ELISA devices possess a detection limit (LOD) of 10 pg mL^{-1} and a dynamic range of 5 orders of magnitude for detection of α -FP; LOD of 100 pg mL^{-1} and a dynamic range of 4 orders of magnitude for detection of HBeAg and HBsAg in human serum.

A dextran-modified PDMS microfluidic ELISA device with a unique architecture for eliminating crossover interference was fabricated. The dextran functionalization of the PDMS was done using a simple, economical and fast flow-through process and demonstrated great enhancement to the hydrophilicity and efficient covalent immobilization of proteins on the PDMS microchannel surface. The device was used to simultaneously detect multiple important biomarkers, which include IL-5, HBsAg and IgG, via a flow-through process, showing excellent sensitivity of 100 pg mL^{-1} and a dynamic range of 4 orders of magnitude. These results are much better than those obtained using hydrophobic PDMS based microfluidic ELISA devices or the plasma-treated PDMS microfluidic immunoassay device with covalently attached protein probes. Moreover, the PDMS devices fabricated in this work were used in the colorimetric detection of proteins, whereby the results obtained were in good agreement with the absorbance measurements and could also be observed clearly by the naked eye.

The next research highlight is the exploration into a new and economic approach to one-step fabrication of a protein array and ELISA microfluidic device with a UV-curable resin. The resin formulation was optimized and characterized to achieve good protein immobilization capacity and

microstructure fabrication processability. The UV-cured film formed by the epoxy resin demonstrated efficient covalent protein immobilization through the reactions of its epoxy functional groups with the amines of proteins. The printing buffer, immobilized probe concentration, and incubation times with the target solution were optimized for the best performance. The protein immobilization kinetics was studied by fitting the experimental results with the kinetic equation. The microarray showed an extremely low standard deviation error, a broad dynamic range of 5 orders of magnitude, and a detection limit of 10 pg mL^{-1} for IgG. The developed microfluidic immunoassay device demonstrates a detection limit of 100 pg mL^{-1} . In addition, the device developed in this work demonstrates the feasibility of a microfluidic device for colorimetric immunoassays that are visible to the naked human eye. This work provides a simple and economical method for the one-step fabrication of protein arrays and microfluidic devices, and could have broad applications in both biomedical sciences and clinical diagnoses.

In brief, we investigated the modification of the PDMS microfluidic device with hydrophilic polymers for enhancement of both hydrophilicity and immobilization efficiency of proteins through their functional groups, e.g. abundant hydroxyl groups. Subsequently, we investigated a novel UV-curable resin for one-step fabrication of epoxy group functionalized microstructures which can be competently used for highly sensitive protein microarrays and flow-through ELISA applications. In addition, we explored a colorimetric method (based on a colored end-product of the PDMS immunoassay) which allowed the results to be read directly by the naked eye for assessment (negative or positive), possibly without using any detection equipment. Detection of pathogenic targets by microfluidic ELISA was investigated on the fabricated plastic microfluidic platforms. Biomarkers related to infectious diseases, such as hepatitis B virus infection, IL-5, were specifically studied.

9.2. Future work

In this PhD project work, the new approaches were used to fabricate inexpensive, sensitive and portable microfluidic ELISA devices for practical applications. Future study can be directed towards several critical aspects for

exploration of scientific knowledge and then further improvement of the device performance.

Although the devices developed in this work show better or comparable sensitivity in comparison to the prior arts, but it needs further improvement (down to a few pg mL^{-1}) for clinic diagnoses, especially for early disease detection. The further improvement relies greatly on the scientifically understanding the device. The surface immobilization may further be modified for highly efficient probe attachment. The prevention of non-specific protein adsorption can increase the signal to noise ratio for enhancing the sensitivity of the flow-through ELISA. Besides hydrophobic-hydrophobic interaction, non-specific protein adsorption is affected by several factors such as protein properties (size, charge, shape, composition and pH), surface properties (charge, chemistry and topology), co-adsorption of low-molecular-weight ions, intermolecular forces between adsorbed molecules, and strength of functional group bonds. AFM can be used to study the interaction of protein and polymer surface for further extending our knowledge in non specific protein adsorption. Impedance spectroscopy can be integrated with ELISA to investigate new detection of the effects of type of probes, steric effect and charge of the analyte and on the detection sensitivity and specificity. The more challenge task for increase S/N ratio is to reduce or eliminate interferences from the real sample, such as blood, saliva and urine, of which the interferences may become more complicated. Thus, alleviating the interference is worthy to study on both sample pretreatment and data process.

The results demonstrate that one-step fabrication of miniaturized bio-analyzers with UV-curable resin is an attractive research direction for preparation of functionalized surfaces for highly efficient assays. The further effort will continue to use one-step fabrication approach to make low-cost, sensitive and reliable microfluidic device which is competent to automatically and efficiently conduct sampling, mixing and detection within the plurality of test sites for multiple screening immunoassays. The flow-through ELISA procedure will be particularly studied to realize a one-step immunoassay which is without washing process.

The most important work in the future particularly goes to fabricate prototype device targeting a number of fatal diseases for both scientific and commercial

success.

Abbreviate

α -FP: alpha-fetoprotein

AFM: atomic force microscopy

APCs: antigen-presenting cells

APTES: 3-aminopropyltriethoxysilane

BioMEMS: biological microelectromechanical systems

CB: carbonate-bicarbonate buffer capsules

CEA: carcinoembryonic antigen

DEX: dextran

EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

EHD: electrohydrodynamic

ELISA: Enzyme-linked Immunosorbent assay

EOF: electroosmotic flow

FTIR: fourier transform infrared spectroscopy

GA: glutaraldehyde

HBV: hepatitis B virus

HBsAg: hepatitis B surface antigen

HBeAg: hepatitis B e antigen

HBcAg: hepatitis B core antigen

HLA: human leukocyte antigen

HRP: horseradish peroxidase

HAS: human serum album

IA: Immunoassay

IgG: immunoglobulin G

IL-5: interleukin-5

MHC: major histocompatibility complex

MHD: magnetohydrodynamic

μ APD: microavalanche photodiode

μ CP: micro-contact printing

μ TAS: micro-Total Analysis System

PBS: phosphate buffered saline

PBMC: peripheral blood mononuclear cell

PC: polycarbonate

PCR: polymerase chain reaction
PDMS: poly (dimethylsiloxane)
PEMs: polyelectrolyte multilayers
PET: polyethylene terephthalate
PGMEA: propylene glycol methyl ether acetate
PMMA: Polymethyl methacrylate
Poly Si: polycrystalline silicon
PVA: Poly (vinyl alcohol)
RIA: radioimmunoassay
RIE: reactive ion etching
RT: room temperature
SAA: succinic acid anhydride
Si: Single crystal silicon
SPI: solid-phase immunoassays
SUVs: small unilamellar vesicles
TBS: tris buffered saline
TLM: thermal lens microscopy
TMB: 3,3,5,5'- tetramethyl benzidine
XPS: X-ray photoelectron spectroscopy
ZPC: zero-point of charge

Reference

1. Gosling, J. P., *Immunoassay: A Practical Approach*. Gosling, J.P. ed.; Oxford University Press: 2000; p 304.
2. McCreedy, T., Fabrication techniques and materials commonly used for the production of microreactors and micro total analytical systems. *Trends Anal. Chem.* **2000**, 19, 396.
3. Madou, M. J., *Biomedical analysis*. CRC Press: 1997.
4. Laurell, T.; Rosengren, L., A micromachined enzyme reactor in <110> -oriented silicon. *Sensors Actuat. B* **1994**, 19, 614.
5. Laurell, T.; Drott, J.; Rosengren, L., Silicon wafer integrated enzyme reactors. *Biosens. Bioelectron.* **1995**, 10, 289.
6. van den Berg, A.; Lammerink, T. S. J., Microsystem technology in chemistry and life science. **1998**, 194, 21.
7. Manz, A.; Graber, N.; Widmer, H. M., Miniaturized total chemical analysis systems: A novel concept for chemical sensing. *Sensors Actuat. B* **1990**, 1, 244.
8. Haswell, S.; Skelton, V., Chemical and biochemical microreactors *Trends Anal. Chem.* **2000**, 19, 389.
9. Schwarz, A.; Rossier, J. S.; Bianchi, F.; Reymond, F.; Ferrigno, R.; Girault, H. H., Micro-TAS on polymer substrates micromachines by laser photoablation. *Proc. Micro-TAS* **1998**, 241.
10. Xia, Y.; Whitesides, G. M., Soft lithography. *Angew. Chem., Int. Ed.* **1998**, 37, 550.
11. Kim, K. S.; Park, J. K., Magnetic force-based multiplexed immunoassay using superparamagnetic nanoparticles in microfluidic channel. *Lab Chip* **2005**, 5, (6), 657.
12. Davidsson, R.; Genin, F.; Bengtsson, M.; Laurell, T.; Emneus, J., Microfluidic biosensing systems Part I. Development and optimisation of enzymatic chemiluminescent [micro sign]-biosensors based on silicon microchips. *Lab Chip* **2004**, 4, (5), 481-.
13. Sato, K.; Tokeshi, M.; Kimura, H.; Kitamori, T., Determination of carcinoembryonic antigen in human sera by integrated bead-bed immunoassay in a microchip for cancer diagnosis. *Anal. Chem.* **2001**, 76, (3), 1213.
14. Khademhosseini, A.; Suh, K. Y.; Jon, S.; Eng, G.; Yeh, J.; Chen, G.-J.; Langer, R., A soft lithographic approach to fabricate patterned microfluidic channels. *Anal. Chem.* **2004**, 76, (13), 3675-.
15. Yu, L.; Li, C. M.; Zhou, Q., Efficient probe immobilization on poly (dimethylsiloxane) for sensitive detection of proteins. *Front. Biosci.* **2005**, 10, 2848.
16. Liu, Y. S.; Li, C. M.; Yu, L.; Chen, P., Optimization of printing buffer for protein microarrays based on aldehyde-modified glass slides. *Front. Biosci.* **2007**, 12, 3768.
17. Wu, R.; Hu, S.; Xiao, Y.; Li, Z.; Shi, D.; Bi, D., Development of indirect enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection and quantification of antibodies against avian influenza virus. *Vet Res Commun.* **2007**, 31, (5), 631-641.
18. He, Q.; Manopo, I.; Lu, L.; Leung, B. P.; Chng, H. H.; Ling, A. E.; Chee, L. L.; Chan, S.-W.; Ooi, E. E.; Sin, Y. L.; Ang, B.; Kwang, J., Novel immunofluorescence assay using recombinant nucleocapsid-spike fusion protein as antigen to detect antibodies against Severe Acute Respiratory Syndrome coronavirus. *Clin. Diagn. Lab. Immunol.* **2005**, 12, (2), 321-328.
19. Huang, L.-J.; Liu, C.-Y.; Chu, S.-C.; Wong, W.-W.; Lin, Y.-C.; Liu, W.-T.; Chan, Y.-J., Predictive value of two commercial human immunodeficiency virus serological tests in cases with indeterminate Western blot results. *J Microbiol Immunol Infect.* **2006**, 39, (3219-24), 219-224.
20. Erdenebaatar, J.; Sugar, S.; Yondondorj, A.; Nagabayashi, T.; Syuto, B.; Watarai, M.; Makino, S.; Shirahata, T., Serological differentiation of Brucella-vaccinated and -infected domesticated animals by the agar gel immunodiffusion test using Brucella polysaccharide in mongolia. *J Vet Med Sci.* **2002**, 64, (9), 839-841.
21. Herrmann, M.; Veres, T.; Tabrizian, M., Enzymatically-generated fluorescent detection in micro-channels with internal magnetic mixing for the development of parallel microfluidic ELISA. *Lab Chip* **2006**, 6, (4), 555.
22. Takatsu, K.; Tominaga, A.; Harada, N.; Mita, S.; Matsumoto, M.; Takahashi, T.; Kikuchi, Y.; Yamaguchi, N., T cell-replacing factor (TRF)/Interleukin 5 (IL-5): Molecular and functional properties. *Immunological Reviews* **1988**, 102, (1), 107-.
23. Secor, W. E.; Stewart, S. J.; Colley, D. G., Eosinophils and immune mechanisms. VI. The synergistic combination of granulocyte-macrophage colony-stimulating factor and IL-5 accounts for eosinophil-stimulation promoter activity in *Schistosoma mansoni*- infected mice. *J Immunol*

1990, 144, (4), 1484-.

24. Huang, C. S.; Chen, S. J.; Chung, R. L.; Tang, R. B., Serum interleukin-5 measurements for monitoring acute asthma in children. *J Asthma*. **2005**, 42, (4), 297-300.
25. Pittock, S. J.; Lennon, V. A.; de Seze, J.; Vermersch, P.; Homburger, H. A.; Wingerchuk, D. M.; Lucchinetti, C. F.; Zéphir, H.; Moder, K.; Weinshenker, B. G., Neuromyelitis optica and non organ-specific autoimmunity. *Arch Neurol*. **2005**, 65, (1), 78-83.
26. Voldman, J.; Gray, M. L.; Schmidt, M. A., Microfabrication in biology and medicine. *Annu. Rev. Biomed. Eng.* **1999**, 1, (1), 401-.
27. Lion, N.; Reymond, F.; Girault, H. H.; Rossier, J. S., Why the move to microfluidics for protein analysis? *Curr Opin Biotechnol* **2004**, 15, (1), 31-37.
28. Lin, F. Y. H.; Sabri, M.; Alirezaie, J.; Li, D. Q.; Sherman, P. M., Development of a nanoparticle-labeled microfluidic immunoassay for detection of pathogenic microorganisms. *Clin. Diag. Lab Immunol* **2005**, 12, (3), 418.
29. Yuan, H.; Mullett, W. M.; Pawliszyn, J., Biological sample analysis with immunoaffinity solid-phase microextraction. *Analyst* **2001**, 126, (8), 1456-.
30. Sander, G. H. W.; Manz, A., Chip-based microsystems for genomic and proteomic analysis. *Trends Anal. Chem.* **2000**, 19, (6), 364.
31. Kopp, M. U.; de Mello, A. J.; Manz, A., Chemical amplification: continuous-flow PCR on a chip. *Science* **1998**, 280, 1046.
32. Chiem, N.; Harrison, D. J., Microchip-based capillary electrophoresis for immunoassays: analysis of monoclonal antibodies and theophylline. *Anal. Chem.* **1997**, 69, (3), 373.
33. Harrison, D. J.; Fluri, K.; Seiler, K.; Fan, Z.; Effenhauser, C. S.; Manz, A., Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. *Science* **1993**, 261, (895).
34. Chiem, N. H.; Harrison, D. J., Microchip systems for immunoassay: an integrated immunoreactor with electrophoretic separation for serum theophylline determination. *Clin. Chem.* **1998**, 44, (3), 591-.
35. Fintschenko, Y.; Wilson, G. S., Flow injection immunoassays: a review. *Mikrochimica Acta* **1998**, 129, (1-2), 7-.
36. Rossier, J. S.; Schwarz, A.; Reymond, F.; Ferrigno, R.; Bianchi, F.; Girault, H. H., Microchannel networks for electrophoretic separations. *Electrophoresis* **1999**, 20, 727.
37. Tambe, N. S.; Bhushan, B., Micro/nanotribological characterization of PDMS and PMMA used for BioMEMS/NEMS applications. *Ultramicroscopy* **2005**, 105, (1-4), 238.
38. Xiang, Q.; Hu, G.; Gao, Y.; Li, D., Miniaturized immunoassay microfluidic system with electrokinetic control. *Biosens. Bioelectron.* **2006**, 21, (10), 2006.
39. Lee, W.; Lim, S. S.; Choi, B. K.; Choi, J. W., Protein array fabricated by microcontact printing for miniaturized immunoassay. *J Microbio. Biotech.* **2006**, 16, (8), 1216.
40. Bange, A.; Halsall, H. B.; Heineman, W. R., Microfluidic immunosensor systems. *Biosens. Bioelectron.* **2005**, 20, (12), 2488.
41. Sasakura, Y.; Kanda, K.; Fukuzono, S., Microarray techniques for more rapid protein quantification: Use of single spot multiplex analysis and a vibration reaction unit. *Anal. Chim. Acta* **2006**, 564, (1), 53.
42. MacBeath, G.; Schreiber, S. L., Printing proteins as microarrays for high-throughput function determination. *Science* **2000**, 289, 1760.
43. Tao, S. C.; Chen, C. S.; Zhu, H., Applications of protein microarray technology. *Comb Chem High Throughput Screen.* **2007**, 10, (8), 706.
44. Angenendt, P.; Glöckler, J.; Sobek, J.; Lehrach, H.; Cahill, D. J., Next generation of protein microarray support materials: Evaluation for protein and antibody microarray applications. *J Chromatogr A* **2003**, 1009, (1-2), 97-.
45. Zhou, Y.; Andersson, O.; Lindberg, P.; Liedberg, B., Protein microarrays on carboxymethylated dextran hydrogels: Immobilization, characterization and application. *Mikrochimica Acta* **2004**, 147, (1-2), 21.
46. Grasso, V.; Lambertini, V.; Ghisellini, P.; Valerio, F.; Stura, E.; Perlo, P.; Nicolini, C., Nanostructuring of a porous alumina matrix for a biomolecular microarray. *Nanotechnology* **2006**, 17, (3), 795.
47. Zhou, X. C.; Zhou, J. Z., Protein microarrays on hybrid polymeric thin films prepared by self-assembly of polyelectrolytes for multiple-protein immunoassays. *Proteomics* **2006**, 6, (5), 1415.
48. Tamarit-Lopez, J.; Morais, S.; Puchades, R.; Maquieira, A., Use of polystyrene spin-coated compact discs for microimmunoassaying. *Anal. Chim. Acta.* **2008**, 609, (1), 120-.

49. Daniela, H.; Patrick, M.; Christine, V.; Dominique, H.; Frédéric, R.; Joël, S. R., Disposable microfluidic ELISA for the rapid determination of folic acid content in food products. *Anal. Bioanal. Chem.* **2007**, 387, (1), 267.
50. Lim, C. T.; Zhang, Y., Bead-based microfluidic immunoassays: The next generation. *Biosens. Bioelectron.* **2007**, 22, (7), 1197.
51. Bernard, A.; Michel, B.; Delamarche, E., Micromosaic Immunoassays. *Anal. Chem.* **2001**, 73, (1), 8-.
52. Hatch, A.; Kamholz, A. E.; Hawkins, K. R.; Munson, M. S.; Schilling, E. A.; Weigl, B. H.; Yager, P., A rapid diffusion immunoassay in a T-sensor. *Nature Biotech* **2001**, 19, (5), 461-.
53. Hu, W.; Li, C. M.; Cui, X.; Dong, H.; Zhou, Q., In situ studies of protein adsorptions on poly(pyrrole-co-pyrrole propylic acid) film by electrochemical surface plasmon resonance. *Langmuir* **2007**, 23, (5), 2761.
54. Tsai, W. C.; Hsieh, C. K., QCM-based immunosensor for the determination of ochratoxin A. *Anal. Lett.* **2007**, 40, (10), 1979.
55. Rahman, M. A.; Shiddiky, M. J. A.; Park, J. S.; Shim, Y. B., An impedimetric immunosensor for the label-free detection of bisphenol A. *Biosens. Bioelectron.* **2007**, 22, (11), 2464-2470.
56. Vareiro, M. L. M.; Liu, J.; Knoll, W.; Zak, K.; Williams, D.; Jenkins, A. T. A., Surface plasmon fluorescence measurements of human chorionic gonadotrophin: role of antibody orientation in obtaining enhanced sensitivity and limit of detection. *Anal. Chem.* **2005**, 77, (8), 2426.
57. Pamme, N.; Koyama, R.; Manz, A., Counting and sizing of particles and particle agglomerates in a microfluidic device using laser light scattering: application to a particle-enhanced immunoassay. *Lab Chip* **2003**, 3, (3), 187-.
58. Chabinyk, M. L.; Chiu, D. T.; McDonald, J. C.; Stroock, A. D.; Christian, J. F.; Karger, A. M.; Whitesides, G. M., An integrated fluorescence detection system in poly(dimethylsiloxane) for microfluidic applications. *Anal. Chem.* **2001**, 73, (18), 4491-.
59. Roos, P.; Skinner, C. D., A two bead immunoassay in a micro fluidic device using a flat laser intensity profile for illumination. *Analyst* **2003**, 128, (6), 527-.
60. Zhu, L.; Zhang, Q.; Feng, H.; Ang, S.; Chau, F. S.; Liu, W.-T., Filter-based microfluidic device as a platform for immunofluorescent assay of microbial cells. *Lab Chip* **2004**, 4, (4), 337.
61. Yadavalli, V. K.; Pishko, M. V., Biosensing in microfluidic channels using fluorescence polarization. *Anal. Chim. Acta* **2004**, 507, (1), 123-128.
62. Moorthy, J.; Mensing, G. A.; Kim, D.; Mohanty, S.; Eddington, D. T.; H., T. W.; Johnson, E. A.; Beebe, D. J., Microfluidic tectonics platform: A colorimetric, disposable botulinum toxin enzyme-linked immunosorbent assay system. *Electrophoresis* **2004**, 25, (10-11), 1705.
63. Lin, F. Y. H.; Sabri, M.; Erickson, D.; Alirezaie, J.; Li, D.; Sherman, P. M., Development of a novel microfluidic immunoassay for the detection of *Helicobacter pylori* infection. *Analyst* **2004**, 129, (9), 823.
64. Michalzik, M.; Wilke, R.; Buttgenbach, S., Miniaturized QCM-based flow system for immunosensor application in liquid. *Sens. Actuators B.* **2005**, 111, 410.
65. Luo, C. X.; Fu, Q.; Li, H.; Xu, L. P.; Sun, M. H.; Ouyang, Q.; Chen, Y.; Ji, H., PDMS microfluidic device for optical detection of protein immunoassay using gold nanoparticles. *Lab Chip* **2005**, 5, (7), 726.
66. Zhang, M.; Gorski, W., Electrochemical sensing based on redox mediation at carbon nanotubes. *Anal. Chem.* **2005**, 77, (13), 3960.
67. Rossier, J.; Reymond, F.; Michel, P. E., Polymer microfluidic chips for electrochemical and biochemical analyses. *Electrophoresis* **2002**, 23, (6), 858.
68. Diaz-Gonzalez, M.; Gonzalez-Garcia, M. B.; Costa-Garcia, A., Recent advances in electrochemical enzyme immunoassays. *Electroanalysis* **2005**, 17, (21), 1901.
69. Ko, J. S.; Yoon, H. C.; Yang, H.; Pyo, H.-B.; Chung, K. H.; Kim, S. J.; Kim, Y. T., A polymer-based microfluidic device for immunosensing biochips. *Lab Chip* **2003**, 3, (2), 106-.
70. Dong, H.; Li, C. M.; Zhou, Q.; Sun, J. B.; Miao, J. M., Sensitive electrochemical enzyme immunoassay microdevice based on architecture of dual ring electrodes with a sensing cavity chamber. *Biosens. Bioelectron.* **2006**, 22, (5), 621.
71. Dong, H.; Li, C. M.; Zhang, Y. F.; Cao, X. D.; Gan, Y., Screen-printed microfluidic device for electrochemical immunoassay. *Lab Chip* **2007**, 7, 1752.
72. Henares, T. G.; Mizutani, F.; Hisamoto, H., Current development in microfluidic immunosensing chip. *Anal. Chim. Acta* **2008**, 611, (1), 17-30.
73. Yakovleva, J.; Davidsson, R.; Bengtsson, M.; Laurell, T.; Emneus, J., Microfluidic enzyme immunosensors with immobilised protein A and G using chemiluminescence detection. *Biosens.*

Bioelectron. **2003**, 19, (1), 21-.

74. Marinova, K. G.; Christova, D.; Tcholakova, S.; Efremov, E.; Denkov, N. D., Hydrophobization of glass surface by adsorption of poly(dimethylsiloxane). *Langmuir* **2005**, 21, (25), 11729.
75. Wu, D. P.; Luo, Y.; Zhou, X. M.; Dai, Z. P.; Lin, B. C., Multilayer poly(vinyl alcohol)-adsorbed coating on poly(dimethylsiloxane) microfluidic chips for biopolymer separation. *Electrophoresis* **2005**, 26, (1), 211.
76. Kurkuri, M. D.; Aminabhavi, T. M., Poly(vinyl alcohol) and poly(acrylic acid) sequential interpenetrating network pH-sensitive microspheres for the delivery of diclofenac sodium to the intestine. *J Control. Release* **2004**, 96, (1), 9-.
77. Prakash, S.; Long, T. M.; Selby, J. C.; Moore, J. S.; Shannon, M. A., "Click" modification of silica surfaces and glass microfluidic channels. *Anal. Chem.* **2007**, 79, (4), 1661-1667.
78. Toepke, M. W.; Beebe, D. J., PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip* **2006**, 6, (12), 1484.
79. Wu, D.; Zhao, B.; Dai, Z.; Qin, J.; Lin, B., Grafting epoxy-modified hydrophilic polymers onto poly(dimethylsiloxane) microfluidic chip to resist nonspecific protein adsorption. *Lab Chip* **2006**, 6, (7), 942.
80. Chen, H. Y.; Elkasabi, Y.; Lahann, J., Surface modification of confined microgeometries via vapor-deposited polymer coatings. *J. Am. Chem. Soc.* **2006**, 128, (1), 374.
81. Asberg, P.; Nilsson, K. P. R.; Inganas, O., Surface energy modified chips for detection of conformational states and enzymatic activity in biomolecules. *Langmuir* **2006**, 22, (5), 2205.
82. Sui, G.; Wang, J.; Lee, C. C.; Lu, W.; Lee, S. P.; Leyton, J. V.; Wu, A. M.; Tseng, H. R., Solution-phase surface modification in intact poly(dimethylsiloxane) microfluidic channels. *Anal. Chem.* **2006**, 78, (15), 5543.
83. Miksa, D.; Irish, E. R.; Chen, D.; Composto, R. J.; Eckmann, D. M., Dextran functionalized surfaces via reductive amination: Morphology, wetting, and adhesion. *Biomacromolecules* **2006**, 7, (2), 557.
84. Wang, A.-J.; Xu, J.-J.; Chen, H.-Y., Proteins modification of poly(dimethylsiloxane) microfluidic channels for the enhanced microchip electrophoresis. *J Chromatogr A* **2006**, 1107, (1-2), 257.
85. Thorslund, S.; Sanchez, J.; Larsson, R.; Nikolajeff, F.; Bergquist, J., Functionality and stability of heparin immobilized onto poly(dimethylsiloxane). *Colloids Surf. B.* **2005**, 45, (2), 76.
86. Xia, Y.; McClelland, J. J.; Gupta, R.; Qin, D.; Zhao, X.-M.; Sohn, L. L.; Celotta, R. J.; Whitesides, G. M., Replica molding using polymeric materials. A practical step toward nanomanufacturing. *Adv.Mater.* **1997**, 9, 147.
87. Malek, C. G. K., Laser processing for bio-microfluidics applications (part I). *Anal. Bioanal. Chem.* **2006**, 385, (8), 1351.
88. Bartnik, A.; Fiedorowicz, H.; Jarocki, R.; Juha, L.; Kostecki, J.; Rakowski, R.; Szczurek, M., Micromachining of organic polymers by X-ray photo-etching using a 10 Hz laser-plasma radiation source. *Microelectronic Eng.* **2005**, 78-79, 452.
89. Cheng, J. Y.; Wei, C. W.; Hsu, K. H.; Young, T. H., Direct-write laser micromachining and universal surface modification of PMMA for device development. *Sens. Actuators B.* **2004**, 99, (1), 186-.
90. Chung, C. K.; Lin, Y. C.; Huang, G. R., Bulge formation and improvement of the polymer in CO₂ laser micromachining. *J Micromech Microeng* **2005**, 15, (10), 1878.
91. Fiedorowicz, H.; Bartnik, A.; Bittner, A.; Juha, L.; Krasa, J.; Kubat, P.; Mikolajczyk, J.; Rakowski, R., Micromachining of organic polymers by direct photo-etching using a laser plasma X-ray source. *Microe. Eng.* **2004**, 73-74, 336-.
92. Kapur, R.; Rudolph, A. S., Cellular and cytoskeleton morphology and strength of adhesion of cells on self-assembled monolayers of organosilanes. *Exp.Cell Res.* **1998**, 224, 275.
93. Dadsetan, M.; Jones, J. A.; Hiltner, A.; Anderson, J. M., Surface chemistry mediates adhesive structure, cytoskeletal organization, and fusion of macrophages. *J Biomed. Mater. Res. A.* **2004**, 71A, (3), 439-.
94. Ratner, B. D.; Bryant, S. J., Biomaterials: Where we have been and where we are going. *Annu. Rev. Biomed. Eng.* **2004**, 6, (1), 41.
95. Sethuraman, A.; Han, M.; Kane, R. S.; Belfort, G., Effect of surface wettability on the adhesion of proteins. *Langmuir* **2004**, 20, (18), 7779.
96. Mansur, H. S.; Oréfice, R. L.; Vasconcelos, W. L.; Lobato, Z. P.; Machado, L. J. C., Biomaterial with chemically engineered surface for protein immobilization. *J Mater Sci. Mater Medic* **2005**, 16, (4), 333.

97. Yu, L.; Li, C. M.; Zhou, Q.; Luong, J. H. T., Poly(vinyl alcohol) functionalized poly(dimethylsiloxane) solid surface for immunoassay. *Bioconjug. Chem.* **2007**, 18, (2), 281-.
98. Burton, Z.; Bhushan, B., Hydrophobicity, adhesion, and friction properties of nanopatterned polymers and scale dependence for micro- and nanoelectromechanical systems. *Nano Lett.* **2005**, 5, (8), 1607.
99. Berdichevsky, Y.; Khandurina, J.; Guttman, A.; Lo, Y. H., UV/ozone modification of poly(dimethylsiloxane) microfluidic channels. *Sens. Actuators B.* **2004**, 97, (2-3), 402-.
100. Efimenko, K.; Wallace, W. E.; Genzer, J., Surface modification of sylgard-184 poly(dimethyl siloxane) networks by ultraviolet and ultraviolet/ozone treatment. *J. Colloid Interface Sci.* **2002**, 254, (2), 306-.
101. Efimenko, K.; Crowe, J. A.; Manias, E.; Schwark, D. W.; Fischer, D. A.; Genzer, J., Rapid formation of soft hydrophilic silicone elastomer surfaces. *Polymer* **2005**, 46, (22), 9329.
102. Morra, M.; Occhiello, E.; Garbassi, F.; Maestri, M.; Bianchi, R.; Zonta, A., The characterization of plasma-modified polydimethylsiloxane interfaces with media of different surface energy. *Clin. Materials* **1990**, 5, (2-4), 147-.
103. Fritz, J. L.; Owen, M. J., Hydrophobic recovery of plasma-treated polydimethylsiloxane. *J. Adhes.* **1995**, 54, (1), 33 -
104. Owen, M. J.; Smith, P. J., Plasma treatment of polydimethylsiloxane. *J. Adhes. Sci. Technol.* **1994**, 8, 1063.
105. Tserepi, A.; Gogolides, E.; Tsougeni, K.; Constantoudis, V.; Valamontes, E. S., Tailoring the surface topography and wetting properties of oxygen-plasma treated polydimethylsiloxane. *J. Appl. Phys.* **2005**, 98, (11).
106. Bodas, D.; Khan-Malek, C., Hydrophilization and hydrophobic recovery of PDMS by oxygen plasma and chemical treatment - An SEM investigation. *Sens. Actuators B.* **2007**, 123, (1), 368.
107. Vesenka, J.; Guthold, M.; Tang, C. L.; Keller, D.; Delaine, E.; Bustamante, C., Substrate preparation for reliable imaging of DNA molecules with the scanning force microscope. *Ultramicroscopy* **1992**, 42-44, (Part 2), 1243-.
108. Stubbings, D.; Bubb, M. O.; Conradie, J. D., Two methods for the introduction of amino groups into agarose-based matrices: Their use in immunoaffinity chromatography. *Anal. Biochem.* **1993**, 210, (1), 159-.
109. Heiney, P. A.; Gruneberg, K.; Fang, J.; Dulcey, C.; Shashidhar, R., Structure and growth of chromophore-functionalized (3-aminopropyl)triethoxysilane self-assembled on silicon. *Langmuir* **2000**, 16, (6), 2651-2657.
110. Sumanda, B.; De, P. P.; Tripathy, D. K.; De, S. K., 3-Aminopropyltriethoxysilane as a promoter in the crosslinking of carboxylated nitrile rubber by surface-oxidized carbon black. *J. Appl. Polym. Sci.* **1996**, 61, (10), 1813-.
111. Shimizu, I.; Yoshino, A.; Okabayashi, H.; Nishio, E.; Oconnor, C. J., Kinetics of interaction of 3-aminopropyltriethoxysilane on a silica gel surface using elemental analysis and diffuse reflectance infrared Fourier transform spectra. *J. Chem. Soc., Faraday Trans.* **1997**, 93, (10), 1971-.
112. Simon, A.; Cohen, T.; Bouhacina, M. C.; Porte, J. P.; Aime, C.; Baquey, Study of two grafting methods for obtaining a 3-Aminopropyltriethoxysilane monolayer on silica surface. *J. Colloid Interface Sci.* **2002**, 251, 278.
113. Howarter, J. A.; Youngblood, J. P., Surface modification of polymers with 3-Aminopropyltriethoxysilane as a general pretreatment for controlled wettability. *Macromolecules* **2007**, 40, (4), 1128.
114. Phely-Bobin, T. S.; Muisener, R. J.; Koberstein, J. T.; Papadimirakopoulos, F., Site-specific self-assembly of Si/SiO_x nanoparticles on micropatterned poly(dimethylsiloxane) thin films. *Synthetic Metals* **2001**, 116, (1-3), 439-.
115. Diaz-Quijada, G. A.; Wayner, D. D. M., A simple approach to micropatterning and surface modification of poly(dimethylsiloxane). *Langmuir* **2004**, 20, (22), 9607-.
116. Makamba, H.; Hsieh, Y. Y.; Sung, W. C.; Chen, S. H., Stable permanently hydrophilic protein-resistant thin-film coatings on poly(dimethylsiloxane) substrates by electrostatic self-assembly and chemical cross-linking. *Anal. Chem.* **2005**, 77, (13), 3971.
117. Kamande, M. W.; Fletcher, K. A.; Lowry, M.; Warner, I. M., Capillary electrochromatography using polyelectrolyte multilayer coatings. *J. Sep. Sci.* **2005**, 28, (8), 710.
118. Peterson, S. L.; McDonald, A.; Gourley, P. L.; Sasaki, D. Y., Poly(dimethylsiloxane) thin films as biocompatible coatings for microfluidic devices: Cell culture and flow studies with glial cells. *J. Biomed Mater Res. A* **2005**, 72A, (1), 10.

119. Eteshola, E.; Leckband, D., Development and characterization of an ELISA assay in PDMS microfluidic channels. *Sens. Actuator B* **2001**, 72, (2), 129-.
120. Llopis, S. L.; Osiri, J.; Soper, S. A., Surface modification of poly(methyl methacrylate) microfluidic devices for high-resolution separations of single-stranded DNA. *Electrophoresis* **2007**, 28, (6), 984.
121. Hu, S.; Ren, X.; Bachman, M.; Sims, C. E.; Li, G. P.; Allbritton, N., Surface modification of poly(dimethylsiloxane) microfluidic devices by ultraviolet polymer grafting. *Anal. Chem.* **2002**, 74, (16), 4117-.
122. Tsukagoshi, T.; Kondo, Y.; Yoshino, N., Surface modification of poly(oligoethylene oxide methacrylate) for resisting protein adsorption. *Colloids Surf. B.* **2007**, 54, (1), 94.
123. Roman, G. T.; Culbertson, C. T., Surface engineering of poly(dimethylsiloxane) microfluidic devices using transition metal sol-gel chemistry. *Langmuir* **2006**, 22, (9), 4445.
124. Bodas, D.; Khan-Malek, C., Formation of more stable hydrophilic surfaces of PDMS by plasma and chemical treatments. *Microe. Eng.* **2006**, 83, (4-9), 1277.
125. Li, J. F.; An, S. Z.; Li, D. X.; Jiao, Y., UV curing polymer materials for making laser glass. *Polym. Adv. Technol.* **1999**, 10, (9), 525-.
126. Chu, L.-Q.; Knoll, W.; Förch, R., Plasma polymerized epoxide functional surfaces for DNA probe immobilization. *Biosens. Bioelectron.* **2008**, 24, (1), 118-.
127. Bae, S. C.; Lee, H.; Lin, Z.; Granick, S., Chemical imaging in a surface forces apparatus: Confocal Raman spectroscopy of confined poly(dimethylsiloxane). *Langmuir* **2005**, 21, (13), 5685.
128. NanoScope®, Command Referece Manual. *Digital Intstruments Inc., Santa Barbara, CA* Chapter 14.
129. Crampton, N.; Bonass, W. A.; Kirkham, J.; Thomson, N. H., Formation of aminosilane-functionalized mica for atomic force microscopy imaging of DNA. *Langmuir* **2005**, 21, (17), 7884.
130. NanoScope®, Force Imaging. In Inc., V. I., Ed. 2004; Vol. Version 5.
131. Wallace, J. M.; Fox, P. F., Rapid spectrophotometric and fluorimetric methods for monitoring nitrogenous (proteinaceous) compounds in cheese and cheese fractions: a review. *Food Chem.* **1998**, 62, (2), 217-224.
132. Zhu, Y. B.; Chian, K. S.; Chan-Park, M. B.; Mhaisalkar, P. S.; Ratner, B. D., Protein bonding on biodegradable poly(L-lactide-co-caprolactone) membrane for esophageal tissue engineering. *Biomaterials* **2006**, 27, (1), 68.
133. Penney, D. P.; Powers, J. M.; Frank, M.; Willis, C.; Churukian, C., Analysis and testing of biological stains - The Biological Stain Commission procedures. *Biotech. Histochem.* **2002**, 77, (5-6), 237-.
134. Yu, T. T.; Shoichet, M. S., Guided cell adhesion and outgrowth in peptide-modified channels for neural tissue engineering. *Biomaterials* **2005**, 26, (13), 1507.
135. Konings, B. L. J. C.; Pelssers, E. G. M.; Verhoeven, A. J. C. M.; Kamps, K. M. P., Covalent coupling of antibodies to hydrophilic core-shell particles. *Colloids and Surfaces B: Biointerfaces* **1993**, 1, (2), 69-73.
136. Elwing, H.; Nilsson, B.; Svensson, K.-E.; Askendahl, A.; Nilsson, U. R.; Lundstr 鰳, I., Conformational changes of a model protein (complement factor 3) adsorbed on hydrophilic and hydrophobic solid surfaces. *Journal of Colloid and Interface Science* **1988**, 125, (1), 139-145.
137. Greg, H. T., *Bioconjugate Techniques*. Academic Press, Inc.: 1995; p 785 pages.
138. Thermo, S., EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) Instruction. In Thermo Fisher Scientific Inc.: 2007.
139. Li, C. M. Microchip and method for detecting molecules and molecular interactions. 13.10., 2005.
140. Nikon, ACT-2U Application for Digital Camera Ver.1.3. *Nikon Inttech CO. LTD. Japan*, 67.
141. Diamandis, E. P.; Christopoulos, T. K., *Immunoassay*. Academic Press: New York, 1996.
142. Price, C. P.; Newman, D. J., *Principles and Practice of Immunoassay*. 2nd ed.; Stockton Press: New York, 1997.
143. Lesaichere, M. L.; Lue, R. Y. P.; Chen, G. Y. J.; Zhu, Q.; Yao, S. Q., Intein-mediated biotinylation of proteins and its application in a protein microarray. *J.Am.Chem.Soc.* **2002**, 124, 8768.
144. Micheli, L.; Radoi, A.; Guarrina, R.; Massaud, R.; Bala, C.; Moscone, D.; Palleschi, G., Disposable immunosensor for the determination of domoic acid in shellfish. *Biosens. Bioelectron.* **2004**, 20, 190.
145. Buijs, J.; Lichtenbelt, J., W.Th.; Norde, W.; Lyklema, J., Adsorption of monoclonal IgGs

- and their F(ab')₂ fragments onto polymeric surfaces. *Colloids Surf. B.* **1995**, 5, 11.
146. Chen, S.; Liu, L.; Zhou, J.; Jiang, S., Controlling antibody orientation on charged self-assembled monolayers. *Langmuir* **2003**, 19, 2859.
 147. Xu, H.; Lu, J. R.; Williams, D. E., Effect of surface packing density of interfacially adsorbed monoclonal antibody on the binding of hormonal antigen human chorionic gonadotrophin. *J. Phys. Chem. B* **2006**, 110, (4), 1907.
 148. Linder, V.; Verpoorte, E.; Thormann, W.; de Rooij, N. F.; Sigrüst, H., Surface biopassivation of replicated poly(dimethylsiloxane) microfluidic channels and application to heterogeneous immunoreaction with on-chip fluorescence detection. *Anal. Chem.* **2001**, 73, (17), 4181-.
 149. Kamysny, A.; Lagerge, S.; Partyka, S.; Relkin, P.; Magdassi, S., Adsorption of native and hydrophobized human IgG onto silica: isotherms, calorimetry, and biological activity. *Langmuir* **2001**, 17, (26), 8242-.
 150. Grogan, C.; Raiteri, R.; O'Connor, G. M.; Glynn, T. J.; Cunningham, V.; Kane, M.; Charlton, M.; Leech, D., Characterisation of an antibody coated microcantilever as a potential immuno-based biosensor. *Biosens. Bioelectron.* **2002**, 17, 201.
 151. Yakovleva, J.; Davidsson, R.; Lobanova, A.; Bengtsson, M.; Eremin, S.; Laurell, T.; Emneus, J.; 2002, Microfluidic enzyme immunoassay using silicon microchip with immobilized antibodies and chemiluminescence detection. *Anal. Chem.* **2002**, 74, 2994.
 152. Butler, J. E., Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays. *Methods* **2000**, 22, 4.
 153. Wang, J.; McGuire, J., Surface tension kinetics of the wild type and four synthetic stability mutants of T4 phage lysozyme at the air-water interface. *J. Colloid Interface Sci.* **1997**, 185, (2), 317-323.
 154. Joshi, O.; Lee, H. J.; McGuire, J.; Finneran, P.; Bird, K. E., Protein concentration and adsorption time effects on fibrinogen adsorption at heparinized silica interfaces. *Colloids Surf. B.* **2006**, 50, (1), 26.
 155. Flach, C. R.; Brauner, J. W.; Taylor, J. W.; Baldwin, R. C.; Mendelsohn, R., External reflection ftir of peptide monolayer films in-situ at the air/water interface - experimental-design, spectra-structure correlations, and effects of hydrogen-deuterium exchange. *Biophys J.* **1994**, 67, (1), 402-.
 156. Yuan, W.; Dong, H.; Li, C. M.; Cui, X.; Yu, L.; Lu, Z.; Zhou, Q., pH-controlled construction of chitosan/alginate multilayer film: Characterization and application for antibody Immobilization. *Langmuir* **2007**, 23, (26), 13046-13052.
 157. Sapsford, K. E.; Ligler, F. S., Real-time analysis of protein adsorption to a variety of thin films. *Biosens. Bioelectron.* **2004**, 19, (9), 1045.
 158. Kim, D. T.; Blanch, H. W.; Radke, C. J., Direct imaging of lysozyme adsorption onto mica by atomic force microscopy. *Langmuir* **2002**, 18, (15), 5841-.
 159. Agnihotri, A.; Siedlecki, C. A., Time-dependent conformational changes in fibrinogen measured by atomic force microscopy. *Langmuir* **2004**, 20, (20), 8846-.
 160. Noh, H.; Vogler, E. A., Volumetric interpretation of protein adsorption: Mass and energy balance for albumin adsorption to particulate adsorbents with incrementally increasing hydrophilicity. *Biomaterials* **2006**, 27, (34), 5801.
 161. Sjöholm, I., Protein A from *Staphylococcus aureus*: spectropolarimetric and spectrophotometric studies. *Eur. J. Biochem* **1975**, 51, 55.
 162. Polzius, R.; Schneider, T.; Biert, F. F.; Bilitewski, U.; Koschinski, W., Optimization of biosensing using grating couplers: immobilization on tantalum oxide waveguides. *Biosens. Bioelectron.* **1996**, 11, 503.
 163. Coleman, P. L.; Walker, M. M.; Milbrath, D. S.; Stauffer, D. M., Immobilization of Protein A at high density on azlactone-functional polymeric beads and their use in affinity chromatography. *J. Chromatogr A* **1990**, 512, 345.
 164. Dubrovsky, T.; Tronin, A.; Dubrovskaya, S.; Vakula, S.; Nicolini, C., Immunological activity of IgG Langmuir films oriented by protein A sublayer. *Sens. Actuator B* **1995**, 23, 1.
 165. Ohnishi, S.; Murata, M.; Hato, M., Correlation between surface morphology and surface forces of protein A adsorbed on mica *Biophys J.* **1998**, 74, (1), 455.
 166. Coen, M. C.; Lehmann, R.; Groning, P.; Biemann, M.; Galli, C.; Schlapbach, L., Adsorption and bioactivity of protein A on silicon surfaces studied by AFM and XPS *J Colloid Interface Sci.* **2001**, 233, (2), 180.
 167. Lee, W.; Oh, B. K.; Bae, Y. M.; Paek, S. H.; Lee, W. H.; Choi, J. W., Fabrication of self-assembled protein A monolayer and its application as an immunosensor. *Biosens.*

- Bioelectron.* **2003**, 19, 185.
168. Pietrasanta, L. I.; Thrower, D.; Hsieh, W.; Rao, S.; Stemmann, O.; Lechner, J.; Carbon, J.; Hansma, H., Probing the *Saccharomyces cerevisiae* centromeric DNA (CEN DNA)-binding factor 3(CBF3) kinetochore complex by using atomic force microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, 96, (7), 3757-3762.
 169. Bagchi, P.; Birnbaum, S. M., Effect of pH on the adsorption of immunoglobulin G on anionic poly(vinyltoluene) model latex particles. *J Colloid Inter Sci* **1981**, 83, (2), 460-.
 170. Schneider, S. W.; Larmer, J.; Henderson, R. M.; Oberleithner, H., Molecular weights of individual proteins correlate with molecular volumes measured by atomic force microscopy. *Pflugers Arch-Eur J. Physiol* **1998**, 435, (3), 362-367.
 171. Yokoyama, Y.; Ishiguro, R.; Maeda, H.; Mukaiyama, M.; Kameyama, K.; Hiramatsu, K., Quantitative analysis of protein adsorption on a planar surface by Fourier transform infrared spectroscopy: lysozyme adsorbed on hydrophobic silicon-containing polymer. *J Colloid Interface Sci.* **2003**, 268, (1), 23-.
 172. Clark, A. J.; Kotlicki, A.; Haynes, C. A.; Whitehead, L. A., A new model of protein adsorption kinetics derived from simultaneous measurement of mass loading and changes in surface energy. *Langmuir* **2007**, 23, (10), 5591.
 173. Dubrovsky, T.; Tronin, A.; Dubrovskaya, S.; Guryev, O.; Nicolini, C., Langmuir films of Fc binding receptors engineered from protein A and protein G as a sublayer for immunoglobulin orientation. *Thin Solid Films* **1996**, 284-285, 698-702.
 174. Zhang, M.; Desai, T.; Ferrari, M., Proteins and cells on PEG immobilized silicon surfaces. *Biomaterials* **1998**, 19, (953).
 175. Pesek, J. J.; Matyska, M. T.; Pik Fong, F., Evaluation of the silanization/Hydrosilation process for the synthesis of chiral stationary phases. *Chromatographia* **2001**, 53, (11), 635-.
 176. Allan, J. S.; Coligan, J. E.; Barin, F.; McLane, M. F.; Sodroski, J. G.; Rosen, C. A.; Haseltine, W. A.; Lee, T. H.; Essex, M., Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* **1985**, 228, (4703), 1091-.
 177. Faucher, S.; Martel, A.; Sherring, A.; Bogdanovic, D.; Malloch, L.; Kim, J. E.; Bergeron, M.; Sandstrom, P.; Mandy, F. F., A combined HIV-1 protein bead array for serology assay and T-cell subset immunophenotyping with a hybrid flow cytometer: A step in the direction of a comprehensive multitasking instrument platform for infectious disease diagnosis and monitoring. *Cytometry B.* **2006**, 70B, (3), 179.
 178. Eriksson, K.; Nordstrom, I.; Horal, P.; Jeansson, S.; Svennerholm, B.; Vahlne, A.; Holmgren, J.; Czerkinsky, C., Amplified elispot assay for the detection of HIV-specific antibody-secreting cells in subhuman primates. *J. Immunol. Methods* **1992**, 153, (1-2), 107-.
 179. Robinson, J. E.; Holton, D.; Liu, J.; McMurdo, H.; Murciano, A.; Gohd, R., A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of rival glycoproteins in microtiter wells coated with concanavalin A. *J. Immunol. Methods* **1990**, 132, (1), 63-.
 180. Dawkins, R.; Leelayuwat, C.; Gaudieri, S.; Tay, G.; Cattley, S.; Martinez, P.; Kulski, J., Genomics of the major histocompatibility complex: haplotypes, duplication, retroviruses and disease. *Immunol. Rev.* **1999**, 167, 275-.
 181. Carrington, M., Recombination within the human MHC. *Immunol. Rev.* **1999**, 167, 245-.
 182. Lemonnier, F. A., The human major histocompatibility complex (HLA). *Biochimie* **1985**, 67, (1), R11-.
 183. Trivedi, V. B.; Dave, A. P.; Dave, J. M.; Patel, B. C., Human leukocyte antigen and its role in transplantation biology. *Transplantation Proc.* **2007**, 39, (3), 688.
 184. Panigrahi, A.; Gupta, N.; Siddiqui, J. A.; Margoob, A.; Bhowmik, D.; Guleria, S.; Mehra, N. K., Monitoring of anti-HLA and anti-major histocompatibility complex class I related chain A antibodies in living related renal donor transplantation. *Transplantation Proc.* **2007**, 39, (3), 759-760.
 185. Pandha, H.; Rigg, A.; John, J.; Lemoine, N., Loss of expression of antigen-presenting molecules in human pancreatic cancer and pancreatic cancer cell lines. *Clin. Exp. Immunol.* **2007**, 148, (1), 127-.
 186. Garcia-Lora, A.; Algarra, I.; Garrido, F., MHC class I antigens, immune surveillance, and tumor immune escape. *J Cel Physiol* **2003**, 195, (3), 346-.
 187. Chil, A.; Sikorski, M.; Bobek, M.; Jakiel, G.; Marcinkiewicz, J., Alterations in the expression of selected MHC antigens in premalignant lesions and squamous carcinomas of the uterine cervix. *Acta Obstetricia Et Gynecologica Scandinavica* **2003**, 82, (12), 1146-.
 188. Gregorius, K.; Mouritsen, S.; Elsner, H. I., Hydrocoating: a new method for coupling

- biomolecules to solid phases. *J. Immunol. Methods* **1995**, 181, (1), 65.
189. Belder, D.; Degee, A.; Husmann, H.; Kohler, F.; Ludwig, M., Cross-linked poly(vinyl alcohol) as permanent hydrophilic column coating for capillary electrophoresis. *Electrophoresis* **2001**, 22, 3813.
190. Belder, D.; Ludwig, M., Surface modification in microchip electrophoresis *Electrophoresis* **2003**, 24, 3595.
191. Kozlov, M.; Quarmyne, M.; Chen, W.; McCarthy, T. J., Adsorption of poly(vinyl alcohol) onto hydrophobic substrates. A general approach for hydrophilizing and chemically activating surfaces. *Macromolecules* **2003**, 36, (16), 6054-.
192. Lavanchy, D., Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures *J. Viral Hepatitis* **2004**, 11, (2), 97.
193. Hsiao, V. K. S.; Waldeisen, J. R.; Zheng, Y. B.; Lloyd, P. F.; Bunning, T. J.; Huang, T. J., Aminopropyltriethoxysilane (APTES)-functionalized nanoporous polymeric gratings: fabrication and application in biosensing. *J. Mater. Chem.* **2007**, 17, (46), 4896-4901.
194. Ombelli, M.; Eckmann, D. M.; Composto, R. J., Dextran grafted silicon substrates: Preparation, characterization and biomedical applications. *Mat. Res. Soc. Symp. Proc.* **2003**, 774, 07.22.
195. Barbani, N.; Bertoni, F.; Ciardelli, G.; Cristallini, C.; Silvestri, D.; Coluccio, M. L.; Giusti, P., Bioartificial materials based on blends of dextran and poly(vinyl alcohol-co-acrylic acid). *Eur Polym J* **2005**, 41, (12), 3004-3010.
196. Fan, Q. G.; Lewis, D. M.; Tapley, K. N., Characterization of cellulose aldehyde using fourier transform infrared spectroscopy. *J Appl Polym Sci* **2001**, 82, (5), 1195-1202.
197. Eteshola, E.; Balberg, M., Microfluidic ELISA: On-chip fluorescence imaging *Biomed. Microdevices* **2004**, 6, (1), 7-.
198. Baker, J., R.Jr The immune response in chronic hepatitis B virus infection: The "Core" of the problem? *Hepatology* **1992**, 16, (2), 498-500.
199. Rodella, A.; Galli, C.; Terlenghi, L.; Perandin, F.; Bonfanti, C.; Manca, N., Quantitative analysis of HBsAg, IgM anti-HBc and anti-HBc avidity in acute and chronic hepatitis B. *J Clin. Virol.* **2006**, 37, (3), 206-212.
200. Bai, Y.; Koh, C. G.; Boreman, M.; Juang, Y. J.; Tang, I. C.; Lee, L. J.; Yang, S. T., Surface modification for enhancing antibody binding on polymer-based microfluidic device for enzyme-linked immunosorbent assay. *Langmuir* **2006**, 22, (22), 9458.
201. Lai, C. L.; Ratziu, V.; Yuen, M.-F.; Poynard, T., Viral hepatitis B. *Lancet* **2003**, 362, (9401), 2089-2094.
202. Phillips, K. S.; Cheng, Q., Microfluidic immunoassay for bacterial toxins with supported phospholipid bilayer membranes on poly(dimethylsiloxane). *Anal. Chem.* **2005**, 77, (1), 327.
203. MacBeath, G., Printing proteins as microarrays for high-throughput function determination. *Science* **2000**, 289, (5485), 1760-1763.
204. Derwinski, K.; Gheber, L. A.; Preininger, C., A comparative analysis of polyurethane hydrogel for immobilization of IgG on chips. *Analytica Chimica Acta* **2007**, 592, (2), 132-138.
205. Nijdam, A. J.; Cheng, M. M. C.; Geho, D. H.; Fedele, R.; Herrmann, P.; Killian, K.; Espina, V.; Petricoin, E. F.; Liotta, L. A.; Ferrari, M., Physicochemically modified silicon as a substrate for protein microarrays. *Biomaterials* **2007**, 28, (3), 550-558.
206. Yang, M.; Li, W.; Wang, G. G.; Zhang, J. Q., Preparation and characterization of a novel microporous PE membrane supporting composite gel polymer electrolyte. *Solid State Ionics* **2005**, 176, (37-38), 2829.
207. Nam, Y.; Branch, D. W.; Wheeler, B. C., Epoxy-silane linking of biomolecules is simple and effective for patterning neuronal cultures. *Biosens. Bioelectron.* **2006**, 22, (5), 589.
208. McDonald, J. C.; Duffy, D. C.; Anderson, J. R.; Chiu, D. T.; Wu, H.; Schueller, O. J. A.; Whitesides, G. M., Fabrication of microfluidic systems in poly (dimethylsiloxane). *Electrophoresis* **2000**, 21, 27.
209. Decker, C., Light-induced crosslinking polymerization. *Polymer Int.* **2002**, 51, (11), 1141-.
210. Jin, Y. H.; Cho, Y. H.; Schmidt, L. E.; Leterrier, Y.; Manson, J. A. E., A fast low-temperature micromolding process for hydrophilic microfluidic devices using UV-curable acrylated hyperbranched polymers. *J Micromech Microeng* **2007**, 17, (6), 1147.
211. Bui, L. N.; Thompson, M.; McKeown, N. B.; Romaschin, A. D.; Kalman, P. G., Surface Modification of the Biomedical Polymer Poly(Ethylene-Terephthalate). *Analyst* **1993**, 118, (5), 463-474.
212. Brogan, K. L.; Shin, J. H.; Schoenfisch, M. H., Influence of surfactants and antibody immobilization strategy on reducing nonspecific protein interactions for molecular recognition

- force microscopy. *Langmuir* **2004**, 20, (22), 9729-.
213. Deng, Y.; Zhu, X. Y.; Kienlen, T.; Guo, A., Transport at the air/water interface is the reason for rings in protein microarrays. *J.Am.Chem.Soc.* **2006**, 128, (9), 2768.
214. Ajikumar, P. K.; Ng, J. K.; Tang, Y. C.; Lee, J. Y.; Stephanopoulos, G.; Too, H.-P., Carboxyl-Terminated Dendrimer-Coated Bioactive Interface for Protein Microarray: High-Sensitivity Detection of Antigen in Complex Biological Samples. *Langmuir* **2007**, 23, (10), 5670-5677.
215. Ressine, A.; Ekstrom, S.; Marko-Varga, G.; Laurell, T., Macro-/nanoporous silicon as a support for high-performance protein microarrays. *Analytical Chemistry* **2003**, 75, (24), 6968-6974.
216. Takatsu, K.; Tominaga, A.; Harada, N.; Mita, S.; Matsumoto, M.; Takahashi, T.; Kikuchi, Y.; Yamaguchi, N., T Cell-Replacing Factor (TRF)/Interleukin 5 (IL-5): Molecular and Functional Properties. *Immunological Reviews* **1988**, 102, (1), 107-135.
217. Secor, W. E.; Stewart, S. J.; Colley, D. G., Eosinophils and immune mechanisms. VI. The synergistic combination of granulocyte-macrophage colony-stimulating factor and IL-5 accounts for eosinophil-stimulation promoter activity in *Schistosoma mansoni*- infected mice. *J Immunol* **1990**, 144, (4), 1484-1489.
218. Zhang, J.; Li, C. M.; Chan-Park, M., B. ; Zhou, Q.; Gan, Y.; Qin, F.; Ong, B.; Chen, T., Fabrication of thin-film organic transistor on flexible substrate via ultraviolet transfer embossing. *Appl Phys Lett* **2007**, 90, (24), 243502.

Publications

1. **L. Yu**, C.M. Li, Y.S. Liu, Y. Gan One-step fabrication of sensitive UV-curable epoxy-based immunoassay microarray and microfluidic device, *Biosensor and Bioelectronics*, DOI:10.1016/j.bios.2009.03.006
2. **L. Yu**, C.M. Li, J. Gao, Y.S. Liu, W. Wang Flow-through functionalized PDMS microfluidic channels with dextran derivative for ELISAs, *Lab-on-a-Chip* DOI:10.1039/B816018J
3. **L. Yu**, C.M. Li, Z.S. Lu, Y. Gan, Y.S. Liu. Comformative and quantitative studies of adsorption of protein A on poly (dimethylsiloxane) surface by AFM *Nanotechnology*, submitted
4. **L. Yu**, C.M. Li, Q. Zhou, John H.T. Luong Poly(vinyl alcohol) functionalized poly(dimethylsiloxane) solid surface for immunoassay *Bioconjugated Chemistry*, **2007** 18: 281-284.
5. **L. Yu**, C.M. Li, Q. Zhou, Y. Gan, Q.L. Bao: Functionalization of multi-walled carbon nanotubes for affinity ligands. *Nanotechnology*, **2007** 18, 115614, 2007.
6. **L. Yu**, C. M. Li and Q. Zhou Efficient probe immobilization on poly (dimethylsiloxane) for sensitive detection of protein. *Frontiers in Bioscience*, **2005** 10, 2848-2855.
7. W. Chen, C.M. Li, **L. Yu**, Z.S. Lu, Q. Zhou, In Situ AFM Study of Electrochemical Synthesis of Polypyrrole/Au Nanocomposite. *Electrochemistry Communications*, **2008** 10, 1340-1343.
8. T. Xu, Z.H. Wang, J.M. Miao, **L. Yu**, C.M. Li Micro-machined piezoelectric membrane based immunosensor array. *Biosensor and Bioelectronics*, **2008**.24, 638
9. Y.S. Liu, C. M. Li, **L. Yu**, P. Cheng: Optimization of printing buffer for protein microarray based on aldehyde-modified glass slides. *Frontiers Biosciences*, **2007** 12, 3768-3773.
10. W.Y. Yuan; H.Dong; C.M. Li; X.Q. Cui; **L. Yu**; Z.S. Lu; Q.Zhou pH-controlled construction of chitosan/alginate multilayer film: characterization and application for antibody immobilization. *Langmuir*, **2007** 23, (26),13046-,2007
11. Z.H. Wang, J.M. Miao, T. Xu, **L. Yu**, C.M. Li Piezoelectric Biosensors Based on Flexural Mode Micromachined Diaphragm *IEEE International Conference of Nano/Micro Engineered and Molecular Systems* **2008**

12. C.M. Li, **L. Yu**, J. Gao, and J.M. Miao Plastic flow-through ELISA array protein chip
International conference on materials for advanced technologies.**2005**
13. **L. Yu**, C. M. Li, Q. Zhou, W.N. Chen Carbon nanotubing based affinity chromatography for immunoglobulin purification *International conference on materials for advanced technologies*. **2005**
14. C.M. Li, W. Chen, **L. Yu** Advanced Nanoystems for Wireless Biomedical Applications *International conference on advances in network science*.**2005**
15. C. M. Li; **L. Yu** Colorimetric polymeric immunoassay microfluidic devices. technique disclosure