Surface chemistry- and nanomaterials-enhanced high performance protein microarrays

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Surface Chemistry- & Nanomaterials-Enhanced
High Performance Protein Microarrays

LIU YINGSHUAI

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**Surface Chemistry- and Nanomaterials-Enhanced
High Performance Protein Microarrays**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2011

Surface Chemistry- and Nanomaterials-Enhanced High Performance Protein Microarrays

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Abstract

There still remain great challenges to improve performance of protein microarrays in practical applications. Objectives of this project are to develop novel surface chemistry- and nanomaterials-based signal enhancement strategies, and to explore how they improve performance of protein microarrays.

First, a poly(glycidyl methacrylate) brush is prepared and used as a supporting matrix, which provides high protein loading capacity, for improving performance of protein microarrays. Printing buffer, probe concentration, and immobilization kinetics are systematically investigated to elucidate fundamentals of protein attachment. The high performance is demonstrated by detections of hepatitis B virus surface antigen (HBsAg) in both phosphate buffered saline (PBS) and human serum.

Second, a poly[glycidyl methacrylate-co-poly(ethylene glycol) methacrylate] (P(GMA-co-PEGMA)) brush is *in situ* synthesized on a poly(methyl methacrylate) (PMMA) substrate and silica nanoparticles (SNP) to synergistically amplify sensitivity of protein microarrays. In this design, the brush-coated SNP could carry a large number of reporter molecules at a single binding event to amplify detection signals, whereas the brush-coated PMMA offers high probe loading capacity and low nonspecific protein adsorption. The sensitivity of protein array is significantly improved.

Third, a unique ZnO nanorods-coated substrate is developed to immobilize a large amount of probe molecules and also to directly amplify the microarray

fluorescent signals. Two important cancer biomarkers, carcinoembryonic antigen (CEA) and α -fetoprotein (AFP), are analyzed in human serum with a detection limit of 1 pg mL^{-1} , which is comparative to or better than that of ELISA. The advanced ZnO nanorods-based substrate is inexpensive, mass producible, and compatible with well-established microarray printing techniques, providing great potentials for developing economical and sensitive protein arrays.

Fourth, a highly sensitive flow-through microarray immunoassay device is developed using a P(GMA-co-PEGMA) brush as a 3-D flexible matrix to achieve large probe loading capacity and a high bioactivity while allowing easy target access to probes in the brush. A limit of detection (LOD) of $1\text{-}10 \text{ pg mL}^{-1}$ for detected targets is one to two orders better than those of reported flow-through immunoassays. The sensitive detection should be mainly ascribed to the P(GMA-co-PEGMA) brush-enabled high probe loading capacity, low nonspecific adsorption, and high Ab-Ag binding efficiency. The integrated microfluidic device is further used to detect a cancer biomarker CEA in serum and achieves a LOD of 10 pg mL^{-1} , demonstrating its great potentials for clinical applications.

Briefly, this research project not only investigated new surface modification and signal amplification strategies to improve the sensitivity of protein microarrays, but also explored scientific insights into the effects of surface chemistry and nanomaterials on the performance improvement.

Chapter 1 Introduction

1.1 Introduction

DNA microarrays (DNA or gene chips) have been successfully developed, resulting in great impacts on genomic research and diagnostics. However, the genetic investigation by DNA arrays cannot provide sufficient information about proteins, which play many critical roles in various biological processes and are the structure molecules in organisms. In addition, due to mRNA processing and post-translational modification the number of protein products is about 300,000, which is as 10 times as the number of genes predicted by the human genome project ¹. Thus, high-throughput protein assays become very essential for proteomes.

A protein microarray is a high-throughput proteomic tool, which is made by immobilizing different protein probe molecules on a piece of solid substrate (glass, plastic, metal et al.) at separate locations in an ordered manner. The miniaturized array chip has a number of merits including high-throughput screening capability, low sample consumption, short assay time, and high reliability over conventional protein analysis platforms, such as two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and enzyme-linked immunosorbent assay (ELISA). In the last decade, the protein microarray has been used for various applications in fundamental bioscience ²⁻⁴, drug discovery ^{5, 6}, and diagnostics ⁷⁻⁹.

Despite tremendous research efforts, only few protein microarrays are successfully commercialized compared with DNA microarrays. Currently, protein microarrays are mainly used in researches for analysis of limited number of proteins owing to the following reasons: protein with 3-D structure is more sensitive to its surrounding environment than DNA; there is no “PCR-like” technique for protein amplification and it is very hard to produce antibodies as capture proteins due to the lack of an efficient preparation method.

Next generation of protein microarrays should meet requirements of practical applications for sensitive, high-throughput protein screening. Novel probe immobilization and efficient signal amplification need to be developed to improve the sensitivity of protein microarrays. In addition, low cost mass production of antibody and/or alternative capture biomolecules such as aptamers is greatly desirable for high-throughput protein screening. This thesis focuses on the improvement of protein microarray sensitivity.

1.2 Motivations

There is a big challenge to developing highly sensitive protein microarrays for fully achieving their potentials in practical applications, especially in early diagnosis. Motivation of this project is to investigate surface chemistry- and nanomaterials-enabled signal enhancement for significant improving the performance, especially sensitivity, of protein microarrays.

Surface chemistry plays a critical role in chip-based protein sensing. Although many strategies have been developed to modify various substrates for protein immobilization¹⁰⁻¹², they always suffer from low attachment efficiency, severe nonspecific adsorption, poor bioactivity, bad target accessibility, multistep activation processes, and specific modifications of substrate and/or protein. *In situ* grafted (co)polymer brushes, whose physicochemical properties can be easily tailored by adjusting the monomer components or polymerization conditions, provide a promising matrix for highly sensitive protein microarrays.

Mainstream strategies for signal amplification are based on enzyme-catalyzed chemical or biochemical reactions¹³⁻¹⁵. Unfortunately, these methods involve unstable and expensive enzymes/reagents, complicated processes, and down-stream reactions. Some nanopatterned plasmonic noble metal films have demonstrated their fluorescence enhancing capability. The nanopatterned materials-based signal amplification is an alternative way to improve the performance of protein microarrays. However, the disadvantages of this strategy are poor compatibility with mature array printing techniques, heterogeneity in the microscope slide scale, and expensive equipment and/or clean room requirements. This project intends to develop a versatile, economic solution-based nanomaterial synthesis and deposition approach for nanomaterials-based microarray signal amplification.

1.3 Objectives of this study

The main objective of this research project is to improve performance of the protein microarrays by investigating surface chemistry- and nanomaterials-enabled signal enhancement. Specifically, the thesis focuses on development of novel polymer brush matrixes for efficient probe immobilization and improved immunobinding, and investigation of nanomaterials-enabled signal enhancement via directly amplifying fluorescent emission or increasing load of reporters. The surface chemistry for protein nonfouling, protein immobilization capacity and kinetics, and specific recognition binding efficiency are studied to elucidate fundamentals of the performance enhancement.

1.3.1 Photografted polymer brush for high protein loading capacity

Polymeric materials such as PMMA, an alternative to glass and silicon, can be used as attractive substrates for protein microarrays due to their advantages including low cost, low intrinsic fluorescence, easy modification, suitability for mass production, and possibility for integrating microelectronics technology onto the chip surface. Several strategies, such as aminolysis and plasma treatment, have been developed to functionalize polymeric substrates for protein immobilization^{16, 17}, but they either have yielded a low functional group density or have involved unstable intermediates such as Schiff's base¹⁸. This study intends to synthesize a PGMA brush, which bears

high density of epoxy groups, on PMMA surface using “grafting from” approach for efficient and stable protein immobilization.

1.3.2 Copolymer brush-assisted synergistic signal amplification

Copolymer brushes, which can benefit from each component, have emerged as promising functional materials for broad applications. With the rapid development of nanoscience and nanotechnology, various nanomaterials with novel physicochemical properties have been innovated in recent years and have demonstrated great potentials for sensitive biosensing. This project aims to improve sensitivity of protein microarrays via synergistic signal enhancement enabled by P(GMA-co-PEGMA) brush-coated both substrate and SNP.

1.3.3 ZnO nanorods-substrate for direct signal enhancement

Fluorescence is the prevailing readout of various sensors, particularly array chips^{13, 19}. One of the most promising ways to improve performance of protein microarrays is to develop an advanced substrate, which can directly enhance the fluorescence. Nanopatterned metals or metal oxides are widely used as substrates to enhance fluorescent emission²⁰⁻²⁶. However, fabrication of uniform and slide-scale nanopatterns remains a challenge. The purpose of this work is to fabricate a ZnO nanorods-decorated glass slide by a scalable and inexpensive method for improvement of protein microarray sensitivity.

1.3.4 Unique polymer brush for highly sensitive flow-through microarray immunoassay

Microfluidic flow-through immunoassay is an attractive method for a fast and inexpensive protein analysis, but it still suffers from low sensitivity due to the low probe loading capacity and poor immunobinding efficiency. In this study, a P(GMA-co-PEGMA) brush is used as a flexible matrix to immobilize capture proteins with high loading capacity and bioactivity while allowing targets and reporters to easily access the probes for efficient antibody-antigen (Ab-Ag) binding, thus significantly improving the sensitivity of flow-through microarray immunoassay.

1.4 Organization

The purpose of this study is to investigate surface chemistry and signal amplification for improving the performance of protein microarrays. The dissertation is organized as follows.

Chapter 1 introduces background, motivations and objectives of the PhD research project.

Chapter 2 comprehensively reviews recent advances in protein immobilization, signal amplification, and integration of microarray with microfluidics for performance improvement of protein chips. Major challenges and future perspectives of protein chips are also briefly discussed.

Chapter 3 includes the experimental materials, apparatuses, and methods used in this thesis project.

Chapter 4 investigates a photografted GMA polymer brush-assisted performance improvement of protein microarrays. The brush preparation, characterization, printing buffer optimization, and protein binding kinetics are studied. Microarray performance is evaluated by detection of hepatitis B virus surface antigen (HBsAg).

Chapter 5 describes a strategy that synergistically enhances protein microarray signal through P(GMA-co-PEGMA) brush-enhanced immobilization of both probe and reporter. Substrate modifications and SNP derivatives are characterized, and synergistic amplification and performance enhancement are demonstrated.

Chapter 6 explores the use of a ZnO nanorods-coated glass slide as an advanced substrate to improve performance of protein microarrays. High performance in terms of low detection limit and wide dynamic range are demonstrated by detection of two cancer biomarkers.

Chapter 7 presents a highly sensitive flow-through microarray device. 3-D, flexible P(GMA-co-PEGMA) brush was used as a supporting matrix to enhance probe loading capacity and immunobinding efficiency. Fast and highly sensitive detections are achieved in both buffers and human serum.

In Chapter 8, general conclusions are drawn and several possible future works are proposed.

Chapter 2 Literature survey

A protein microarray is fabricated by immobilizing different probes on a piece of solid substrate in a highly ordered array via physical adsorption²⁷⁻³¹, covalent bonding³²⁻³⁶ or bioaffinity assistance³⁷⁻⁴⁰. In the array, each spot or a group of spots for redundant design contains a probe to specifically capture its target protein. Therefore, hundreds or thousands of proteins may be simultaneously analyzed on a miniaturized substrate. The history of protein microarrays can be traced back to the 1980s, when Ekins and colleagues first demonstrated parallel microspot-based ligand binding assays⁴¹⁻⁴³.

Although protein microarrays have become powerful tools for proteomic studies in various fields^{2-4, 6, 44-47}, performance improvement is still in great demand to fully achieve their potentials in practical applications. An advanced supporting matrix and effective signal amplification are very important for improving the performance. The former enables efficient and appropriate probe immobilization to facilitate bio-recognition binding, while the latter directly enhances the detection signal. With dramatic developments in microfabrication, nanotechnologies, and biotechnologies, enormous progress has been made, particularly in improving biosensing sensitivity. This chapter mainly reviews advances in protein biochip technologies with emphasis on novel protein immobilization strategies and nanomaterials-assisted signal amplifications. Prominent progress in integration of protein microarrays with

microfluidic platforms is briefly discussed. Major challenges and future perspectives are also addressed.

2.1 Emerging strategies for efficient protein immobilization

No matter what kind of protein chips is constructed, proteins have to be immobilized on a substrate surface as capture molecules. Efficient protein immobilization strongly depends on surface chemistry, one of the most important factors for fabrication of high performance protein microarrays. Ideally, proteins are homogeneously immobilized on a substrate with high loading capacity, minimum nonspecific adsorption, uniform orientation, natural conformation for bioactivity, and good accessibility to analytes. Improper attachment may result in partial or complete loss of protein bioactivity due to steric hindrance or conformation changes. Nanomaterials- and polymer coating-assisted protein immobilization have emerged as attractive strategies for fabrication of protein microarrays. The advances in these two areas are discussed in this section.

2.1.1 Nanomaterials-assisted loading capacity enhancement

Probe loading capacity, an essential factor in protein assays, is often poor due to the limited surface area of a planar substrate. Thus, enlargement of a specific surface area is an effective way to improving protein loading. With rapid developments in nanotechnology, many kinds of nanomaterials have been synthesized. The large

surface-to-volume ratio of nanomaterials makes them highly suitable for immobilizing large numbers of proteins while retaining their bioactive conformation.

Figure 2.1: SEM images of the anodized porous silicon surface (a, top view & cross-section) and schematic illustration for covalent protein immobilization in polyelectrolytes-modified porous alumina membranes^{48, 49}. Reprinted with permission from American Chemical Society.

Porous silicon (Figure 2.1a) with large surface area has emerged as a novel substrate onto which protein probes are immobilized with a high protein loading capacity for forward- and reverse-phase protein microarrays^{31, 49, 50}. Besides the high surface area, the nanoporous substrate possesses many merits including low autofluorescence, good biocompatibility, and excellent chemical resistance. However,

the high cost of silicon limits its applications, especially for clinical diagnosis, in which disposable protein chips are highly desirable ⁵¹. Additional drawbacks are instability and low bioactivity of the immobilized probe due to weak physical adsorption.

Anodized porous alumina also has been used as a 3-D substrate for protein immobilization ^{48, 52}. Rather than using physical attachment, Dai and colleagues modify the porous support with polyelectrolyte multilayers terminated with poly(acrylic acid), which was then activated by EDC/NHS for covalent protein anchoring (Figure 2.1b) ⁴⁸. High protein loading capacity and low nonspecific adsorption are achieved on this substrate, resulting in LOD of 0.02 ng mL⁻¹ for Cy5-labeled IgG. The porous structure, combined with its good hydrophilicity, may cause large spot diameter (around 2 mm), thus reducing array spot density ⁴⁸. In addition, the film is difficult to be handled because it is thin and fragile. To address this problem, it has been attached to glass slide via a thin layer of polydimethylsiloxane (PDMS) or paraffin ⁵².

Physically nanoroughened or nanostructured surfaces are alternatives to porous substrates for providing a large specific surface area. Nijdam et al. reported that physicochemically modified silicon prepared by reactive ion etching (RIE) and chemical silanization, provided comparable protein loading and detection sensitivity with nitrocellulose ^{53, 54}. More recently, nanostructured quartz has been fabricated using two-step RIE to improve protein binding capacity and target accessibility ⁵⁵. By

combining the 3-D nanostructure with viral particle-assisted oriented probe immobilization, attomolar sensitivity is achieved for detection of *E. coli* enterotoxin marker LT-B.

In contrast to expensive silicon and quartz, plasma- or RIE-nanostructured polymeric substrates such as PMMA, Zeonex and polycarbonate (PC) are employed as protein microarray supports ^{16, 56}. These nanostructures can be fabricated and controlled without template requirements. More importantly, they may be applied to various materials, both inorganic and organic.

Nanomaterials, which come in a variety of shapes including spherical nanoparticle, nanocluster, nanorod, and nanopillar, have attracted great interest among engineers and researchers in many fields, especially biosensing ⁵⁷. Silica nanoparticle (SNP) is one of the most promising materials due to its good stability, easy modification, excellent water dispersibility, and high surface-to-volume ratio ^{58, 59}. A micropatterned multilayer assembly of SNPs on a PEG-coated glass slide was prepared to create a protein microarray supporting matrix, that allowed six times the protein loading of a corresponding planar substrate ⁶⁰. Moreover, proteins are selectively attached to the SNP assembly, while nonspecific adsorption in other area is minimized by the PEG coating, resulting in high signal-to-noise ratio and improved detection limitation. SNPs have also been used for protein micropatterning and peptide microarray fabrication ^{61, 62}. ZnO nanorod has demonstrated great potential for biosensing applications because of its low cost, easy synthesis, and fluorescence

enhancing capability^{19, 63}. Cluster-assembled TiOx nanostructures⁶⁴ and 3-D silica nanopillar bundles⁶⁵ have been developed to allow more protein immobilization in protein detection.

In addition to pure nanomaterials, various nanocomposites, which can benefit from their different components, are employed to modify substrates. Marquette et al. employ copolymer-coated nanobead as carriers of probe molecules for protein microarray preparation⁶⁶. High sensitivity is achieved through the combination of a large specific surface area provided by nanobeads and efficient protein immobilization enabled by the copolymer coating. Polypyrrole-pyrrolepropylic acid-gold nanocomposite⁶⁷, gold nanoparticle/SiO₂/ thionine nanocomposite⁶⁸, and SNPs/TiO₂ sol-gel nanocomposite⁶⁹ also were developed to enhance probe immobilization for performance improvement of electrochemical immunosensors.

2.1.2 Polymeric coating for superior protein immobilization

Surface modification with small molecules alone or in combination with short crosslinkers is the main approach for protein immobilization in heterogeneous immunoassays^{12, 15, 70}. In comparison with small molecules, functional polymers offer many advantages for protein attachment^{36, 71, 72}. First, polymers carry abundant functional groups to covalently immobilize high density of proteins. Second, it is easy to control surface properties by selecting suitable polymers and thus minimizing nonspecific protein adsorption. Moreover, the long chain of polymers can work as a

spacer between probe and substrate to reduce steric hindrance, and to improve accessibility of probes to analytes.

Glass is one of the most common substrates used for protein chips. The prevailing way to preparing reactive coatings on glass surface is based on formation of self-assembled monolayers (SAMs) of alkylchlorosilanes or alkylalkoxysilanes, which bear a variety of active groups like amine, thiol, and epoxy³². The silanization method is relatively inexpensive, and easy to prepare and handle, but the low loading capacity and serious biofouling restricts its application in sensitive protein assays⁷³.

Recently, polymeric coating-based strategies have been developed to functionalize glass slides for efficient protein attachment. Antibody microarrays with high signal-to-noise ratio have been demonstrated on a homobifunctional amino-PEG-coated glass slide³⁵. Although the PEG layer can effectively reduce nonspecific protein adsorption, only limited active sites are provided by the linear polymer⁷⁴. In contrast, Cretich et al. fabricated protein microarrays on a multifunctional random copolymer-coated substrate, on which probe molecules could be covalently immobilized at high density with native conformation, while minimizing nonspecific adsorption⁷⁵. The combinatorial merits contribute to the high detection sensitivity.

Dendrimers are a class of synthetic polymers characterized by a highly branched 3-D structure, which provides numerous functional groups at the chain ends. Due to the attractive features, dendrimers have demonstrated their great potential for various

applications, especially in biosensing area. Dendritic poly(amidoamine) (PAMAM), with high density of amino groups, is used to modify glass surface, following by activation with disuccinimidylglutarate (DSG) or 1,4-phenylenediisothiocyanate (PDITC) for covalent immobilization of DNA and protein with a high loading capacity ⁷⁶. Similarly, a carboxyl-terminated dendrimer is used as a substrate coating (Figure 2.2) to create high sensitive and selective microarray immunoassays ³⁶. Hyperbranched polymers, with a 3-D structure similar to dendrimers, can be easily synthesized with a simple process, providing a promising material for substrate functionalization ⁷⁷.

Figure 2.2: Schematic illustration of preparation (a) and atomic force microscope image (b) of the carboxyl-terminated dendrimer-coated glass slide ³⁶. Reprinted with permission from American Chemical Society.

In addition, *in situ* prepared polymer brushes work as attractive alternatives to presynthesized polymers/copolymers because their thickness, roughness, and hydrophilicity can be easily controlled by adjusting monomer components and polymerization conditions. Recently, polymer brushes of poly[oligo(ethylene glycol) methacrylate] and poly(dimethylacrilamide-co-N,N- acryloyloxysuccinimide) were grafted from glass slides and used as supporting matrixes to achieve high performance chip-based immunoassays^{78, 79}.

Glass slides modified with hydrogels or membranes such as agarose⁷³, polyacrylamide^{27, 80}, polyurethane⁸¹, and nitrocellulose²⁹ have been prepared for protein microarray applications. Layer-by-layer self-assembly of polyelectrolytes, a simple and robust method to modify various substrates, provides higher protein loading capacity over 2-D planar surfaces⁸². At the same time, the aqueous environment in gel is favorable for maintaining the native confirmation of proteins. However, noncovalent attachment and large signal variations may lead to poor performance reproducibility²⁷. The 3-D matrix also works as a barrier to diffusion of the target and/or reporter; thus, a long incubation time is required to carry out biorecognition bindings³⁰.

Gold thin film has been widely employed as a substrate for protein chips. SAMs, formed by spontaneous adsorption of alkanthiols, are a well-known architecture for gold surface modification. However, like silanized glass, the alkanthiol SAMs have a number of shortcomings.

Figure 2.3: Covalent protein immobilization on polyCBA-coated gold surface⁸³. Reprinted with permission from American Chemical Society.

A poly(acrylic acid) (PAA) brush-coated gold surface activated with EDC/NHS, or derivatized with nitrilotriacetate (NTA)-Cu²⁺ complexes provides about two, or 15, monolayers immobilization of bovine serum albumin (BSA), respectively⁸⁴.

Jiang's group prepared an ultralow fouling and functionalizable poly(carboxybetaine acrylamide) (polyCBA) film on a surface Plasmon resonance (SPR) chip. The film allowed proteins to be covalently immobilized via EDC/NHS activation (Figure 2.3)⁸³. High performance immunoassay has been demonstrated by detection of a cancer biomarker in undiluted human blood plasma. More recently, a poly[oligo(ethylene glycol) methacrylate-co-glycidyl methacrylate] (POEGMA-co-GMA) brush was grafted from a gold surface and used as a unique supporting matrix for SPR imaging (SPRi) protein arrays, showing efficient protein loading and low nonspecific adsorption⁸⁵. In contrast to PAA and polyCBA brushes, the POEGMA-co-GMA brush facilitates covalent protein immobilization via the

epoxy functional group from GMA component without additional activation requirement. The OEGMA moiety was copolymerized to adjust surface properties and to prevent biofouling, thus improving the signal-to-noise ratio and eliminating false positive response.

Alternatively, conductive poly(pyrrole-co-pyrrole propylic acid) composite film was electrochemically produced on a gold surface to create a label-free SPR immunosensor, in which pyrrole propylic acid provided carboxyl group for covalent protein immobilization and pyrrole improved the film conductivity ⁸⁶. The research demonstrated that protein immobilization capacity of the composite film was significantly higher than that of an 11-mercaptopundecanoic acid SAM.

Due to their low cost and superior chemico-physical properties, polymeric substrates have become attractive supports for protein sensing, especially for microarray and microfluidic immunoassays. PDMS is of particular interest in the fabrication of microfluidic devices since it can be easily casted in a suitable mold to obtain a desired replicate with high fidelity. However, proteins tend to be nonspecifically adsorbed on pristine PDMS due to its high hydrophobicity, leading to undesirably strong biofouling.

Hydrophilic PDMS surfaces have been engineered through various approaches such as chemical oxidation followed by PEG coating ⁸⁷, multilayer polyelectrolytes modification ⁸⁸, surfactant coating ⁸⁹, and photografting with hydrophilic monomers ⁹⁰ to minimize nonspecific protein adsorption. PDMS is also coated by hydrophilic

functional polymers (i.e. poly(vinyl alcohol) and dextran derivative, Figure 2.4), which can not only introduce high density of functional groups for covalent protein binding, but also improve the hydrophilicity of PDMS to prevent biofouling^{91, 92}. Highly sensitive immunoassays have been performed on these hydrophilic PDMS surfaces.

Figure 2.4: Schematic illustration for flow-through modification of PDMS microchannel with dextran derivative⁹². Reprinted with permission from Royal Society of Chemistry.

Besides PDMS, organic polymeric materials such as PMMA⁹³, polyethylene terephthalate (PET)⁹⁴, polycarbonate (PC)⁹⁵, and cyclic polyolefin⁹⁶ have also been employed as supports of protein chips. Conventionally, these substrates are

functionalized by saponification, aminolysis, plasma treatment, and surface reduction for protein immobilization^{18, 97-100}. However, these methods are energy- and labor-consuming, and cannot provide a high density of functional groups.

Amine-rich poly(ethyleneimine) (PEI) has been covalently attached to a PMMA surface via amination to introduce large amounts of amino groups, followed by activation with glutaraldehyde for protein binding^{18, 101}. The modification greatly improves antibody binding capacity and performance of the PMMA-based microfluidic ELISA for IgG and Escherichia coli O157:H7 detection. Liu's group constructed a PMMA microfluidic chip, which was modified with (BMA)_x-(MAOPTMS)_y copolymer, followed by Al₂O₃ sol-gel, to encapsulate antibodies¹⁰². The hybrid material-modified surface is favorable for preserving the bioactivity of immobilized antibodies and preventing nonspecific adsorption. Site-controlled protein immobilization on a modified PMMA surface via combining chip IEF with low-temperature sol-gel technology, called IEF-GEL, also was proposed by the same group¹⁰³.

Promisingly, protein microarrays and microfluidic devices are directly fabricated with a UV-curable epoxy resin, which contains an intrinsic epoxy functional group for protein coupling without tedious and time-consuming modification processes¹⁰⁴. The epoxy resin-based microfluidic immunoassay device demonstrates a lower detection limit and a broader dynamic range compared with PDMS microfluidic devices made by more complicated fabrication processes.

Table 1. Summary of advanced protein immobilization approaches with their pros and cons.

Strategies	Substrate and modification	Pros	Cons	Reference
Nanomaterials-assisted immobilization	Porous silicon/alumina	High loading capacity Low autofluorescence	High cost Low producibility	48-50, 52
	Nanoroughened or nanostructured substrate	High loading capacity Suitable for various substrates and no template needed	High energy consumption Requirement of special equipment	16, 53-56
	Nanomaterials coating	High protein loading capacity Ease of fabrication	Nonuniform coating Low reproducibility	63-65, 105
Polymeric coating-based immobilization	Polymer or copolymer coating	High density immobilization with native conformation Low nonspecific adsorption	Requirement of activation Poor coating controllability	35, 75, 91, 92, 101
	Dendrimer coating	High density immobilization	Multi-step synthesis process of dendrimer	36, 76
	Hydrogel or polyelectrolyte film	High loading capacity High bioactivity	Large signal variation Long incubation time	27, 73, 81, 82
	Polymer/copolymer brush grafting	High loading capacity Good controllability of surface properties High bioactivity and accessibility	Multi-step preparation of brush	79, 83-85, 106

In summary, considering all these factors, polymer brush-based functionalization is the most efficient and promising strategy among all polymeric modification approaches. 3-D polymer brushes provide sufficient accessible volume to bind a large amount of probes equal to many monolayers of proteins¹⁰⁷. The long and flexible chains are more favorable for preserving native protein conformation and improving immunobinding efficiency via free adjusting their shapes^{85, 107}. Compared to presynthesized polymers, properties of *in situ* prepared polymer brushes can be tailored easily through changes of monomer species, concentration and ratio.

2.2 Nanomaterials-assisted signal amplification of protein chips

Sensitive protein detection is still a challenging task due to lack of “PCR-like” techniques for protein amplification. Several methods such as ELISA^{108, 109}, enzyme-catalyzed reporter deposition (CARD)¹⁴, enzyme-linked chemiluminescence, rolling circle amplification (RCA)¹¹⁰, and gold nanoparticle (Au NP)-assisted scanometric assay^{111, 112} have been established for improving the sensitivity of immunoassays. However, their application in miniaturized, highly parallel assays is restricted by their complicated processes and needs for well-trained professionals.

Although fluorescence, chemiluminescence, absorbance, and electrochemical methods can be used to monitor recognition binding in protein detection, fluorescence is the prevailing readout, particularly on high-throughput platforms such as microarrays^{13, 113}. Since enzyme- and nucleic acid-based fluorescence amplification strategies have been reviewed elsewhere¹³, this section only focuses on new progresses in nanomaterials-enabled fluorescence amplification for protein chips.

2.2.1 Nanostructured substrate-enabled fluorescence enhancement

To improve performance of fluorescence-based protein detection, one of the most straightforward ways is to engineer an advanced substrate, which can significantly enhance the fluorescent signal. The most popular substrate is noble metal-based plasmonic film featured with various nanopatterns. The nanostructured noble metal-based enhancement is ascribed to increase of fluorescence quantum yield,

decrease of lifetime, and improvement of energy transfer through interaction of fluorophore with surface Plasmon resonance in the substrate ¹¹⁴. Nanoroughened gold patterns ²³, colloidal gold self-assembled monolayers ¹¹⁴, and nanoporous gold ¹¹⁵ are engineered as plasmonic substrates, resulting in an optimal factor of fluorescence enhancement ranging from 15 to 45. Alternatively, silver nanostructures including nanoisland films ^{25, 116}, fractal-like structures ¹¹⁷, 1-D gratings, and 2-D nanohole arrays ¹¹⁸⁻¹²⁰ have demonstrated good fluorescence-enhancing capacity.

Fluorescence may be partially quenched on bare metals due to the electron or nonradiative energy transfer from attached fluorophores to metal supports ¹²¹. Various dielectric layers such as alumina ^{122, 123}, silica ^{20, 21, 124}, polymers ¹²⁵, and amorphous carbon ¹²¹ have been employed to separate fluorophores from metal structures to reduce the quenching effect and to optimize the enhancing factor. Although significant fluorescence enhancement is successfully achieved by the tailored noble metal nanostructures, the high cost of these materials and complicated fabrication processes limit their practical applications.

Plasmonic enhancement strongly depends on diversity of nanostructures, from metal type, geometry, size and distribution of regimes, and dielectric environment, to the distance between fluorophore and metal surface. Thus, it is challenging to fabricate uniform metal structures on the scale of a microscope slide with precisely controlled characteristics for microarray applications. Moreover, the amplification effect also relies on the overlap of Plasmon resonance of engineered nanostructures

with excitation and emission band of fluorophores²⁰. Thus, only the signal from fluorophores with specific spectral characteristics can be effectively enhanced.

Instead of noble metal, photonic crystals have been constructed to amplify fluorescent signals via enhancement of electromagnetic fields and redirection of fluorophore emission towards photon detection instrumentation (Figure 2.5), enhancing signal by over 100 times^{22, 126}. A microarray immunoassay with improved performance for cytokine detection was realized on the photonic crystal surface²². Similar to the plasmonic surfaces, the unique structure of photonic crystals must be prepared using a complicated process with expensive equipment.

Figure 2.5: Schematic of photonic crystal-enabled fluorescence enhancement²². The amplification is ascribed to increased electromagnetic fields and directionally enhanced emission profile. Reprinted with permission from American Chemical Society.

ZnO, an economical metal oxide, has received considerable attention in a variety of fields due to its desirable optical and electrical properties. Nanoscale ZnO has demonstrated a strong fluorescence-enhancing capability, which may be explained by changes in photonic mode density and/or reduction in self-quenching of fluorophores¹¹³. Fluorescence-based ultrasensitive detection of DNA and protein has been performed on nanoscale ZnO platforms^{63, 127}. However, these assays were carried out on prepatterned ZnO nanorod arrays, which were prepared through a multistep microfabrication process. Furthermore, the prepatterned substrates have poor compatibility with well-established microarray fabrication techniques, thus limiting their applications in high-throughput protein assays.

2.2.2 Multilabeled nanoparticle-assisted fluorescence enhancement

Multilabeled nanoparticle-assisted signal enhancement has been developed as an alternative to enzymatic ELISA and RCA. The nanoparticle reporter, incorporated with numerous dye molecules and coated with detection antibody or streptavidin, can specifically bind to captured analytes to introduce many fluorescence emitters at a single binding event, thus enhancing signal intensity. Since no further reaction is required after probing with multilabeled nanoparticle reporter, the observed signal are directly proportional to the concentration of analytes in sample, providing an accurate quantitative analysis.

Lanthanide chelates, mainly of europium (Eu), have been widely used as inorganic reporters in various assays due to their intrinsic long-life fluorescent emissions at visible wavelengths ¹²⁸. Nichkova et al. carried out a microarray immunoassay using Eu-doped gadolinium oxide (Eu:Gd₂O₃) nanoparticles as fluorescent label onto which poly(L-lysine) (PLL) was coated to facilitate recognition antibody conjugation and to avoid aggregation of nanoparticles ¹²⁹.

Polystyrene nanoparticles incorporated with Eu-chelate were also prepared for signal enhancement of an antibody microarray (Figure 2.6) ⁵⁰. About 1 order of magnitude improvement in sensitivity is achieved by Eu-doped polystyrene nanoparticles compared with Eu-chelate without nanoparticles. Similarly, tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate (Rubpy) dye-encapsulated SNP was used as a label, demonstrating a LOD of 0.1 ng mL⁻¹ for interleukin-6 detection ¹³⁰. The drawbacks of this scheme are potential dye leakage and aggregation of nanoparticles, which may cause low reproducibility and increased background. At high concentration, the sensor surface limits nanoparticle binding because of its much bigger size compared to antibody, leading to a narrow dynamic range. Furthermore, under saturation conditions, the number of bound nanoparticles is much less than that of detection antibodies; thus, the enhancing factor decreases ¹³¹.

Figure 2.6: schematic of protein microarray assays based on europium chelate-labeled streptavidin (a) and europium chelate-incorporated polystyrene nanoparticle, coated with streptavidin (b) ⁵⁰. Reprinted with permission from American Chemical Society.

Recently, a polymerization-based amplification adapted from DNA microarrays has been demonstrated for use in sensitive antibody microarrays ¹³². After introducing photoinitiator to each microspot through specific biorecognition binding, photopolymerization was carried out through contact of the chip with the mixture of monomer and fluorescent nanoparticles. With growth of the polymer film, fluorescent nanoparticles are trapped to form a microscopically visible and highly fluorescent film, which can be detectable by naked eyes and qualified by fluorescent microscopy. The

work provides an alternative nonenzymatic scheme. Although this approach eliminates brittle and expensive enzymes, down-stream chemical reactions are still involved.

2.3 Integrated microarrays and microfluidics

Protein microarrays can be easily fabricated and analyzed using well-established equipment and software for high-throughput protein assays. However, static microarrays normally require long incubation time to achieve optimal signal intensity due to the diffusion-controlled biorecognition binding on chip surface ^{79, 133}. Moreover, ligands must travel a long distance to bind with appropriate surface-attached partners, further slowing the assay speed ¹³⁴. Microfluidics can convert static incubation to dynamic one to accelerate the assay process by improving mass transport. Currently, most microfluidics-based multiplexed immunoassays are performed by using fluidic network itself to segregate different capture molecules ^{92, 135, 136}. Therefore, high-throughput assays are difficult to be carried out with microfluidics not only due to complicated layouts with many microchannels, inlets and outlets, but also because of the lack of capable equipment and software for acquiring and analyzing the massive amounts of data.

Figure 2.7: Merged microarray with microfluidic device. (a) Detailed description of the microfluidic cartridge; (b) Chemiluminescent image of a human IgG biochip ¹³⁷. Reprinted with permission from Elsevier.

Microfluidic flow-through array biochips fabricated by coupling microarray to microfluidics is a very promising trend for highly parallel protein analysis. They provide unique advantages including further reduction of sample/reagent consumption, shorter analysis time, and better high-throughput capability compared with conventional static arrays ^{137, 138}. Some integration examples have been successfully developed to carry out flow-through microarray immunoassays, demonstrating a number of superiorities to individual platform (Figure 2.7) ^{79, 137}. However, in these cases, the microfluidic structures are fabricated by either photolithography or hard

mask-dependent soft-lithography. Thus, expensive equipment and clean room are required, resulting in high fabrication costs and low production capacity.

For practical application, especially in clinical diagnosis, cost-effective and mass producible microfluidic array chips are in high demand for rapid and parallel protein detections. As alternatives to conventional silicon and glass, organic polymeric substrates offer a variety of merits in terms of low cost, excellent mass producibility, easy functionalization, and good transparency. In addition to materials and fabrication processes, sensitivity and specificity of flow-through platforms must be improved to meet the requirements of important applications.

Automation of sample processing and manipulation can be easily realized by microfluidic systems via a specific layout design and computer-based precise controls, eliminating labor-intensive and time-consuming operation processes involved in conventional microtitre-based ELISA and protein microarrays. Thus, integration of the microarrays with microfluidics can simultaneously achieve parallel processing of multiple samples and high-throughput array assays, providing great potential for automatic, broad screening.

2.4 Future perspectives

Unlike DNA, 3-D proteins are prone to denaturation and very sensitive to their surrounding environment. Thus, appropriate substrate surface and optimal conjugation

is quite essential in protein chip fabrication and applications. Ideally, probe proteins should be stably and directionally immobilized on a substrate without loss of bioactivity and with minimum biofouling¹¹. Though enormous efforts have been dedicated to improving protein immobilization efficiency, achievement of all requirements on one substrate is still a big challenge. Overall, multifunctional polymer coating is a promising strategy for protein chip construction, in which the polymers can not only adjust substrate surface properties to preserve native conformation of attached protein and to prevent nonspecific adsorption, but also provide high protein loading capability to improve the sensitivity of protein chips. In addition, the flexible polymer chains could freely change their shape to offer favorable orientation for efficient biorecognition binding on the chip surface.

With the rapid development of nanotechnology, a variety of nanomaterials with novel physicochemical properties have been innovated in recent years. Nanomaterials-enabled signal amplification of protein microarrays has become an attractive alternative to the prevailing enzyme-catalytic strategies, eliminating expensive and unstable enzymes/reagents, as well as the need for additional biochemical reactions. Both nanostructured substrate-enabled direct amplification and nanomaterials-assisted high reporter loading contribute to signal enhancement. The compatibility, biofunctionality, and amplification capability of nanomaterials must be continuously investigated to discover simple, sensitive, inexpensive, and reliable

protein assays for future proteomic applications. Thus, further research should be devoted to nanomaterials-based performance improvement of protein chips.

Advances in microfabrication greatly speeds up the miniaturization and integration of protein chips. Although numerous attempts have been made to package different components into a single unit, building a fully functional chip to conduct a complete assay still remains big challenge. Eventually, protein assays could be carried out by an autonomous lab-on-a-chip system, which would be capable of performing sample preparation and manipulation, biorecognition reaction, washing, signal read-out, and even data analysis. The success of such a high performance lab-on-a-chip system depends on the development of superior surface chemistry, effective signal amplification, and a fully integrated chip platform. In a word, disposable lab-on-a-chips with customer-friendly operation systems hold great promise for high-throughput protein screening in both fundamental research and pharmaceutical/clinical applications.

Chapter 3 Experimental

3.1 Materials and instrumentations

3.1.1 Chemicals

Hexamethylenediamine, ethylenediamine, glycidyl methacrylate (GMA), glycerol, triton[®] X-100, polyoxyethylene (20) sorbitan monolaurate (Tween 20), ethanol, methanol, butanediol diglycidyl ether (BDGE), poly(ethylene glycol) methacrylate (PEGMA, $M_n = 360$), triethylamine (TEA, 99.5%), 2-bromoisobutyryl bromide (BIB, 98%), 2, 2'-bipyridyl (Bipy, $\geq 99\%$) and copper (I) bromide (CuBr, 99.999%), tetrahydrofuran (THF), (3-glycidoxypopyl)trimethoxy silane (GPTMS), (3-aminopropyl)triethoxy silane (APTES), tetraethoxysilane (TEOS), zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$, $\geq 98\%$), ascorbic acid and copper (II) bromide (CuBr_2 , 99.999%) were purchased from Sigma-Aldrich. 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, hydrazide (BODIPY[®] FL hydrazide) was supplied by Invitrogen. All chemicals were of analytical or higher grade and used as received without further treatment.

3.1.2 Biomolecules and buffers

Goat IgG, rabbit IgG, rat IgG, cyanine 3 (Cy3)-labeled rabbit anti-goat IgG, biotinylated goat anti-rabbit IgG, Cy3-labeled goat anti-rabbit IgG, bovine serum albumin (BSA), human serum (male, AB), phosphate buffered saline (PBS, 0.01 M

pH 7.4) and Tris-buffered saline with 0.05% Tween 20 (TBS, pH 8.0) were purchased from Sigma-Aldrich. Human IgG, Alexa Fluor[®] 546 goat anti-mouse IgG, Cy3-tagged goat anti-human IgG and Alexa Fluor[®] 546-conjugated goat anti-rat IgG were ordered from Invitrogen. Hepatitis B surface antigen (HBsAg), anti-HBsAg and biotin-labeled anti-HBsAg were received from US Biological. Carcinoembryonic antigen (CEA), α -fetoprotein (AFP), mouse anti-CEA, rabbit anti-CEA, mouse anti-AFP, and rabbit anti-AFP were supplied by Fitzgerald. Cy3-conjugated streptavidin was obtained from GE healthcare. Blocker[™] casein in TBS containing 0.05% Tween 20 was purchased from Pierce. The deionized (DI) water used in all experiments was produced by Q-Grad[®]1 system, Millipore Corporation, USA.

3.1.3 Instrumentations

- Field emission-scanning electron microscope (FE-SEM, JOEL JSM-6700F) was employed to characterize nanomaterials.
- Surface topography of various substrates was monitored by atomic force microscopy (AFM, Nanoman, Veeco, USA).
- Attenuated total reflection-Fourier transform infrared (ATR-FTIR, Thermo Nicolet, Madison, WI) spectroscopy was utilized to record IR spectra.
- The contact angle was measured by FTA4000 (First Ten Angstroms, Portsmouth).
- XRD pattern was recorded by D8 advance X-ray diffractometer (Bruker AXS, German) with Cu K α radiation ($\lambda = 0.15418$ nm).

- VersArray chip writerTM compact system (Bio-Rad, USA) was used to perform microarray contact printing.
- Fluorescence images were acquired by ScanArray GX microarray scanner (PerkinElmer, USA) equipped with ScanArray[®] Express analysis software.
- Zeta potential and hydrodynamic size were measured using Zetasizer Nano Series (Malvern instruments, UK).
- UV flood equipment (shuttered UV system, 400 W) was employed to irradiate sample for photopolymerization.
- ProXPRESS 2-D proteomic imaging system (PerkinElmer, USA) was used for characterization of surface functional group.

3.2 Methodology

3.2.1 AFM imaging

Topography of various substrate surfaces were examined with AFM (SPM 3100, Veeco instruments Inc., USA) in tapping mode. The scan area was $20\ \mu\text{m} \times 20\ \mu\text{m}$. All images were acquired in open air.

3.2.2 SEM imaging

The morphology and size of synthesized nanomaterials was characterized by FE-SEM. 5 μL of well-dispersed SNP suspension was dropped on a piece of aluminum foil, followed by drying for overnight in vacuum oven. A small piece of ZnO nanorods-coated glass slide was tailored by diamond cutter. The samples were coated by platinum using a sputter before viewing.

3.2.3 FTIR analysis

The IR spectra of PMMA slides and their derivatives were recorded by using a Nicolet 5700 instrument (Thermo Electron Corporation) equipped with an attenuated total reflection (ATR) accessory. The PMMA slide was placed onto the ATR crystal and pressed tightly to obtain good spectra. The analysis was performed by 16 scans with a resolution of 1 (data spacing is 0.482 cm^{-1}).

The IR spectra of bare SNPs and brush-SNPs were collected with a Nicolet 5700 spectrometer through the KBr pellet method. Before measuring, the SNPs and copolymer brush-coated SNPs were dried in vacuum for overnight. The spectra were then recorded from 4000 to 400 cm^{-1} with a 1 cm^{-1} resolution.

3.2.4 Contact angle measurement

The contact angle was measured immediately after dropping $5\text{ }\mu\text{L}$ of DI water on various substrates using FTA 1000 series drop shape instrument (First Ten Angstroms, Virginia). Five different measurements were performed on each substrate. The mean value of 5 measurements was used for figure plot.

3.2.5 Protein microarray printing

Probe proteins were firstly transferred into 384-microtitre plate as printing source. The robotic contact printing was performed by VersArray chipwriterTM (BIO-RAD, USA) equipped with stealth microspotting pins. Around 0.3 nL of sample per spot

was deposited onto substrates at RT and 60% humidity. The printed slides were then incubated overnight at RT to allow formation of covalent bond between amine group on proteins and epoxy group on substrates.

3.2.6 Imaging and data analysis

The ready-to-measure slides were scanned with ScanArray GX Microarray Scanner (Perkin Elmer) to obtain fluorescent images. Signal intensity of each spot was quantified by using ScanArray[®] Express analysis software. The intensity was local background corrected mean, which was used for downstream statistical analysis. The acquired data were inputted to Origin 7.0 for further analysis and plots.

Chapter 4 Photografted Poly(glycidyl methacrylate) brush on Substrate for Performance Improvement of Protein Microarray ^a

4.1 Introduction

Protein microarrays are generally fabricated on glass^{33, 34} or silicon^{31, 53} surfaces. Alternatively, polymeric materials such as PET¹⁰⁶, PMMA^{18, 139-141}, PDMS^{91, 92}, PC¹⁴², and epoxy resin⁵¹ have been utilized as substrates for DNA/protein microarrays or microfluidic biochips. The particular advantages of these synthetic polymers are their low cost, suitability for mass production, and possibility for integrating microelectronic components onto the surface of array chips^{18, 136, 140}. For developing a fluorescence-based protein microarray, PMMA has many additional merits including low intrinsic fluorescence (Figure 4.1), transparency and easy modification. Although several approaches are available to modify PMMA surface for protein detections, they are either yielded a low surface density of functional groups or involve unstable intermediates such as schiff's base¹⁸.

In this chapter, a simple, robust and efficient surface modification strategy was investigated to functionalize PMMA, which was then used as a substrate for developing a high performance protein microarray. GMA, with both acrylate and

^a Reproduced in part with permission from Y. S. Liu, C. M. Li, et al., *Med. Chem. Commun.*, 2010, 1, 132.
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epoxy group, was selected as a monomer for photopolymerization. The former was employed as an active site for polymerization in the presence of photoinitiator under UV irradiation, and the latter served as an anchor for covalent immobilization of proteins via secondary amine bond formed between epoxy and amino group. The amine linkage is stable and no chemical reduction is required, showing advantages over aldehyde-mediated protein attachment. Through polymerization, a layer of GMA polymer “brushes” was produced on PMMA surface, resulting in abundant functional groups and enlarged surface area for efficient protein immobilization.

Figure 4.1: Intrinsic fluorescence of PMMA under 543 nm and 633 nm, which are the most two common excitations in microarray scanner.

4.2 Preparation and measurement

4.2.1 Preparation of PGMA-PMMA and GA-PMMA

PGMA-PMMA: The PGMA-PMMA slides were prepared step-by-step under mild chemical conditions (Figure 4.2). 1-mm thick PMMA sheets were tailored into

75 mm × 25 mm × 1 mm (L × W × H) slides. For thoroughly cleaning, the slides were immersed in 2-propanol for 30 min and then cleaned for 5 min in an ultrasonic bath, followed by intensive rinsing with ethanol and DI water. The cleaned PMMA slides were aminated according to previously described procedure¹³⁹. Specifically, the slides were incubated with 10% hexamethylenediamine aqueous solution for 2 h at room temperature (RT), followed by rinsing with DI water and drying under nitrogen stream. The aminated PMMA slides were soaked in 5% (V/V) GMA monomer solution (prepared in 50% ethanol) for 2 h at RT to produce an acrylate-activated PMMA surface. The acrylate groups provide active sites for GMA photografting.

Figure 4.2: Schematic illustration for preparation of PGMA-PMMA substrate. The dimension in the scheme is not drawn to scale.

For photopolymerization, the acrylate-activated PMMA slides were immersed in nitrogen-purged GMA monomer solution (5% V/V in 50% ethanol-DI water), which contained 5 mM sodium persulfate as a photoinitiator. And then, the mixture was irradiated for 15 min with UV flood equipment, followed by thoroughly washing with ethanol and DI water to remove unpolymerized monomer and uncoupled oligomer or polymer.

GA-PMMA: The aminated PMMA slides prepared above were incubated with 5% aqueous glutaraldehyde (GA) solution for 2 h at RT. The resulting GA-PMMA slides were then completely rinsed with DI water and dried under nitrogen flow.

4.2.2 Fabrication of GPTMS-glass

Microscope glass slides were firstly cleaned and hydroxylated in piranha solution (a mixture of concentrated H_2SO_4 and 30% H_2O_2 , 7/3) for 2 h at 80 °C, followed by three-time washing with DI water and ethanol respectively. The cleaned slides were immersed in 5% (v/v) GPTMS ethanol solution for 1 h at RT to introduce epoxy groups onto the slide surface, and then thoroughly rinsed with ethanol. The resulting slides were dried under nitrogen flow and baked in a vacuum oven at 110 °C for 2 h.

4.2.3 Printing buffer and immobilization kinetics investigation

For printing buffer optimization, 0.01 M PBS with 2.5%-40% glycerol and 0.001%-0.012% triton X-100 was used for printing. The optimal buffer was employed in other experiments. The printing was performed as described in section 3.2.9.

Protein coupling reaction was carried out in a humid chamber for 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 h respectively for immobilization kinetics investigation. 8 h incubation was used for other experiments. After post-printing incubation, the arrayed slides were rinsed 3 times for 2 min each with TBS to remove unbound proteins. Subsequently, all slides were immersed in BlockerTM casein in TBS for 1 h to passivate unreacted functional groups.

4.3 Results and discussion

4.3.1 Characterization of substrates by AFM and ATR-FTIR

Figure 4.3A shows that the pristine PMMA exhibits a flat and featureless surface morphology, of which the root-mean-square roughness (R_q) is 3.2 ± 1.2 nm. After amination treatment, there is no significant change in surface morphology (R_q 2.5 ± 0.5 nm). However, the PGMA-PMMA surface illustrates a drastic morphology change compared to that of pristine and aminated PMMA, and its R_q sharply increases to 20.5 ± 1.6 nm. The results indicate that GMA photopolymers are successfully grafted, producing a homogeneous and nanostructured 3-D like functional layer on PMMA surface. For comparison, surface topography of GA-PMMA and GPTMS-glass, functionalized by the common used aldehyde activation and silanization methods for PMMA and glass slides respectively, are also characterized by AFM. As shown in Figure 4.3D & E, GA-PMMA is quite smooth

with $R_q 1.7 \pm 0.10$ nm and GPTMS-glass is featured by small dots with $R_q 3.8 \pm 0.16$ nm. Apparently, the obtained roughened surface provides larger specific surface area for high protein loading capacity compared to GA-PMMA and GPTMS-glass⁵³. In addition, the polymer brushes can reduce steric hindrance during Ab-Ag reaction due to the flexibility of polymer chains. Thus, the shortcoming of epoxy assisted random immobilization is overcome in certain extent.

Figure 4.3: AFM topographic images (20 μm × 20 μm). (A) Pristine PMMA; (B) Aminated-PMMA; (C) PGMA-PMMA; (D) GA-PMMA; (E) GPTMS-glass; (F) Roughness comparison of each surface.

Figure 4.4: ATR-FTIR spectra of pristine PMMA (dot line) and PGMA-PMMA (solid line).

ATR-FTIR spectra were recorded to characterize the functional groups on pristine and grafted PMMA. The typical IR spectrum of pristine PMMA with the characteristic peaks below 2000 cm^{-1} is shown in Figure 4.4 (dot line). The peak at 1720 cm^{-1} could be assigned to C=O stretching of the esters. The absorption bands at 1271 , 1240 cm^{-1} and 1189 , 1141 cm^{-1} could be ascribed to C-O and C-O-C stretching, respectively. Because the GMA photopolymer has the similar molecular structure with the PMMA, most of the characteristic peaks discussed above are also presented in the spectrum of PGMA-PMMA. It can be clearly seen that a new and distinct absorption peak at 908 cm^{-1} appears on the spectrum of PGMA-PMMA (solid line), which could be assigned to the epoxy group stretching. However, there is no noticeable absorption peak at the same wavenumber for the pristine PMMA (dot line).

The results confirm that the GMA photopolymer is successfully grafted. In addition, the introduced epoxy groups on PMMA surface can provide high protein loading capacity through covalent probe immobilization, which is one of the most important factors for high performance protein microarray⁵³.

4.3.2 Protein loading evaluation

Figure 4.5: Fluorescence intensity as a function of protein binding on GA-PMMA, GPTMS-glass and PGMA-PMMA surface. Fluorescence intensity of each group is calculated from 60 microspots on 5 parallel substrates.

Protein binding characteristics was investigated by printing Alexa Fluor® 546-conjugated IgG on PGMA-PMMA to evaluate its potentiality as a substrate for high performance protein microarray. Simultaneously, same investigation was carried out on GA-PMMA and GPTMS-glass. As shown in Figure 4.5, the fluorescence intensity on PGMA-PMMA is significantly higher than that on GA-PMMA functionalized with the conventional method^{18, 139} ($P < 0.01$) and on GPTMS-glass

modified with the silanization strategy ³² ($P < 0.01$). The improvement by two times and 20% clearly demonstrates the good protein binding capacity of PGMA-PMMA slide. As discussed above, the high protein loading should be attributed to the large specific surface area with introduced functional groups provided by PGMA-PMMA ⁶⁶.

4.3.3 Optimization of printing buffer

Printing buffer plays a very important role in protein microarray fabrication by affecting the quality of microspot, stability of immobilized protein, and protein binding capability of substrate. To obtain the best performance of protein microarrays, 0.01 M PBS with different concentrations of glycerol and triton X-100 was used as printing buffer for spotting goat IgG onto PGMA-PMMA surface. The microspot quality and signal intensity were examined by fluorescent scanner after incubating the arrayed slides with Cy3-labeled rabbit anti-goat IgG.

Figure 4.6: Affect of glycerol and triton X-100 as printing buffer additives on microspot morphology (A) and signal intensity (B).

Nano- or subnanoliter volume of protein solution is delivered onto substrate during protein microarray fabrication. A hygroscopic glycerol was used as supplements of the protein printing buffer to prevent dehydration of printed protein solution. Furthermore, glycerol could affect the size and morphology of printed protein microspot. As shown in Figure 4.6A, the spot size gradually decreases with the increase of glycerol concentration in the printing buffer ¹⁴³. However, high concentration of glycerol significantly reduces signal intensity (Figure 4.6B) because its high viscosity retards the protein diffusion in printing buffer ³³. Therefore, the optimal amount of glycerol is determined to be 2.5%, which can efficiently prevent printing buffer dehydration and give the highest signal intensity.

Nonuniform or irregular spot profile such as ring-like and smearing can result in poor protein microarray reproducibility and low reliability. Recently, ring-like formation in protein microarrays was eliminated by adding certain amount of competitive surfactant triton X-100 to printing buffer ¹⁴⁴. In this study, typical smearing spots appear on PGMA-PMMA (Figure 4.6A, rows 1-6) when there is no or a low level of triton X-100 presented in the printing buffer. The smearing profile is completely avoided using higher level of triton X-100 in printing buffer (Figure 4.6A, rows 7-15). However, too much triton X-100 results in significant decrease of signal intensity (Figure 4.6B). This is possibly because that triton X-100, as a non-ionic detergent, can block protein adsorption on the substrate and can damage protein bioactivity ¹⁴⁵⁻¹⁴⁷. Figure 4.6B shows that the signal intensity firstly increases and then

decreases with the increasing of triton X-100 concentration. The highest fluorescence intensity is observed when 0.003% triton X-100 is applied. Considering the effect of glycerol and triton X-100, the optimal printing buffer is determined to be 0.01 M PBS with 2.5% glycerol and 0.003% triton X-100. Therefore, the optimal printing buffer will be employed in following experiments.

4.3.4 Protein immobilization kinetics

Protein immobilization kinetics on the PGMA-PMMA surface was investigated to determine efficient immobilization time, which is an essential parameter for protein microarrays fabrication. On PGMA-PMMA, protein probes were immobilized covalently via the reaction between epoxy groups on the substrate surface and the amino groups on protein molecules. Therefore, the immobilization progress can be simplified as a chemical reaction:

Where P is probe protein in the printing solution, A is available anchor site on the substrate surface, and PA is the complex of immobilized probe and anchor sites. k_a is association rate constant, and k_d is dissociation rate constant.

According to the proposed reaction in equation (1), the immobilization rate can be expressed as follows:

$$\frac{d[PA]}{dt} = k_a[P][A]_t - k_d[PA] \quad (2)$$

The concentration of P in printing solution is assumed to be constant with time for simplifying the reaction. Thus, fluorescence intensity is proportional to the concentration of PA on the substrate surface. The signal intensity vs immobilization time can be expressed by the following equation¹⁴⁸:

$$F_t = E^*(1 - e^{-k_s t}) + F_0 \quad (3)$$

With

$$E^* = \frac{k_a C F_{\max}}{(k_a C + k_d)},$$

$$k_s = k_a C + k_d.$$

Where F is the fluorescence intensity, $F_{\max}$ is the maximum intensity at a certain concentration, F_0 is the intensity at $t = 0$, C is the concentration of protein probe in printed solution.

Figure 4.7 shows time-dependent binding curves for Alexa Fluor® 546-labeled goat anti-mouse IgG at concentrations of $25 \mu\text{g mL}^{-1}$, $125 \mu\text{g mL}^{-1}$, and $250 \mu\text{g mL}^{-1}$. The equation (3)-based fitting curves (solid lines) are in reasonable agreement with the experimental data (scatter points). It suggests that protein immobilization on PGMA-PMMA surface can be expressed by the proposed reaction. The parameters E^* , k_s and F_0 were obtained by the fitting, and then k_a and k_s were further calculated to be $88.5 \text{ M}^{-1}\text{S}^{-1}$ and $6.03 \times 10^{-5} \text{ S}^{-1}$, respectively. Therefore, k_a/k_s defined as the affinity coefficient (K) between P and A is equal to $1.47 \times 10^6 \text{ M}^{-1}$.

Figure 4.7: Protein binding curves (scatter points) and fitting curves (solid lines) with different printing concentrations of Alexa fluor® 546-labeled IgG on PGMA-PMMA. Fluorescence intensity of each is calculated from 60 microspots on 5 parallel substrates.

According to the experimental results and the theoretical simulation, the protein immobilization depends on the concentration of spotted proteins. For $250 \mu\text{g mL}^{-1}$, the amount of immobilized protein reaches more than 94% of the theoretical maximum after 6-8 h incubation. In other experiments, 8 h was employed as efficient time for achieving optimal protein immobilization.

4.3.5 Optimization of capture antibody concentration

Protein immobilization directly affects sensitivity and dynamic range of the protein microarrays. Thus, a maximum immobilization must be achieved on given

substrate to obtain the best assay performance. Since protein probes (mostly antibodies or antigens) are very expensive and the available concentration is limited, concentration-dependent protein immobilization on PGMA-PMMA is investigated to find an efficient protein concentration. A series of double-diluted Alexa Fluor® 546-conjugated goat anti-mouse IgG in range from 1 mg mL⁻¹ to 7.8 µg mL⁻¹ were printed on PGMA-PMMA. Fluorescence intensity as a function of printed protein concentration is shown in Figure 4.8.

Figure 4.8: Fluorescence intensity as a function of concentrations of printed Alexa 546-labeled anti-mouse IgG (0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 µg mL⁻¹ in optimal printing buffer) on PGMA-PMMA. Fluorescence intensity of each concentration is calculated from 60 microspots on 5 parallel substrates.

Based on immobilization kinetics described in equation (2), the fluorescence intensity *vs* printed protein concentrations could be given by the Langmuir adsorption isotherm at equilibrium state:

$$F_{eq} = \frac{K[P]F_0}{1 + K[P]} \quad (4)$$

Where K is the affinity coefficient, defined as $K = k_a / k_d$, F_{eq} is the equilibrium signal intensity at a certain concentration and F_0 is the signal for a complete coverage.

The experimental results (scatter points) were fitted based on equation (4). A typical Langmuir plot is shown in Figure 4.8 with an affinity coefficient K (k_a/k_s) of $4.29 \times 10^6 \text{ M}^{-1}$ and maximum fluorescence intensity of 32000 a.u.. The fluorescence intensity reaches 90% of the maximal value when $250 \text{ } \mu\text{g mL}^{-1}$ of Alexa fluor® labeled IgG was printed on the PGMA-PMMA. This indicates that the protein immobilization is nearly saturated at this concentration. In consideration of both efficiency and costs, $250 \text{ } \mu\text{g mL}^{-1}$ was used as optimal concentration in microarray fabrication. In addition, the value of affinity coefficient K is reasonably consistent with that ($1.47 \times 10^6 \text{ M}^{-1}$) calculated from immobilization kinetics in part 4.3.4. The results from kinetics and thermodynamics demonstrate that protein binding on the PGMA-PMMA surface follows the proposed reaction.

4.3.6 Detection of HBsAg in PBS

Hepatitis viruses have caused a serious worldwide health problem. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two main types of the viruses, which

often cause chronic liver diseases, cirrhosis, and hepatocellular carcinoma ¹⁴⁹. For clinical diagnosis, protein products (antigens) of viruses are the primary diagnostic biomarkers in screening of potential patient blood ¹⁵⁰. To investigate the performance of protein microarrays on PGMA-PMMA, we fabricated antibody microarray by printing anti-HBsAg as probe for sandwich immunoassay of target HBsAg. After post-printing incubation and blocking, decimally diluted HBsAg in range from 10 $\mu\text{g mL}^{-1}$ to 1 pg mL^{-1} was applied onto each array, while the diluent PBS was added to another array as a negative control.

Figure 4.9: Dose-response curves of HBsAg detection in PBS with protein microarrays on PGMA-PMMA (A), GA-PMMA (B) and GPTMS-glass (C). Signal intensity of each group used in this experiment is calculated from 60 microspots on 5 parallel substrates.

A typical sigmoid dose-response curve is plotted with double-logarithmic axes as shown in Figure 4.9A, which indicates the dynamic range for HBsAg detection on PGMA-PMMA is 10 pg mL^{-1} to $1 \text{ } \mu\text{g mL}^{-1}$ ($k = 0.452$). The LOD is determined to be 10 pg mL^{-1} by three SDs⁸². For comparison, we also fabricated same protein microarrays on GA-PMMA and GPTMS-glass, and performed an immunoassay for HBsAg detection under identical conditions to that of protein microarrays on PGMA-PMMA. The dose-response curves of HBsAg on GA-PMMA and GPTMS-glass were plotted respectively (Figure 4.9B & C). The dynamic ranges are 1 ng mL^{-1} to $1 \text{ } \mu\text{g mL}^{-1}$ for GA-PMMA ($R^2 = 0.993$, $k = 0.447$) and 100 pg mL^{-1} to $1 \text{ } \mu\text{g mL}^{-1}$ for GPTMS-glass ($R^2 = 0.990$, $k = 0.412$). The LOD of HBsAg assays on GA-PMMA and GPTMS-glass are calculated to be 1 ng mL^{-1} and 100 pg mL^{-1} , respectively. It is clearly shown that the PGMA-PMMA provides a broader dynamic range and lower LOD than GA-PMMA and GPTMS-glass.

4.3.7 Detection of HBsAg in human serum

To further demonstrate the potential for diagnostic applications, HBsAg assay was performed by PGMA-PMMA-based protein microarray in diluted human serum. It can be seen in Figure 4.10 that the dynamic range covers five orders of magnitude in the range between 10 pg mL^{-1} and $1 \text{ } \mu\text{g mL}^{-1}$ ($R^2 = 0.980$), and LOD can be down to 10 pg mL^{-1} . Compared to the results obtained in PBS, same LOD and dynamic range are achieved in the biological samples, while the sensitivity reflected by slope

of the linear range shows a slight decrease ($k = 0.398$), demonstrating its great potential for practical clinical diagnosis. In comparison with conventional one i.e. ELISA¹⁵¹ (LOD: 0.2 ng mL^{-1}) and chromatography (LOD: 1 ng mL^{-1} , www.unilatem.com), the present platform offers many merits such as higher sensitivity, high-throughput, and small sample and reagent consumption, all of which are very important for diagnostics.

Figure 4.10: Microarray sandwich immunoassay on PGMA-PMMA for HBsAg detection in human serum. (A) Fluorescence images of protein microarrays exposed to different concentrations of HBsAg; (B) Dose-response curve for detection of HBsAg in human serum. Signal intensity of each concentration is calculated from 60 microspots on 5 parallel substrates.

4.4 Conclusions

In summary, a simple but efficient strategy is described, which is capable of photografting GMA brush from PMMA for improving the performance of plastic-based protein microarray. The optimal printing buffer and suitable printing concentration are determined to be 0.01 M PBS with 2.5% glycerol + 0.003% triton X-100 and $250\ \mu\text{g mL}^{-1}$ on the new substrate, respectively. The efficient immobilization time is 8 h according to the results of protein immobilization kinetics study. Lower LOD and wider dynamic range are demonstrated by microarray immunoassay of HBsAg on PGMA-PMMA substrate compared to GA-PMMA and GPTMS-glass. The performance improvement is ascribed to the large specific surface area and high density of functional groups from the grafted GMA photopolymer brush for efficient probe immobilization. Furthermore, high performance is also achieved for detection of HBsAg in 10% human serum, demonstrating its great potential for clinical diagnosis.

Chapter 5 Synergistic Signal Amplification of Protein Microarray by Polymer Brush-Immobilized Probe and Reporter for Sensitive Detection

5.1 Introduction

Bioarrays have become a powerful tool and provided broad applications in biomedical and biotechnological fields due to their prominent advantages¹⁵²⁻¹⁵⁵. However, protein microarray technology has no ‘PCR-like’ amplification method, thus seriously limiting its important applications particularly in early diagnosis of cancers¹⁰⁵. In the past years, various strategies including enzymatic catalysis^{14, 35}, RCA¹⁵, and Au NP-assisted scanometry^{111, 112} have been explored to improve protein microarray sensitivity. The enzyme-based amplification has achieved LOD of 0.5 and 80 pg mL⁻¹ using methods of CARD¹⁴ and enzyme-linked chemiluminescence³⁵ respectively, but the former shows nonlinearity at high target concentrations¹³ and the latter requires specific instrument to conduct detections in solution. Furthermore, both approaches suffer from high cost and instability of enzymes/reagents¹⁵⁶. RCA method achieves different LODs depending on the detected target proteins^{15, 110}, but the complicated operation processes require skillful labors in professional labs¹⁴ and it could have poor dose-response¹⁵⁷. Recently, Au NP-assisted scanometric assay has been developed and used on protein microarray platform^{111, 112}. Unfortunately, the

post-deposition of silver or gold on Au NP may result in low reproducibility due to the variety of deposition conditions in different operations. Thus, there is a great demand for a versatile, inexpensive, and easy-operational strategy to amplify the detection signal of protein microarray. Nanoparticles serving as carriers of protein and /or DNA have been studied to efficiently amplify detection signals, particularly in solutions ¹⁵⁸. However, a polymer brush-modified nanoparticle based approach has not been reported yet.

Protein immobilization on substrate plays a critical role in heterogeneous immunoassays for protein detection ^{33, 92, 148, 159, 160}. Polymer coatings offer many advantages such as high protein binding capacity and efficient capture of analytes. Hyperbranched dendrimer ³⁶, layer-by-layer assembled polyelectrolytes ^{48, 82} and polyurethane hydrogel ⁸¹ have been used to modify glass and porous alumina substrate, achieving high protein loading capacity. Presynthesized copolymers with active ester have also been reported as functional coating for protein microarray ^{66, 161}. Alternatively, coatings with 3-D polymer/copolymer brushes have been developed as an attractive approach to functionalize a variety of substrates. Poly(carboxylic acid) brushes were prepared on gold surface as a functional coating for protein immobilization ^{84, 162}. Though high capacity binding of protein is achieved on these brushes, multistep activation is required. N-hydroxysuccinimide (NHS) ester and GMA polymer/copolymer brushes have also been synthesized on glass, silicon and polymeric substrates ^{106, 154, 163, 164}. However, only hydrophobic monomers are used

for the preparation of all brushes, resulting in strong surface hydrophobicity, which may cause serious nonspecific protein adsorption. In addition, protein microarray is fabricated on POEGMA brush-coated surface, onto which proteins were physically adsorbed¹⁶⁵. The instability of physical adsorption could limit its applications.

In this chapter, a random copolymer brush P(GMA-co-PEGMA) was *in situ* synthesized on substrate and SNP to efficiently immobilize probe and reporter respectively for the synergistic signal amplification. In the copolymer, each component offers a specific function: GMA branch provides epoxy group for protein covalent immobilization without activation requirement⁵¹, while hydrophilic PEG minimizes nonspecific protein adsorption and improves water dispersibility of SNPs. The schematic illustration for brush preparation and protein attachment on SNP and substrate surface is shown in Figure 5.1. The brush-coated SNP can carry a large number of reporter molecules (second antibodies in this case) at a single protein binding event to remarkably amplify the detection signals, while the P(GMA-co-PEGMA) brush-coated PMMA (P(GMA-co-PEGMA)-PMMA) offers high probe loading capacity for synergistic performance improvement. Up to date, the synergistic signal amplification of protein microarray from both copolymer brush-coated nanoparticle and substrate has not been reported yet.

5.2 Preparation and measurement

5.2.1 Production of P(GMA-co-PEGMA)-PMMA and BDGE-PMMA

P(GMA-co-PEGMA)-PMMA: The P(GMA-co-PEGMA)-PMMA slide was prepared via surface initiated-atom transfer radical polymerization (SI-ATRP, Figure 5.1B), in which the slide was firstly aminated according to the reported work with some modifications¹⁷. Specifically, PMMA slides with dimension of 75 mm × 25 mm × 1 mm (L × W × H) were cleaned with 2-propanol for 5 min under sonication, followed by rinsing with ethanol and DI water. The precleaned PMMA slides were then incubated with 1 M ethylenediamine, prepared in heptanes-tetrahydrofuran (THF) (4:1, V/V), for 3 h at RT. After that, the slides were washed with ethanol and DI water, and dried under nitrogen stream. The aminated slides were immersed in ice-cold solution of 55 mM TEA in heptanes-THF (4:1, V/V), into which BIB (final concentration, 50 mM) was added dropwisely under orbital shaking. The reaction was carried out for 15 min at 0 °C and for another 2 h at RT. Subsequently, the slides were rinsed with ethanol and DI water, and blow-dried with N₂. The initiator-immobilized slides were then immersed in a N₂-purged methanol-water (1:1, V/V) solution, which contained Cu(I)Br (4 mg mL⁻¹), bipyridine (4.6 mg mL⁻¹), and monomers (GMA 7% and PEGMA 3%, V/V). The polymerization was carried out for 1.5 h at RT under nitrogen protection, followed by rinsing with ethanol and DI water, and blow-drying with N₂.

5.2.2 Surface functional groups and protein immobilization assay

The density of functional group on each substrate was analyzed by fluorescence-labeling technique, in which the detected signal was proportional to surface density of epoxy group¹⁶⁶. The fluorescent dye, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, hydrazide specifically reacts with epoxy group to form covalent bond, and then fluorescence signal was acquired by ProXPRESS 2-D proteomic imaging system (PerkinElmer, USA).

For protein binding assay, Alexa Fluor® 546-labeled goat anti-mouse IgG at concentration of 1 mg mL⁻¹ was deposited onto each substrate as described in section 3.2.9. After post-printing incubation and thoroughly washing, the slides were scanned by ScanArray GX Microarray Scanner (PerkinElmer, USA). The acquired signal intensity is proportional to the density of attached protein.

5.2.3 SNP synthesis and modifications

Antibody-conjugated P(GMA-co-PEGMA)-SNPs were prepared according to the procedures shown in Figure 5.1A. Monodisperse SNPs (around 300 nm in diameter) were synthesized followed the well-known Stöber's method¹⁶⁷. 25 mg of dry SNPs powder was redispersed in 5 mL of ethanol (99.86%) with the assistance of sonication. Then, 0.5 mL of APTES was added into the suspension under magnetic stirring. The reaction mixture was stirred for 2 h at RT to introduce amino groups to SNPs surface via silanization. Aminated SNPs were collected by centrifugation at 10000 rpm. The

precipitation was washed three times with THF-toluene (1:1, V/V) by repeating the centrifugation process to remove excess APTES.

For initiator immobilization, the silanized SNPs were suspended in precooled solution of TEA (55 mM) in THF-toluene (1:1, V/V), into which BIB (final concentration, 50 mM) was added dropwisely under magnetic stirring. The reaction was kept for 15 min at 0 °C and for another 2 h at RT. The product was then isolated and washed as described for silanization. Surface initiated polymerization was performed with same procedures for preparation of P(GMA-co-PEGMA)-PMMA. The resulting P(GMA-co-PEGMA)-SNPs were washed with ethanol and 0.01 M PBS three times respectively by centrifugation, and then dried overnight in vacuum oven.

5 mg of dry P(GMA-co-PEGMA)-SNPs was redispersed in 2 mL of 0.01M PBS (pH7.4) for antibody conjugation. Biotinylated goat anti-rabbit IgG was injected into the brush-SNPs solution to yield a final concentration of 200 $\mu\text{g mL}^{-1}$. The resulting mixture was incubated for 12 h at RT under gentle shaking. After that, the antibody conjugated P(GMA-co-PEGMA)-SNPs were thoroughly washed using 0.05 M TBS (with 0.05% Tween 20) for four times by centrifugation, and finally suspended in TBS by gentle sonication. The excess epoxy groups on copolymer brush were blocked by casein in TBS with 0.05% Tween 20.

5.2.4 Bioconjugation of polystyrene nanoparticle (PSNP)

The original amino-PSNPs were firstly activated by GA. In more detail, amino-PSNPs suspension was dropwisely added into 50% GA aqueous solution under magnetic stirring. The resulting mixture was continuously stirred for 3 h at RT, followed by intensive washing with 0.01 M PBS using centrifugation. In between the washing steps, precipitate was resuspended in washing solution by brief sonication. Subsequently, conjugation of biotinylated goat anti-rabbit IgG to PSNPs was carried out with same procedure for antibody conjugation to P(GMA-co-PEGMA)-SNPs. The resulting schiff's bases during activation and bioconjugation were reduced to a stable secondary amine linkage using sodium cyanoborohydride.

5.2.5 Hydrodynamic size and zeta potential characterization

Bare and modified SNPs were well dispersed in DI water using gentle sonication to yield a final concentration of $100 \mu\text{g mL}^{-1}$. Zeta potential and hydrodynamic size were measured at RT with Zetasizer Nano Series (Malvern instruments, UK). Each sample was measured three times, and then mean value was obtained for figure plot.

5.2.6 Protein microarray fabrication

Probe proteins (antibody or antigen) at concentration of $250 \mu\text{g mL}^{-1}$ prepared in printing buffer (0.01 M PBS with 2.5% glycerol and 0.003% triton X-100) were spotted on BDGE-PMMA and P(GMA-co-PEGMA)-PMMA surface by contact

printing as described in section 3.2.9. After post-printing incubation, all slides were rinsed 3 times for 2 min each using TBS to remove unbound proteins, and then immersed in TBS for 2 h to block the unreacted functional groups on substrate.

5.3 Results and discussion

5.3.1 Substrate surface properties and protein immobilization efficiency

Figure 5.2A shows that contact angle on pristine PMMA is around 79° , while a slight drop ($\sim 73^\circ$) is observed after BDGE modification owing to its short chain length and limited polarity. However, the contact angle is drastically decreased to $\sim 50^\circ$ after P(GMA-co-PEGMA) brush coating, indicating good surface wettability. The significantly improved wettability is apparently due to the introduced hydrophilic PEGMA component, which can efficiently prevent nonspecific protein adsorption¹⁶⁵. As shown in Figure 5.2B (red), fluorescence intensity measured from P(GMA-co-PEGMA)-PMMA are nearly three times as high as that from BDGE-PMMA, clearly demonstrating that high density of epoxy groups from GMA component in the 3-D copolymer brush is effectively introduced onto PMMA surface.

Figure 5.2: Surface properties and protein immobilization characterization. (A) Contact angle measurement; (B) Surface functional groups evaluation and protein loading assays. The contact angle is the average of 6 readings taken from different areas of each substrate. The signal intensities for fluorophore are calculated from 3 parallel substrates for each group, while the intensities for Cy3-antibody are obtained by averaging 6 subarrays (3×3 microspots each) from 3 parallel substrates.

The actual protein loading capacity was further examined by measuring their fluorescence of immobilized Cy3-tagged antibody on BDGE-PMMA and P(GMA-co-PEGMA)-PMMA, respectively. Figure 5.2B (blue) displays that fluorescence intensity from the latter are about twice as high as that from the former, demonstrating higher protein immobilization capacity of the P(GMA-co-PEGMA)-PMMA than BDGE-PMMA. The significant improvement is undoubtedly attributed to the grafted copolymer brush^{106, 164, 165}. It is worthy of a note that for these two types of substrate, the enhancing effect of the 3-D brush on introducing epoxy functional group (red) is larger than actual protein attachment (blue). This is very possibly due to the larger size of antibody (IgG) used in the capacity measurement as

compared to the dye molecule used in the functional group characterization, thus leading to certain steric effect to “reduce” the enhancing effect.

5.3.2 Characterization of SNP and its derivatives

Surface topography examined by FE-SEM exhibits that the bare SNPs are ~300 nm in diameter with a quite smooth surface (Figure 5.3A). After brush coating, there is no obvious increase in the size, but highly concentrated small dots are observed to homogeneously distribute on P(GMA-co-PEGMA)-SNPs (Figure 5.3B). It is expected that these dots are formed by copolymer chains through crosslinking or intertwisting with each other during the drying process for SEM sample preparation.

FTIR spectra recorded from SNPs (green) and brush-SNPs (blue) are shown in Figure 5.3C. It can be seen that several characteristic bands appear for P(GMA-co-PEGMA)-SNPs, while no obvious absorption peak is presented for bare SNPs at same wavenumbers. The stretching bands at 3423 and 1102 cm^{-1} can be assigned to hydroxyl (OH) and ether (C-O-C) groups¹⁶⁸ respectively, indicating successful grafting of PEGMA. The absorption peaks at 801 and 950 cm^{-1} , assigned to epoxy ring stretching, demonstrate the presence of GMA on the brush-SNPs¹⁶⁹. The band at 1731 cm^{-1} is ascribed to the stretching of carbonyl group (C=O)¹⁷⁰, which exists in both PEGMA and GMA. The results confirm that P(GMA-co-PEGMA) brush is successfully grafted from SNPs surface with introduced epoxy groups.

Figure 5.3: Characterization of bare SNPs and P(GMA-co-PEGMA)-SNPs. FE-SEM images of bare SNPs (A); P(GMA-co-PEGMA)-SNPs (B); IR spectra of SNPs and P(GMA-co-PEGMA)-SNPs (C).

The brush coating and protein immobilization on SNPs are further verified by measurement of hydrodynamic size and zeta potential. It is shown in Figure 5.4A that the observed average size of bare SNPs is around 440 nm in diameter, which is somewhat larger than that determined by SEM (~300 nm). Apparently, the increase is attributed to the existence of a structured-water layer, which is adjacent to the hydrophilic surface of SNPs and is measured together with nanoparticle as hydrodynamic size 171. After copolymer grafting, the hydrodynamic diameter is

drastically increased from ~ 440 nm to ~ 780 nm (Figure 5.4A), although no notable increase in size is observed from SEM images.

Figure 5.4: Characterization of bare SNPs and their derivatives. (A) hydrodynamic size; (B) zeta potential. Each value is measured from 3 parallel samples with a total of 9 measurements.

It is believed that the grafted polymer brush can freely extend to solvent and separate from each other, resulting in much larger apparent diameter measured by dynamic light scattering. However, the hydrodynamic diameter of brush-coated SNPs decreases to ~ 525 nm after biotinylated antibody conjugation. As discussed above, the copolymer brush carries high density of epoxy groups, which work as active sites for covalent protein attachment. Thus, the antibody with multiple amino groups can serve as crosslinker, which links the polymer chains together and confines their extension and motion in solution, leading to the decrease of apparent size.

Zeta potential of bare SNPs and their derivatives were also examined in aqueous solution. As shown in Figure 5.4B, the zeta potential of bare SNPs is around -28 mV, resulting from abundant hydroxyl groups (Si-OH “silanol”) on SNPs surface, which present negative charge at neutral pH ¹⁷². However, the potential value of $\sim +16$ mV is measured from copolymer brush-grafted SNPs, indicating that net positive charges are presented on their surface. Such drastic change demonstrates successful grafting of GMA, of which epoxy group bears positive charges ¹⁷³. The zeta potential of antibody-conjugated brush-SNPs shifts back to negative value of ~ -15 mV (Figure 5.4B), demonstrating the epoxy functional groups are occupied by biotinylated secondary antibody and blocker reagent. The potential of around -2.5 mV is observed from biotinylated secondary antibody in DI water (Figure 5.4B). Thus, the biotinylated antibody attachment can result in negative shift of surface potential. In addition, casein (PI 4.6) and Tris(hydroxymethyl)-aminomethane (with one amino and three hydroxyl groups) in blocker reagent also have negative charges in neutral condition. Therefore, the conjugation of biotinylated antibody and blocker reagent bring the potential value from $\sim +16$ mV to ~ -15 mV. The measurement of zeta potential also confirms copolymer grafting and protein attachment, which is consistent with the results of hydrodynamic diameter examination.

5.3.3 Signal amplification evaluation

Antigen microarrays were fabricated by printing rabbit IgG on P(GMA-co-PEGMA)-PMMA to evaluate synergistic amplification effect. Three types of targets, biotinylated anti-rabbit IgG, PSNP-conjugated and brush-SNP-conjugated biotinylated anti-rabbit IgGs were applied to three groups of subarrays respectively, followed by incubation with Cy3-labeled streptavidin for signal generation. Figure 5.5 displays the bar plots for signal intensity observed from each group of subarrays under same scanning parameters. The intensity measured from subarrays incubated with brush-SNP-conjugated antibody are about ten times as high as biotinylated anti-rabbit IgG alone (without nanoparticle), while only 3-fold improvement can be achieved with the assistance of PSNP (without brush coating; 220 nm), even the size of which is comparable with SNP. Such remarkable improvement is apparently ascribed to the synergistic amplification by copolymer brushes-enhanced immobilization of both probe and reporter. Generally, the more probe is immobilized on a substrate; the more target is captured from a sample solution. Thus, more target molecules can be captured by efficiently immobilized probes on the brush-coated substrate, resulting in higher signal intensity. On the other hand, much more reporter antibodies are introduced to a microspot at a single Ab-Ag binding event by the brush functionalized-SNP than non-brush-functionalized PSNP^{50, 158}. Moreover, the flexible structure of the brushes on both substrate and nanoparticle could spontaneously adjust their shapes to provide favourite orientation for efficient immunobindings.

Figure 5.5: Evaluation of P(GMA-co-PEGMA) brush-assisted synergistic signal amplification. Fluorescence intensity of each group is calculated from 5 subarrays with a total of 45 microspots ($5 \times 3 \times 3$).

5.3.4 Antibody microarray for CEA immunoassay

CEA has been established as an important biomarker of various human cancers, such as colorectal, breast, lung, and cervical cancer^{174, 175}. As a model, sandwich immunoassay was performed on P(GMA-co-PEGMA)-PMMA with P(GMA-co-PEGMA)-SNPs-assisted signal amplification for CEA detection in 10% human serum (Figure 5.6). The dose-dependent curve with double logarithmic axes is shown in Figure 5.7A, while fluorescent image of each subarray is displayed in Figure 5.7B. The dynamic range from 10 pg mL^{-1} to 100 ng mL^{-1} for CEA detection can be obtained based on the typical sigmoid dose-response relation. LOD is determined to

be 10 pg mL^{-1} according to the well-known definition of $M_b + 3S_d$, where M_b and S_d is the mean signal and standard deviation of negative control, respectively ¹⁷⁶.

Figure 5.6: Schematic illustration of sandwich immunoassay with P(GMA-co-PEGMA) brush-assisted synergistic signal amplification. (Drawn not to scale)

Figure 5.7: Dose-dependent curve for CEA detection (A) and fluorescent images for CEA detection on P(GMA-co-PEGMA)-PMMA with SNPs-assisted signal amplification (B). Signal intensity for each concentration is calculated from 9 microspots (3×3)

For comparison, same antibody microarrays were fabricated on BDGE-PMMA. CEA detection was carried out under the detection conditions identical to that used for P(GMA-co-PEGMA)-PMMA-based microarray but without using brush-SNPs for signal amplification. The obtained dose-response curve in Figure 5.7A (green) shows an LOD of 1 ng mL^{-1} and a dynamic range from 1 ng mL^{-1} to $1 \mu\text{g mL}^{-1}$, which are much worse than that with use of the synergistic signal amplification from both probe and reporter brushes. The 2 orders improvement of the LOD can be attributed to the high protein (both probe and reporter proteins) loading capacity resulted from copolymer brushes coated on both array substrate and SNPs. Such high sensitivity could also be partially ascribed to flexible structure of the brushes, which could provide favourite protein orientations by spontaneously adjusting their shapes for efficient target and reporter binding. This versatile and efficient amplification method can also render 5 orders of dynamic range, which is better or comparable with other reported sensitive protein microarrays. Owing to the much bigger size of SNP used in this approach compared to antibody, the SNP-based reporter binding event is limited by the available surface area in particular for the detection of analytes at high concentrations¹³¹. That is why its maximum concentration (100 ng mL^{-1}) in dynamic range is lower than that observed under direct detection scheme. This work demonstrates the improved microarray performance in terms of low LOD and broad dynamic range, providing a universal, inexpensive, and easy operational method for efficient and non-enzymatic signal enhancement of protein microarrays.

5.4 Conclusions

In summary, a synergistic signal amplification strategy is investigated by using a P(GMA-co-PEGMA) brush-coated substrate and SNP for high loading capacity of probe and reporter to improve the performance of protein microarray. Among the brush, GMA provides active site for covalent protein attachment, while PEGMA can effectively minimize nonspecific protein adsorption. Synergistic signal amplification effect is clearly demonstrated on microarray format. Two orders improvement of sensitivity enabled by synergistic amplification is realized for CEA detection as compared to BDGE-PMMA without any amplification. This work provides a versatile, inexpensive, and easy-operational signal amplification strategy for sensitive and reliable protein microarray, which is of great importance in early diagnosis of various fatal diseases such as cancers.

Chapter 6 ZnO Nanorods-Enhanced Fluorescence for Sensitive Biomarker Detection without Additional Reporter-Amplification

6.1 Introduction

High-throughput antibody microarray technology has sparked intensive research interest in detection of various biomarkers in biological fluids such serum, plasma, urine, and saliva^{105, 177-179}. The sensitivity of the antibody microarrays is extremely critical for early diagnosis of diseases, therapeutic treatment in due course and accurate drug discovery/validations; however, currently it greatly relies on the reporter signal amplifications based on enzymatic reactions or nanostructured tags, which either result in low density microarrays or require specific platforms such as microwells or microchannels to avoid diffusion/convection mass transport-caused crossover interferences^{14, 15, 180, 181}. For examples, ELISA microarrays use enzymatic reaction to amplify the signals for required detection sensitivity, but they need microwells-based platform such as the standard 96 wells to perform multiplexed detection, thus offering low density of microarrays^{92, 182, 183}. The current signal amplification methods also require complicated operation procedure, prolonged assay time, and expensive reagents.

^a Reproduced in part with permission from W. H. Hu[#], Y. S. Liu[#], C. M. Li, et al., *Biosens. Bioelectron.*, 2011, 26, 3683. Copyright 2011, Elsevier.

Substrate-enabled signal amplification is a straightforward approach, in which the substrate is not only used to immobilize more probe molecules, but also can directly enhance the fluorescent signals of proximal fluorophores. Thus, there is no crossover interference faced by the conventional reporter amplification methods. Metal-enhanced fluorescence (MEF) has been observed on nanostructured metal materials originated from Plasmon-enhanced adsorption and scattering cross-sections of fluorophores^{23, 118, 184, 185}, but its application is significantly limited by high costs and susceptibility of fluorophore quenching^{186, 187}. A dielectric layer with well-defined thickness is often necessary to spatially separate the fluorophores and the metals to avoid the fluorescence quenching, which is time-consuming and costly. Optical interference coating can also enhance the fluorescent intensity by maximizing the photo-absorption and reflecting the emissive light; nevertheless, it needs to strictly control the thickness of the dielectric layer, thus resulting in high manufacturing expense^{161, 188, 189}.

ZnO nanorod, an economical metal oxide, demonstrates strong fluorescence-enhancing ability for neighboring fluorophores due to its intriguing optical properties^{63, 105, 113, 127, 190}. Moreover, unlike on the metallic surfaces, the enhancement is robust without quenching risk even if the fluorophores are directly attached on the ZnO nanorods. Up to date, no antibody microarray has been fabricated on ZnO-based substrate for biomarker detections. In this chapter, ZnO nanorods were synthesized on glass slides using a reliable chemical bath deposition method and further employed as a unique microarray substrate. By simultaneously providing

fluorescence-enhancing ability and high surface area, the ZnO substrate facilitates sensitive detection of two important biomarkers, CEA and AFP in human serum in a microarray format, demonstrating comparative or lower detection limits than the conventional ELISA without use of any reporter amplification tag/reaction.

6.2 Preparation and measurement

6.2.1 Synthesis and functionalization of ZnO nanorods-coated slides

Microscope slides (25 mm × 75 mm × 1 mm, Fisher Scientific) were cleaned in ethanol and DI water, followed by activating through soaking in 30 mL of freshly-prepared Potassium permanganate (5 mM) solution for 0.5 h (Figure 6.1A). After intensive rinsing with DI water, the slides were put in a beaker (slightly inclined) with 50 mL of 0.1 M zinc nitrate hexahydrate solution containing 4% (v/v) of ammonium hydroxide (28 wt%) and 10% (v/v) of ethanolamine ($\geq 98\%$). The beaker was placed in a water bath and maintained for 1.5 h at 75 °C without agitation. The slides (called ZnO slides hereafter) were then taken out from the solution and rinsed with DI water, followed by drying in gentle N₂ flow. ZnO slides were incubated with 5% (v/v) GPTMS ethanol solution for 2 h, and then cured at 110 °C in vacuum for 2 h (Figure 6.1A).

Figure 6.1: Schematic illustration of the fabrication of ZnO-based antibody microarray (A), and the biomarker detection format (B).

6.2.2 Fabrication of antibody microarrays

250 $\mu\text{g mL}^{-1}$ of monoclonal antibody (anti-CEA or anti-AFP) in 0.01 M PBS containing 2.5% glycerol and 0.004% triton X-100 was used as the printing buffer. Protein microarrays were printed as described in section 3.2.9. After post-printing incubation, all slides were completely washed 3 times for 2 min each with TBS to remove unbound protein molecules. The arrayed-slides were passivated with 10 mg mL^{-1} BSA solution for 1 h at RT to eliminate the nonspecific binding of proteins and finally washed with 0.05 M TBS. The printing buffer was optimized by varying concentrations of glycerol and surfactant in 50 $\mu\text{g mL}^{-1}$ Cy3-labelled anti-goat

IgG/0.01 M PBS. For comparison, fluorescent microarrays were also printed on GPTMS-modified glass substrate using $50 \mu\text{g mL}^{-1}$ Cy3-labelled anti-goat IgG in 0.01 M PBS containing 2.5% glycerol and 0.003% triton X-100 as the ink.

6.2.3 Detection of biomarkers

The antibody microarrays were first incubated with sample solutions containing 10% human serum (in clinical blood diagnosis, 10 folds dilution is a routine process) for 2 h. After rinsing with TBS, they were allowed to sequentially react with the recognition antibody (Rabbit anti-CEA or Rabbit anti-AFP in serum, 500-fold dilution) for 1 h and $2 \mu\text{g mL}^{-1}$ Cy3-labelled anti-rabbit IgG for 0.5 h (Figure 6.1B). After intensive washing with TBS and DI water, the slides were dried at 37°C before fluorescent imaging.

6.3 Results and discussion

6.3.1 Characterization of ZnO nanorods

The ZnO nanorods were synthesized on the permanganate-activated glass slides by a reliable chemical bath deposit method^{191, 192}. Figure 6.2A-B depicts the SEM images of as-prepared ZnO nanorods, showing that the nanorods are successfully deposited on the glass with random orientation. The nanorods display hexagonal faceted structures with sharp tips and varied diameters from 200 to 500 nm. Their

population density across a large surface area is very uniform (Figure 6.2A), which is essential for fabrication of antibody microarrays with low array-to-array and spot-to-spot detection variations. The optical photograph (Figure 6.2C) shows that a homogenous film covers on the slide surface. The diffraction peaks assigned to (100), (002) and (101) facets of wurtzite ZnO can be clearly observed on the XRD pattern (PDF card no. 36-1451) shown in Figure 6.2D, confirming a well crystallized structure of ZnO nanorods. Differing from that of the aligned nanorods where the (002) peak is predominant¹⁰⁵, these three peaks display similar intensity, suggesting that the nanorods rest on the substrate without preferential direction, which is consistent with the random orientation on the SEM observations.

Figure 6.2: Characterization of ZnO nanorods grown on glass. SEM images (A, B); optical photograph (C); and XRD pattern (D).

6.3.2 Signal enhancement evaluation and printing buffer optimization

It is very challenging to print microspots with homogeneous population of attached probe on a solid surface, especially on a nanostructured substrate. We have previously demonstrated that it is hard to print homogeneous microspots on aligned ZnO nanorods but is good on randomly oriented nanorods¹⁰⁵. Protein deposition by robotic spotting system on GPTMS-modified ZnO slides was investigated by using Cy3-labelled anti-goat IgG as the ink. For comparison, GPTMS-modified glass without ZnO nanorods was used as a substrate, of which the fluorescent intensity reached 894 ± 110 a.u. as shown in Figure 6.3B (a). Figure 6.3A (b) illustrates that the microspots printed on the GPTMS-modified ZnO substrate with the fluorescent protein in 0.01 M PBS, showing quasi rectangular with a dimension around $130 \mu\text{m}$ and a fluorescent intensity of 4709 ± 717 a.u. Each spot has a nonuniform intensity with a bright perimeter, indicating that the protein loading density on each spot is low and spatially inhomogeneous. It is believed to be originating from the hydrophobic nature of GPTMS-modified ZnO surface, which allows the top parts of ZnO nanorods to contact the protein solution but prevents further spreading of the solution into other parts of ZnO nanorods. With use of glycerol in the printing buffer to prevent drying of spotted solution, the spot morphology and homogeneity show no significant improvement, as shown in Figure 6.3A (c), even though the average intensity increases slightly (6591 ± 1737 a.u.).

Figure 6.3: Signal enhancement evaluation and printing buffer optimization. Fluorescent images (A) and corresponding fluorescent intensity (B) of microarrays printed with $50 \mu\text{g mL}^{-1}$ of Cy3-conjugated anti-goat IgG in (a) 0.01 M PBS with 2.5% glycerol and 0.003% Triton X-100 as the ink on GPTMS-modified glass; (b) 0.01 M PBS; (c) 0.01 M PBS with 2.5% glycerol, and (d) 0.01 M PBS with 2.5% glycerol and 0.004% Triton X-100 as the ink on GPTMS-modified ZnO nanorods, respectively.

To enhance the utilization efficiency of ZnO surface for protein attachment, the nonionic surfactant Triton X-100 (0.004%, v/v) was added in the printing buffer. The resulted spots exhibit regular roundish shape with a mean diameter of $220 \mu\text{m}$ (Figure

6.3A (d)). The fluorescent intensity drastically increases to 16942 ± 2834 a.u., which is nearly as 20-fold high as that on GPTMS-modified glass. ZnO nanorods-enabled significant signal enhancement is unambiguously attributed to the high specific surface area and the fluorescence-enhancing ability, which results from changes of photonic mode density and/or reduction in self-quenching of fluorophores^{105, 113}. As comparison, around 6 and 5 times signal enhancement is obtained from micropatterned multilayer assembly of SNPs and nanostructured TiO_x cluster respectively, both of which only provide high surface area^{60, 64}. Using the optimized printing buffer (glycerol 2.5%, v/v and Triton X-100 0.004%, v/v), microspots with high and uniform antibody density could be printed on GPTMS-modified ZnO slides for the sequential biomarker detections.

6.3.3 Surface passivation and nonspecific protein adsorption

Figure 6.4: Surface passivation and nonspecific protein adsorption. Fluorescent images of (A) original ZnO slide; (B) BSA-blocked ZnO slide after exposing to $2 \mu\text{g mL}^{-1}$ of Cy3-conjugated anti-goat IgG for 0.5 h; (C) Non-blocking ZnO slide after exposing to $2 \mu\text{g mL}^{-1}$ of Cy3-conjugated anti-goat IgG for 0.5 h. All scale bars are 1 cm.

The nonspecific protein adsorption could significantly affect the detection limit of an antibody microarray in biological fluids such as blood and serum due to various interferences^{85, 148, 159, 160}. In this work, concentrated BSA solution was employed to block the GPTMS-modified ZnO surface for reducing the interferences. The ZnO slides with or without BSA blocking were incubated in 2 $\mu\text{g mL}^{-1}$ of Cy3-conjugated anti-goat IgG for 0.5 h (typically used concentration and incubation time in sandwich immunoassays) to investigate the blocking efficiency. With BSA blocking, the ZnO slide shows a slightly higher background after exposure to the fluorescent protein solution (Figure 6.4B) in comparison to the ZnO slide without exposure (Figure 6.4A), indicating that the nonspecific protein adsorption is efficiently suppressed. On the contrary, the non-blocking ZnO slide displays much higher fluorescent intensity due to the intensive nonspecific binding (Figure 6.4C).

6.3.4 Antibody microarray for detection of biomarkers

The optimized antibody microarrays were used to detect two clinically important cancer biomarkers, CEA and AFP in human serum. AFP is an indicative protein relating to the hepatocellular carcinoma and other chronic liver diseases, while CEA is a critical biomarker to diagnose various cancers and to evaluate the therapeutic treatments as well. The tested fluorescent images of microarray spots for both biomarker assays are shown in Figure 6.5A and B, respectively. The dose-response curves in Figure 6.5C show a dynamic detection range of 5 orders of magnitudes with

a detection limit of 1 pg mL^{-1} over the negative controls (M_b+3S_d definition) for both biomarkers in serum, which are comparable to or even better than that of conventional ELISAs^{179, 193, 194}.

Figure 6.5: Microarray immunoassays of cancer biomarkers on ZnO slides. Representative fluorescent microarray images for CEA (A) and APF (B) detection in 10% human serum and the corresponding dose-response curves (C).

Compared to the immunoassay microarrays constructed on glass, silicon, and plastic substrates, ZnO substrate enables 1 order of magnitude lower detection limits^{51, 106}, which is apparently attributed to the intrinsic fluorescence-enhancement of the ZnO nanorods. One may note that the spots of negative controls in Figure 6.5A, B show weak signals, which are caused by damaged ZnO nanorods produced from the spotter pin. Even though the detection is demonstrated in diluted serum solution (10%) here, the microarray shows similar results when analyzing in 100% serum. The commonly used cut/off values for clinic screening of CEA and AFP are 20 and 5 ng mL⁻¹ in blood^{195, 196}. Therefore, even 1000-fold diluted clinic samples could be efficiently analyzed using ZnO-based antibody microarrays.

6.4 Conclusions

ZnO nanorods were used to fabricate antibody microarrays and have demonstrated a low biomarker detection limit of 1 pg mL⁻¹ and a wide dynamic range of 5 orders of magnitudes without using conventional costly signal amplification methods or specific platforms. The reporter amplification is directly attributed to the ZnO nanorods substrate itself, which eliminates the crossover interference from the conventional enzymatic amplification approach in ELISA and could eventually increase the microarray density and abolish the use of microwells or microchannels for multiplexed immunoassays. Since the fabrication of ZnO nanorods-based microarrays is scalable,

inexpensive and adaptable to the mature spotting technique, it offers great potential for a new type of high performance protein microarrays.

Chapter 7 Highly Sensitive Poly[glycidyl methacrylate-co-poly(ethylene glycol) methacrylate] Brush-based Flow-Through Microarray Immunoassay Device

7.1 Introduction

Although ELISA in a microtitre-plate has become the most prevailing approach for protein-based diagnostics, it suffers from high consumption of sample and reagents, tedious operation, and long assay time. With advances in microfabrication, flow-through immunoassay has been developed as a promising technique to take advantages of fast detection, small consumption of reagents, miniaturization, automation, and much reduced chemical waste for high-throughput protein analyses^{51, 92, 136, 197}. However, the existing flow-through devices don't have sufficient sensitivity for practical applications due to the poor protein probe loading and low Ab-Ag binding efficiency. In addition, it is very challenging to improve the sensitivity in a flow-through device because of the short Ab-Ag interaction time.

Supramolecules such as dextran^{92, 198} and PEI^{18, 101} have been reported as coating materials to provide multiple active sites for improving protein immobilization efficiency in microchannels. In this scheme, only a monolayer of polymer is randomly anchored on the substrate to introduce limited functional groups. The subsequent activation via oxidation or homobifunctional crosslinker could also result in low

activation efficiency and unstable intermediates^{18, 92}. Rigid porous matrixes including polymer monoliths^{197, 199} and organic/inorganic membranes^{48, 200, 201} could provide large surface area for high protein loading capacity to improve the detection sensitivity. Nevertheless, in a flow operation only the probes on the outside surface of the porous matrix could efficiently interact with the targets but those in the inner pores are very difficult to be accessed by the targets in a short contact time.

In situ synthesized 3-D polymer brushes could be well tailored to have high protein attachment, low nonspecific adsorption, and good accessibility⁸⁵. Unlike the rigid porous structures, the one end-tethered flexible polymer brush is mainly floating in solution, allowing targets to easily access both surface and inner site-immobilized protein probes. Various polymer brushes^{83, 106, 163, 202-204} have been synthesized to modify substrate surfaces for sensitivity improvement of static immunoassays. They may be good supporting candidates for improving the sensitivity of flow-through immunoassay devices. Recently, poly(dimethylacrilamide-co-N,N-acryloyloxysuccinimide) (poly(DMA-co-NAS)) brush has been employed as a coating matrix in microfluidic immunoassays⁷⁹, but the LOD only achieved at 9.6 ng mL⁻¹ (4 U mL⁻¹, 1 U mL⁻¹ = ~2.4 ng mL⁻¹)²⁰⁵ for allergen-specific immunoglobulin E (IgE), which is still much worse than that of ELISA method (a few pg mL⁻¹). The low sensitivity could be caused by the possibly poor protein binding efficiency resulted from the fast hydrolysis of N-hydroxysuccinimide (NHS) active group on poly(DMA-co-NAS) in an aqueous reaction media²⁰⁶. Thus, a specially tailored

polymer brush, which provides high protein loading efficiency, low nonspecific adsorption, and efficient Ab-Ag binding, is highly desirable for sensitive flow-through immunoassays in practical applications.

In this chapter, a copolymer brush, P(GMA-co-PEGMA) was prepared as a supporting matrix to efficiently immobilize proteins for improving the sensitivity and specificity of a flow-through array immunoassay device. In the brush, GMA component provides epoxy group for efficiently covalent protein attachment without activation requirement, while PEG moiety can effectively prevent nonspecific adsorption. The device is fabricated by simply laminating the protein-arrayed slide onto a ready-for-bonding double-sided adhesive tape-attached PMMA microfluidic structure. As a demonstration, a parallel microarray panel is designed to perform flow-through immunoassays for good detection flexibility and simultaneous analysis of various samples in an integrated device.

7.2 Preparation and measurement

7.2.1 Preparation of P(GMA-co-PEGMA)-slide

The microarray slide was prepared step-by-step as shown in A-D of Figure 7.1, in which P(GMA-co-PEGMA) brush was grafted from a glass slide by modified purge-free SI-ATRP²⁰⁷. In more detail, plain microscope glass slides with dimension of 75 mm × 25 mm × 1 mm were cleaned with ethanol for 10 min under sonication,

followed by rinsing with DI water. Then, the cleaned slides were hydroxylated by immersing in 3 M potassium hydroxide (KOH) for 2 h at RT. After rinsing and drying, the slides were silanized by incubation with 5% (V/V) APTES ethanol solution for 1 h at RT³³. The silanized slides were thoroughly washed with ethanol and DI water, then blow-dried with compressed air and baked in a vacuum oven at 110 °C for 2 h. The aminated slides were then soaked in ice-cold methylene chloride (CH₂Cl₂) containing 55 mM TEA, into which the initiator BIB (final concentration, 50 mM) was dropwisely added under orbital shaking to react at 0 °C for 15 min and then at RT for another 2 h for initiator immobilization on slides. Subsequently, the slides were rinsed with ethanol and DI water, and dried with nitrogen blow.

For polymerization, a 50 mL of reaction mixture of CuBr₂ (3.35 mg mL⁻¹), Bipy (4.6 mg mL⁻¹), GMA (V/V, 2%), and PEGMA (V/V, 20%) was firstly prepared in 50% (V/V) methanol. The initiator-immobilized slides were immersed into the polymerization solution, and the reaction container was immediately sealed after adding 1 mL of 119 mg mL⁻¹ ascorbic acid. The polymerization was carried out at RT for 6 h, followed by thoroughly rinsing and blow-drying. The GPTMS-slides were fabricated by incubating the hydroxylated glass slides with 5% (V/V) GPTMS ethanol solution, and then curing at 110 °C for 2 h in a vacuum oven¹⁶⁴.

Figure 7.1: Fabrication scheme and photograph of flow-through microarray device. (A) APTES-aminated glass slide; (B) initiator-modified glass slide; (C) P(GMA-co-PEGMA) copolymer brush-coated glass slide; (D) P(GMA-co-PEGMA)-slide with printed protein microarrays; (E) double-sided adhesive tape coated PMMA sheet; (F) PMMA microfluidic structure with top protection layer; (G) top protection layer removed ready-for-bonding PMMA microfluidic platform; (H) integrated flow-through microarray device; (I) photograph of flow-through microarray device.

7.2.2 Fabrication of microfluidic structure

PMMA microfluidic structure was fabricated by laser ablation²⁰⁸. Specifically, a 100- μm thick double-sided adhesive tape (Arclad 8102 transfer adhesive, Adhesives Research, Inc.) was preattached on a 1-mm thick transparent PMMA sheet before operation. A Synrad J48-2w CO₂ laser with a UC-2000 controller was used to cut desired channel patterns. The channels, reaction chambers and inlets/outlets holes were formed by evaporating the polymer controlled by computer. The dry adhesive tape was patterned together with the PMMA sheet. After that, the top protection layer of adhesive tape was removed to get the ready-for-bonding PMMA microfluidic construction, which was then bonded to protein-arrayed glass slide with the assistance of the adhesive tape for an integrated microfluidic array device (Figure 7.1 E-H).

7.2.3 Static microarray immunoassay

After post-printing incubation, the arrayed slides were intensively washed with TBS (containing 0.05% Tween 20, pH 8.0) 3 times for 2 min each to remove unbound probes. Then the remaining active sites on substrate were passivated by TBS for 1 h at RT. The Ab-Ag interaction time was investigated by applying 1 $\mu\text{g mL}^{-1}$ of Cy3-rabbit IgG to each subarray of monoclonal anti-rabbit IgG with different incubation time over a range of 10 to 120 min.

7.2.4 Flow-through microarray immunoassay

To perform microfluidic flow-through assays, the protein-arrayed P(GMA-co-PEGMA)-slide was tightly sealed by the ready-for-bonding microfluidic structure. 500 μL of TBS solution was pumped into each reaction chamber at a flow rate of 50 $\mu\text{L min}^{-1}$ for 10 min to wash away the unbound protein molecules and simultaneously block the unreacted active sites. For direct immunoassays, the solution of fluorophore-labeled analyte(s) flowed into each reaction chamber at a rate of 5 $\mu\text{L min}^{-1}$ for 30 min, followed by a flow-through washing with TBS at 50 $\mu\text{L min}^{-1}$ for 5 min. In the sandwich immunoassay, the analyte CEA with different concentrations in 10% human serum solution was pumped into each reaction chamber at a rate of 5 $\mu\text{L min}^{-1}$ for 30 min. Solution without any CEA was pumped into one chamber as a negative control. Flow-through rinsing was conducted with TBS as described in direct immunoassays. A mixture of CEA recognition antibody and Cy3-tagged secondary antibody was preincubated at 37 °C for 10 min and then injected into each chamber at 5 $\mu\text{L min}^{-1}$ for 30 min, followed by thoroughly rinsing.

7.3 Results and discussion

7.3.1 Protein loading capacity

To evaluate the protein loading capacity, Cy3-conjugated rabbit anti-goat IgG with various concentrations was spotted on P(GMA-co-PEGMA)-slides according to

the printing procedure described above. As a control, same protein microarray was fabricated on GPTMS-slides without the copolymer brush. Fluorescence intensity measured from the P(GMA-co-PEGMA)-slides is much higher than that from GPTMS-slides (Figure 7.2), indicating that higher protein loading is achieved by the copolymer brush. The signal intensity measured from P(GMA-co-PEGMA)-slides is linearly enhanced with increase of the protein concentration until $250 \mu\text{g mL}^{-1}$, after which the signal increase slows down. At the concentration as high as $500 \mu\text{g mL}^{-1}$, the surface protein immobilization is not saturated possibly because of the large number of active sites in the 3-D copolymer brush scaffold⁸⁵.

Figure 7.2: Evaluation of protein loading capacity on P(GMA-co-PEGMA)-slide and GPTMS-slide.

In contrast, for GPTMS-slide, the fluorescence intensity promptly reaches a plateau at $100 \mu\text{g mL}^{-1}$, and no further signal increase can be observed at higher concentrations, apparently due to its limited immobilization surface area and functional groups. To achieve the maximum protein loading, a high probe concentration is normally required. In consideration of expense and efficiency, the optimal probe concentration is determined to be $250 \mu\text{g mL}^{-1}$, at which the fluorescence intensity from P(GMA-co-PEGMA)-slide is higher than that from GPTMS-slide by 4 times, indicating the copolymer brush can significantly improve the detection sensitivity.

7.3.2 Surface hydrophilicity and nonspecific adsorption

Nonspecific adsorption is always a critical factor to affect sensitivity and specificity of protein detection in a biological sample such as serum or blood ¹⁶⁵. It is known that protein with diverse physicochemical properties can be adsorbed on a surface via various driving forces, among which hydrophobic-hydrophobic interaction is the most important one. Control of the substrate hydrophilicity is an effective approach to minimizing the nonspecific protein adsorption. Surface wettability of P(GMA-co-PEGMA)-slides and GPTMS-slides was examined by contact angle measurements. The results (Figure 7.3A) show that smaller contact angle ($46 \pm 0.95^\circ$) is observed on P(GMA-co-PEGMA)-slides than that on GPTMS-slides ($64 \pm 1.6^\circ$), indicating that the copolymer brush coating significantly improves surface

hydrophilicity. This should be attributed to the hydrophilic PEG component, which was previously used alone to reduce nonspecific protein adsorption^{85, 165}.

Figure 7.3: Surface hydrophilicity and nonspecific protein adsorption. (A) Contact angle measurements; (B) Nonspecific protein adsorption evaluation.

The nonspecific protein adsorption was further investigated by deposition of Cy3-conjugated anti-goat IgG on P(GMA-co-PEGMA)-slides and GPTMS-slides,

which were all passivated with BSA in TBS, casein in TBS, and plain TBS respectively. Figure 7.3B shows that the measured fluorescence intensity from P(GMA-co-PEGMA)-slides is remarkably lower than those from GPTMS-slides no matter what blocking agent is used, demonstrating the nonfouling property of the copolymer brush. The excellent resistance to nonspecific protein adsorption should be mainly ascribed to its higher hydrophilicity than GPTMS (Figure 7.3A). Since copolymer brush-coated surface has similar low nonspecific protein adsorption using TBS and BSA as blocking reagent while TBS is inexpensive, TBS is employed as the optimal blocker in the following experiments.

7.3.3 Optimization of Ab-Ag interaction time

The optimal incubation time for a complete Ab-Ag interaction was investigated under both flow-through and static immunoassays. 8 subarrays (4×4 spots per subarray) were printed on P(GMA-co-PEGMA)-slide using monoclonal anti-rabbit IgG as the probe. In flow-through detections, $1 \mu\text{g mL}^{-1}$ Cy3-tagged rabbit IgG was continuously pumped into different reaction chambers, where one microarray chamber was used as a control by pumping in plain buffer, for various incubation times over 0 to 60 min. For the static microarray experiment, a slide containing 8 subarrays was fabricated with monoclonal anti-rabbit IgG as the probe, and $1 \mu\text{g mL}^{-1}$ Cy3-tagged rabbit IgG as the target protein was incubated for different times over 0 to 120 min.

Figure 7.4: Ab-Ag binding under flow-through and static condition (inset): fluorescence intensity vs. Ab-Ag reaction time.

As shown in Figure 7.4, the fluorescence intensity rapidly reaches a plateau value in ~30 min under continuous flow, while it continuously increases under the static condition within 120 min incubation (Figure 7.4, inset), and the former illustrates 4.5 times stronger fluorescence intensity than the latter for a 30-min incubation. The results clearly demonstrate that a flow-environment significantly enhances the mass transport rate to reach a saturation in a short time for the Ab-Ag binding while it is very hard to achieve the plateau value due to the sluggish mass transport process^{79, 133}. According to the result, 30 min Ab-Ag binding time was selected for further flow-through experiments.

7.3.4 Ab-Ag binding efficiency on copolymer brush in flow-through immunoassay

Monoclonal anti-rabbit IgG ($250 \mu\text{g mL}^{-1}$) as a probe was spotted on P(GMA-co-PEGMA)-slides and GPTMS-slides, followed by pumping Cy3-tagged rabbit IgG as the target into each array chamber to investigate Ab-Ag binding efficiency. At the same time, $250 \mu\text{g mL}^{-1}$ of Cy3-tagged rabbit IgG was printed on both substrates. The relative Ab-Ag binding efficiency, determined to be the ratio of fluorescence intensity measured from the probe antibody-captured Cy3-tagged rabbit IgG to that directly measured from the substrate-immobilized Cy3-tagged rabbit IgG, can well present the actually probe-captured target.

Figure 7.5: Ab-Ag binding efficiency on P(GMA-co-PEGMA)-slide and GPTMS-slide. (a) Experimental results; (b) Schematic illustration.

As shown in Figure 7.5a, the binding efficiency achieved on P(GMA-co-PEGMA)-slides is significantly higher than that on GPTMS-slides. The high Ab-Ag binding efficiency of P(GMA-co-PEGMA) brush is very likely ascribed to its floating flexible brush structure, which allows the target molecules not only access the surface anchored probes, but also the inner site-attached probes of the brush (Figure 7.5b). In addition, the floating, long and flexible brush chain as a spacer is more favorable for the immobilized probe proteins to efficiently capture the target proteins^{79, 85}. The good hydrophilicity of the brush can also increase the accessibility of targets to the immobilized probe proteins in the brush. On the planar GPTMS-slide surface, however, proteins are prone to denaturation and suffer from severe steric resistance owing to the close contact between protein and surface, resulting in low Ab-Ag binding efficiency (Figure 7.5b)³⁶.

7.3.5 Integrated flow-through direct immunoassay device

To investigate multi-detection capability and specificity, 4 microarray panels were printed on copolymer brush-coated substrate to fabricate an integrated microfluidic device as described above for detection of anti-rabbit IgG, anti-rat IgG and anti-human IgG. Each panel contains 4×10 spots as shown in Figure 7.6(I). From left to right, every two columns were immobilized with BSA (negative control), rabbit IgG, rat IgG, human IgG, and Cy3-labeled streptavidin (positive control), respectively. Anti-rabbit IgG, anti-rat IgG, and anti-human IgG at 100 ng mL^{-1} were introduced

into chamber A, B and C, respectively. The mixture of these three targets with a same concentration of 100 ng mL^{-1} was pumped into chamber D.

Figure 7.6: Multi-detection configuration and specificity test of flow-through microarray device via direct immunoassays. (I) Fluorescent images; (II) Quantitative bar plots.

Figure 7.6(I) shows that in chamber A strong fluorescence signal is observed from the columns where rabbit IgG is immobilized as the probe. However, no fluorescence is observed from the rat IgG- and human IgG-immobilized columns in chamber A. Similarly, in chamber B and C strong signals are obtained from corresponding columns where rat IgG and human IgG are attached, demonstrating good specificity of the brush-based Ab-Ag reaction (Figure 7.6(I)). Furthermore, in chamber D positive responses are observed from all columns with immobilized probe rabbit IgG, rat IgG and human IgG. The quantitative bar plots of fluorescence intensity vs. analytes for all reaction chambers shown in Figure 7.6(II) illustrate that

the signal intensity of all negative controls and negative responses is significantly lower than that of positive responses from specific Ab-Ag bindings, indicating excellent sensitivity and specificity for this flow-through direct multi-immunoassay.

Figure 7.7: Dose-dependent curves for multiple detections using flow-through microarray device.

Quantitative multiple detections were carried out by direct immunoassay with rabbit IgG, rat IgG and human IgG as probes. 9 groups of cocktails of Cy3-labeled anti-rabbit IgG, Alexa Fluor® 546-tagged anti-rat IgG, and Cy3-conjugated anti-human IgG were introduced into different reaction chambers respectively. Diluent of 0.01 M PBS was employed as a negative control. Figure 7.7 shows the calibration curves of the three targets at concentrations range from 0.1 pg mL^{-1} to 10^8 pg mL^{-1} .

$\mu\text{g mL}^{-1}$. The LODs determined as $M_b + 3S_d$ (where M_b and S_d are mean signal and standard deviation for negative control) ¹⁷⁶ are 1 pg mL^{-1} , 10 pg mL^{-1} and 1 pg mL^{-1} for anti-rabbit IgG, anti-rat IgG, and anti-human IgG, respectively. From the typical sigmoid dose-dependent curves, it can be seen that the dynamic ranges are 1 pg mL^{-1} to 100 ng mL^{-1} for anti-rabbit IgG, 10 pg mL^{-1} to $1 \mu\text{g mL}^{-1}$ for anti-rat IgG, and 1 pg mL^{-1} to $1 \mu\text{g mL}^{-1}$ for anti-human IgG.

The performance in terms of LOD and dynamic range is much better than those of flow-through microarrays for direct detection of anti-human IgG (LOD of $\sim 1 \text{ ng mL}^{-1}$ and dynamic range of 4 orders of magnitude) with probes immobilization on PDMS ¹³⁷ and for IgE detection (LOD: $\sim 9.6 \text{ ng mL}^{-1}$) on poly(DMA-co-NAS) brush-coated glass ⁷⁹, providing 2 orders higher sensitivity than the reported flow-through immunoassays. It also accomplishes a total assay time of 45 min, which is another excellent performance. The rapid and sensitive flow-through immunoassays achieved in this work should be mainly ascribed to P(GMA-co-PEGMA) brush, of which the high hydrophilicity from its PEG component and the binding capability from its GMA component provide high protein loading capacity, low nonspecific adsorption, and high Ab-Ag binding efficiency. In contrast, protein immobilization on the reported (DMA-co-NAS) brush via NHS suffers from hydrolyzation to lose binding capability, resulting in poor LOD although it has inherent advantages of the polymer brush as a protein immobilization substrate.

7.3.6 Integrated flow-through immunoassay device for detection of biomarker in serum

CEA, an important biomarker for various cancers^{174, 175}, was used as a model target to further investigate the capability of the developed flow-through immunoassay device in practical clinical applications. Figure 7.8 shows the double logarithmic dose-dependent curve of CEA over a concentration range from 0.1 pg mL⁻¹ to 10 µg mL⁻¹ diluted in 10% human serum. The LOD is calculated to be 10 pg mL⁻¹ (around 56 fM), while the dynamic range covers five logs from 10 pg mL⁻¹ to 1 µg mL⁻¹ according to the linear fitting range (Figure 7.8), demonstrating its great potential in clinical applications. In addition, for sandwich immunoassays, the P(GMA-co-PEGMA)-assisted flow-through immunoassay array device also shows much better performance than reported microfluidic ELISA devices based on dextran-functionalized PDMS⁹², PEI-modified PMMA¹⁸, UV-curable epoxy resin⁵¹, and porous materials²⁰⁰. Using the integrated microfluidic array device, the total assay time in sandwich immunoassay is about 80 min, which is much shorter than that required by static microarray immunoassays and conventional microtitre plate-based ELISAs. The high sensitivity, inexpensive materials, and easy for mass production of P(GMA-co-PEGMA)-brush-based microfluidic techniques offer a great potential for developing miniaturized and disposable microfluidic lab-on-a-chip for practical clinical applications.

Figure 7.8: Flow-through microarray device for CEA detection in serum. (A) Fluorescent images; (B) Dose-dependent curve.

7.4 Conclusions

A highly sensitive flow-through microarray immunoassay device was developed using GMA-co-PEGMA copolymer brush as a flexible matrix for efficient probe immobilization and Ab-Ag binding. The device is fabricated by simply laminating the

protein-arrayed slide onto a ready-for-bonding double-sided adhesive tape-attached PMMA microfluidic structure. The LOD of 1-10 pg mL⁻¹ achieved for different target proteins are one to two orders better than those of reported flow-through immunoassays. The significantly reduced total assay time is also demonstrated in comparison to the static microarray immunoassay. The rapid and sensitive flow-through immunoassays achieved in this work should be mainly ascribed to the superiorities of P(GMA-co-PEGMA) brush substrate including high protein loading capacity, low nonspecific adsorption, and high Ab-Ag binding efficiency. The integrated P(GMA-co-PEGMA) based microfluidic device was further used to detect an important cancer biomarker (CEA) in serum and achieved an LOD of 10 pg mL⁻¹, demonstrating its great potential for clinical applications. In addition, as a demonstration, a parallel microarray panel is designed to perform flow-through immunoassays for good detection flexibility and simultaneous analysis of multiple samples.

Chapter 8 General Conclusions and Future works

8.1 General conclusions

In this dissertation, new advances in surface modification and nanomaterials-assisted signal amplification for performance improvement of protein chips are reviewed in detail. Recent progress in integration of protein microarray with microfluidic platform is also briefly discussed. To conquer the limitations and drawbacks of reported works, new surface modification and nanomaterials-enabled signal amplification strategies are systematically investigated and employed to improve protein microarray performance.

First, a GMA polymer brush was *in situ* synthesized via UV-induced photopolymerization to functionalize PMMA as a microarray substrate. The proposed approach is not only simple and inexpensive, but also can introduce abundant epoxy functional groups and large specific surface area. The functional polymer brush provided a high protein loading capacity, which resulted in sensitive detection of HBsAg in both PBS buffer and human serum. Optimal printing buffer and probe concentration, as well as protein immobilization kinetics, were also systematically investigated to understand the fundamentals involved in sensitive protein detection on a microarray platform.

Second, a random copolymer brush P(GMA-co-PEGMA) was synthesized by SI-ATRP on PMMA substrate and SNP for synergistic signal enhancement. The

brush-coated PMMA substrate offers high probe protein loading capacity and low nonspecific protein adsorption, while the brush-coated SNP carries a large number of reporter molecules at a single protein binding event. Two orders improvement in detection limitation is achieved through the synergistic enhancing effect compared to a small crosslinker-activated substrate without any amplification. Thus, this work provides a universal approach to inexpensively and significantly improving protein microarray sensitivity.

Third, unique ZnO nanorods-coated slides were used as an advanced substrate for improving the performance of protein microarrays. ZnO nanorods-enabled signal enhancement results from not only the high protein loading capacity, but also the direct fluorescence enhancing capability. ZnO nanorods-based antibody microarrays demonstrate a low biomarker detection limit of 1 pg mL^{-1} and a wide dynamic range of 5 orders of magnitudes without using conventional costly signal amplification methods or specific platforms. The fabrication of ZnO nanorods-coated substrate is scalable, inexpensive and adaptable to the mature spotting technique, offering great potential for a developing a new type of high performance protein microarrays.

Fourth, a highly sensitive flow-through microarray immunoassay device was fabricated using GMA-co-PEGMA copolymer brush as a flexible matrix for efficient probe immobilization and Ab-Ag binding. The LOD of $1\text{-}10 \text{ pg mL}^{-1}$ achieved for detected proteins is one to two orders better than reported flow-through immunoassays. The device also demonstrates significantly reduced total assay time

over the static microarray immunoassay. The rapid and highly sensitive detection should be mainly ascribed to the superiorities of P(GMA-co-PEGMA) brush substrate including high protein loading capacity, low nonspecific adsorption, and high Ab-Ag binding efficiency. The integrated device was further used to detect an important biomarker CEA in serum and achieved an LOD of 10 pg mL^{-1} , demonstrating its great potential for clinical applications.

Table 2. Summary of developed strategies for performance improvement of protein microarray

Strategies applied	(LOD)	Explanation
Photografted GMA brush is synthesized on substrate	10 pg mL^{-1}	The GMA brush provides high protein loading capacity.
P(GMA-co-PEGMA) brush is coated on both substrate and SNP	10 pg mL^{-1}	P(GMA-co-PEGMA) brush enhances the immobilization of both probe and reporter, and also significantly improves immunobinding efficiency.
ZnO nanorod array is coated on microarray substrate	1 pg mL^{-1}	ZnO nanorods not only provide high protein loading capacity, but also directly enhance fluorescent emission.
P(GMA-co-PEGMA) brush is used as supporting matrix for flow-through immunoassay	$1\text{-}10 \text{ pg mL}^{-1}$	P(GMA-co-PEGMA) brush enables high probe loading capacity and improved immunobinding efficiency.

In brief, substrate surface chemistry and nanomaterials-enabled signal amplification were investigated to significantly improve the performance of protein microarrays, especially in terms of sensitivity. The scientific insights into the effect of surface chemistry on protein loading capacity, nonspecific protein adsorption, and biorecognition binding efficiency were also systematically explored in this project. Furthermore, nanomaterials-mediated signal amplification holds great promise for

developing simple, inexpensive, sensitive, and reliable protein microarrays for practical application in various fields.

8.2 Future works

The success of protein chips strongly depends on interdisciplinary research and development in both science and engineering. Although a number of strategies have been explored in previous works and in this thesis project to improve the performance of protein microarrays, further improvement in sensitivity, versatility, reliability, and automaticity are still needed for various practical applications.

Oriented protein immobilization is greatly desirable for sensitive protein microarrays, but unfortunately it has not been achieved by a simple, universal and efficient way up to date. Since antibody is the prevailing probe used in protein microarrays, future work will mainly focus on development of novel polymer brushes with functional groups, which can specifically react with carbohydrate located at Fc portion of antibodies, for oriented antibody immobilization. In addition, engineering stable and inexpensive peptides with specificity to Fc portion of antibodies is an alternative strategy.

Currently, most of protein microarrays are dependent on direct or indirect labeling with fluorophore or enzyme. Although some labelless protein microarrays such as surface Plasmon resonance imaging have been developed, the sensitivity is

quite low, thus limiting their practical applications. In future works, new nanomaterials with unique physicochemical properties will be investigated to efficiently improve the sensitivity of label-free protein microarrays.

For practical applications, an integrated lab-on-a-chip system with arrayed protein sensors is in high demand for automatic and high-throughput protein screening. The miniaturized arrays offer many advantages including high-throughput screening capability, small sample and reagent consumption, but a long incubation time is still needed due to their diffusion controlled mass transport under the static situation. Microfluidics can convert static incubation to dynamic one, but suffer from low-throughput assays. The integration of microarray and microfluidics benefits from each to have fast, automatic and high-throughput protein analysis. The disadvantages of integrated system could come from the time-consuming and high cost fabrication, and lack of flexibility. To address these problems, inexpensive mass production techniques such as screen printing could be developed for fabrication of integrated and standardized lab-on-a-chip systems. In a word, a disposable, fully-functional lab-on-a-chip with customer-friendly operation system holds great promise for the future of high-throughput protein screening in proteomic research and diagnosis.

Abbreviate

AFM: atomic force microscope
AFP: α -fetoprotein
APTES: 3-aminopropyltriethoxysilane
BIB: 2-bromoisobutryl bromide
BSA: bovine serum albumin
BDGE: butanediol diglycidyl ether
Bipy: 2, 2'-bipyridyl
CEA: carcinoembryonic antigen
Cy3: Cyanine 3
DEG: diethylene glycol
ELISA: Enzyme-linked Immunosorbent assay
FTIR: Fourier transform infrared spectroscopy
GA: glutaraldehyde
GMA: glycidyl methacrylate
GPTMS: 3-glycidoxypropyltrimethoxy silane
HBsAg: hepatitis B surface antigen
HRP: horseradish peroxidase
IgG: immunoglobulin G
LOD: limit of detection
NHS: N-hydroxysuccinimide
PEG: polyethylene glycol
PMMA: poly(methyl methacrylate)
POEGMA: poly[oligo(ethylene glycol) methacrylate]
P(GMA-co-PEGMA): poly[glycidyl methacrylate-co-poly (ethylene glycol) methacrylate]
PBS: phosphate buffered saline
SEM: scanning electronic microscope
SI-ATRP: surface initiated-atom transfer radical polymerization
SNP: silica nanoparticle
SPR: surface plasmon resonance
SPRi: surface plasmon resonance imaging
TBS: tris buffered saline
TEA: triethylamine
TEOS: tetraethoxysilane
THF: tetrahydrofuran
XRD: X-ray diffraction

References

1. Schena, M., *Protein Microarrays*. Jones and Bartlett Publishers Sudbury, MA, **2005**, p 2.
2. FromontRacine, M.; Rain, J. C.; Legrain, P., *Nat. Genet.* **1997**, 16, 277-282.
3. Kersten, B.; Possling, A.; Blaesing, F.; Mirgorodskaya, E.; Gobom, J.; Seitz, H., *Anal. Biochem.* **2004**, 331, 303-313.
4. Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M., *Nat. Genet.* **2000**, 26, 283-289.
5. Fang, Y.; Lahiri, J.; Picard, L., *Drug Discov. Today* **2003**, 8, 755-761.
6. Huang, J.; Zhu, H.; Haggarty, S. J.; Spring, D. R.; Hwang, H.; Jin, F. L.; Snyder, M.; Schreiber, S. L., *Proc. Natl. Acad. Sci. U. S. A.* **2004**, 101, 16594-16599.
7. Ressine, A.; Corin, I.; Jaras, K.; Guanti, G.; Simone, C.; Marko-Varga, G.; Laurell, T., *Electrophoresis* **2007**, 28, 4407-4415.
8. Bacarese-Hamilton, T.; Mezzasoma, L.; Ardizzoni, A.; Bistoni, F.; Crisanti, A., *J. Appl. Microbiol.* **2004**, 96, 10-17.
9. Xu, R. Z.; Gan, X. X.; Fang, Y. M.; Zheng, S.; Dong, Q., *Anal. Biochem.* **2007**, 362, 69-75.
10. Cretich, M.; Damin, F.; Pirri, G.; Chiari, M., *Biomol. Eng.* **2006**, 23, 77-88.
11. Rusmini, F.; Zhong, Z. Y.; Feijen, J., *Biomacromolecules* **2007**, 8, 1775-1789.
12. Seong, S. Y.; Choi, C. Y., *Proteomics* **2003**, 3, 2176-2189.
13. Nagl, S.; Schaeferling, M.; Wolfbeis, O. S., *Microchim. Acta* **2005**, 151, 1-21.
14. Woodbury, R. L.; Varnum, S. M.; Zangar, R. C., *J. Proteome Res.* **2002**, 1, 233-237.
15. Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W. P.; Wang, M. J.; Fu, Q.; Shu, Q. P.; Laroche, I.; Zhou, Z. M.; Tchernev, V. T.; Christiansen, J.; Velleca, M.; Kingsmore, S. F., *Nat. Biotechnol.* **2002**, 20, 359-365.
16. Rucker, V. C.; Havenstrite, K. L.; Simmons, B. A.; Sickafoose, S. M.; Herr, A. E.; Shediach, R., *Langmuir* **2005**, 21, 7621-7625.
17. Brown, L.; Koerner, T.; Horton, J. H.; Oleschuk, R. D., *Lab Chip* **2006**, 6, 66-73.

18. Bai, Y. L.; Koh, C. G.; Boreman, M.; Juang, Y. J.; Tang, I. C.; Lee, L. J.; Yang, S. T., *Langmuir* **2006**, 22, 9458-9467.
19. Dorfman, A.; Kumar, N.; Hahm, J., *Adv. Mater.* **2006**, 18, 2685-2690.
20. Bakker, R. M.; Drachev, V. P.; Liu, Z. T.; Yuan, H. K.; Pedersen, R. H.; Boltasseva, A.; Chen, J. J.; Irudayaraj, J.; Kildishev, A. V.; Shalaev, V. M., *New J. Phys.* **2008**, 10, 125022.
21. Bakker, R. M.; Yuan, H. K.; Liu, Z. T.; Drachev, V. P.; Kildishev, A. V.; Shalaev, V. M.; Pedersen, R. H.; Gresillon, S.; Boltasseva, A., *Appl. Phys. Lett.* **2008**, 92, 043101
22. Mathias, P. C.; Ganesh, N.; Cunningham, B. T., *Anal. Chem.* **2008**, 80, 9013-9020.
23. Shankar, S. S.; Rizzello, L.; Cingolani, R.; Rinaldi, R.; Pompa, P. P., *Acs Nano* **2009**, 3, 893-900.
24. Le Moal, E.; Leveque-Fort, S.; Potier, M. C.; Fort, E., *Nanotechnology* **2009**, 20, 225502.
25. Staiano, M.; Matveeva, E. G.; Rossi, M.; Crescenzo, R.; Gryczynski, Z.; Gryczynski, I.; Iozzino, L.; Akopova, I.; D'Auria, S., *Acs Appl. Mater. Interfaces* **2009**, 1, 2909-2916.
26. Im, H.; Lesuffleur, A.; Lindquist, N. C.; Oh, S. H., *Anal. Chem.* **2009**, 81, 2854-2859.
27. Angenendt, P.; Glokler, J.; Murphy, D.; Lehrach, H.; Cahill, D. J., *Anal. Biochem.* **2002**, 309, 253-260.
28. Brian B Haab, M. J. D., and Patrick O Brown, *Genome Biol.* **2001**, 2, Research 0004.0001-0004.0013.
29. Kukar, T.; Eckenrode, S.; Gu, Y. R.; Lian, W.; Megginson, M.; She, J. X.; Wu, D. H., *Anal. Biochem.* **2002**, 306, 50-54.
30. Kusnezow, W.; Hoheisel, J. D., *J. Mol. Recognit.* **2003**, 16, 165-176.
31. Ressine, A.; Ekstrom, S.; Marko-Varga, G.; Laurell, T., *Anal. Chem.* **2003**, 75, 6968-6974.
32. Kusnezow, W.; Jacob, A.; Walijew, A.; Diehl, F.; Hoheisel, J. D., *Proteomics* **2003**, 3, 254-264.
33. Liu, Y. S.; Li, C. M.; Yu, L.; Chen, P., *Front. Biosci.* **2007**, 12, 3768-3773.

34. MacBeath, G.; Schreiber, S. L., *Science* **2000**, 289, 1760-1763.
35. Wolter, A.; Niessner, R.; Seidel, M., *Anal. Chem.* **2007**, 79, 4529-4537.
36. Ajikumar, P. K.; Kiat, J.; Tang, Y. C.; Lee, J. Y.; Stephanopoulos, G.; Too, H. P., *Langmuir* **2007**, 23, 5670-5677.
37. Peluso, P.; Wilson, D. S.; Do, D.; Tran, H.; Venkatasubbaiah, M.; Quincy, D.; Heidecker, B.; Poindexter, K.; Tolani, N.; Phelan, M.; Witte, K.; Jung, L. S.; Wagner, P.; Nock, S., *Anal. Biochem.* **2003**, 312, 113-124.
38. Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M., *Science* **2001**, 293, 2101-2105.
39. Minarik, A.; Humenik, M.; Li, S.; Huang, Y. W.; Krausch, G.; Sprinzl, M., *Chembiochem* **2008**, 9, 124-130.
40. Jenikova, G.; Lao, U. L.; Gao, D.; Mulchandani, A.; Chen, W., *Langmuir* **2007**, 23, 2277-2279.
41. Ekins, R. P.; Chu, F. W., *Clin. Chem.* **1991**, 37, 1955-1967.
42. Ekins, R. P., *J. Pharmaceut. Biomed.* **1989**, 7, 155-168.
43. Ekins, R.; Chu, F.; Biggart, E., *Annales De Biologie Clinique* 1990, 48, (9), 655-666.
44. Knezevic, V.; Leethanakul, C.; Bichsel, V. E.; Worth, J. M.; Prabhu, V. V.; Gutkind, J. S.; Liotta, L. A.; Munson, P. J.; Petricoin, E. F.; Krizman, D. B., *Proteomics* **2001**, 1, 1271-1278.
45. Carlsson, A.; Wingren, C.; Ingvarsson, J.; Ellmark, P.; Baldertorp, B.; Ferno, M.; Olsson, H.; Borrebaeck, C. A. K., *Eur. J. Cancer* **2008**, 44, 472-480.
46. Fang, Y.; Frutos, A. G.; Lahiri, J., *Chembiochem* **2002**, 3, 987-991.
47. Fang, Y.; Frutos, A. G.; Lahiri, J., *J. Am. Chem. Soc.* **2002**, 124, 2394-2395.
48. Dai, J. H.; Baker, G. L.; Bruening, M. L., *Anal. Chem.* **2006**, 78, 135-140.
49. Ressine, A.; Corin, I.; Jaras, K.; Guanti, G.; Simone, C.; Marko-Varga, G.; Laurell, T., *Electrophoresis* **2007**, 28, 4407-4415.

50. Jaras, K.; Tajudin, A. A.; Ressine, A.; Soukka, T.; Marko-Varga, G.; Bjartell, A.; Malm, J.; Laurell, T.; Lilja, H., *J. Proteome Res.* **2008**, 7, 1308-1314.
51. Yu, L.; Liu, Y. S.; Gan, Y.; Li, C. M., *Biosens. Bioelectron.* **2009**, 24, 2997-3002.
52. Kim, J.; Miles, A.; Gale, B. K., *Small* **2010**, 6, 1415-1421.
53. 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., *Biomaterials* **2007**, 28, 550-558.
54. Nijdam, A. J.; Zianni, M. R.; Herderick, E. E.; Cheng, M. M. C.; Prosperi, J. R.; Robertson, F. A.; Petricoin, E. F.; Liotta, L. A.; Ferrari, M., *J. Proteome Res.* **2009**, 8, 1247-1254.
55. Lee, J. H.; Kim, J. S.; Park, J. S.; Lee, W.; Lee, K. E.; Han, S. S.; Lee, K. B.; Lee, J., *Adv. Funct. Mater.* **2010**, 20, 2004-2009.
56. Tsougeni, K.; Tserepi, A.; Constantoudis, V.; Gogolides, E.; Petrou, P. S.; Kakabakos, S. E., *Langmuir* **2010**, 26, 13883-13891.
57. Zhong, W. W., *Anal. Bioanal. Chem.* **2009**, 394, 47-59.
58. Wu, Y. F.; Chen, C. L.; Liu, S. Q., *Anal. Chem.* **2009**, 81, 1600-1607.
59. Tallury, P.; Payton, K.; Santra, S., *Nanomedicine* **2008**, 3, 579-592.
60. Lee, Y.; Park, S.; Park, J.; Koh, W. G., *Biomed. Microdevices* **2010**, 12, 457-464.
61. Souplet, V.; Desmet, R.; Melnyk, O., *Bioconjugate Chem.* **2009**, 20, 550-557.
62. Wang, C.; Zhang, Y.; Seng, H. S.; Ngo, L. L., *Biosens. Bioelectron.* **2006**, 21, 1638-1643.
63. Dorfman, A.; Kumar, N.; Hahm, J. I., *Langmuir* **2006**, 22, 4890-4895.
64. Carbone, R.; De Marni, M.; Zanardi, A.; Vinati, S.; Barborini, E.; Fornasari, L.; Milani, P., *Anal. Biochem.* **2009**, 394, 7-12.
65. Kim, S. Y.; Yu, J.; Son, S. J.; Min, J., *Ultramicroscopy* **2010**, 110, 659-665.
66. Marquette, C. A.; Cretich, M.; Blum, L. J.; Chiari, M., *Talanta* **2007**, 71, 1312-1318.
67. Chen, W.; Lu, Z. S.; Li, C. M., *Anal. Chem.* **2008**, 80, 8485-8492.
68. Zhuo, Y.; Yu, R. J.; Yuan, R.; Chai, Y. Q.; Hong, C. L., *J. Electroanal. Chem.* **2009**, 628, 90-96.

69. Liu, Y.; Jiang, H., *Electroanalysis* **2006**, 18, 1007-1013.
70. Afanassiev, V.; Hanemann, V.; Wolfl, S., *Nucleic Acids Res.* **2000**, 28, e66.
71. Avseenko, N. V.; Morozova, T. Y.; Ataulakhanov, F. I.; Morozov, V. N., *Anal. Chem.* **2001**, 73, 6047-6052.
72. Avseenko, N. V.; Morozova, T. Y.; Ataulakhanov, F. I.; Morozov, V. N., *Anal. Chem.* **2002**, 74, 927-933.
73. Victor Afanassiev, V. H., Stefan Wolfl, *Nucleic Acids Res.* **2000**, 28, e66.
74. Yang, H.; Lopina, S. T., *J. Biomat. Sci.-Polym. E.* **2003**, 14, 1043-1056.
75. Cretich, M.; Pirri, G.; Damin, F.; Solinas, I.; Chiari, M., *Anal. Biochem.* **2004**, 332, 67-74.
76. Benters, R.; Niemeyer, C. M.; Wohrle, D., *Chembiochem* **2001**, 2, 686-694.
77. Mori, T.; Yamanouchi, G.; Han, X.; Inoue, Y.; Shigaki, S.; Yamaji, T.; Sonoda, T.; Yasui, K.; Hayashi, H.; Niidome, T.; Katayama, Y., *J. Appl. Phys.* **2009**, 105, 102020.
78. Angus Hucknall, D.-H. K., Srinath Rangarajan, Ryan T. Hill, William M. Reichert, A. C., *Adv. Mater.* **2009**, 21, 1-4.
79. Cretich, M.; Di Carlo, G.; Giudici, C.; Pokoj, S.; Lauer, I.; Scheurer, S.; Chiari, M., *Proteomics* **2009**, 9, 2098-2107.
80. Lueking, A.; Possling, A.; Huber, O.; Beveridge, A.; Horn, M.; Eickhoff, H.; Schuchardt, J.; Lehrach, H.; Cahill, D. J., *Mol. Cell. Proteomics* **2003**, 2, 1342-1349.
81. Derwinska, K.; Gheber, L. A.; Preininger, C., *Anal. Chim. Acta* **2007**, 592, 132-138.
82. Zhou, X. C.; Zhou, J. Z., *Proteomics* **2006**, 6, 1415-1426.
83. Vaisocherova, H.; Yang, W.; Zhang, Z.; Cao, Z. Q.; Cheng, G.; Piliarik, M.; Homola, J.; Jiang, S. Y., *Anal. Chem.* **2008**, 80, 7894-7901.
84. Dai, J. H.; Bao, Z. Y.; Sun, L.; Hong, S. U.; Baker, G. L.; Bruening, M. L., *Langmuir* **2006**, 22, 4274-4281.
85. Hu, W. H.; Liu, Y. S.; Lu, Z. S.; Li, C. M., *Adv. Funct. Mater.* **2010**, 20, 3497-3503.
86. Hu, W.; Li, C. M.; Dong, H., *Anal. Chim. Acta* **2008**, 630, 67-74.

87. Sui, G. D.; Wang, J. Y.; Lee, C. C.; Lu, W. X.; Lee, S. P.; Leyton, J. V.; Wu, A. M.; Tseng, H. R., *Anal. Chem.* **2006**, 78, 5543-5551.
88. Makamba, H.; Hsieh, Y. Y.; Sung, W. C.; Chen, S. H., *Anal. Chem.* **2005**, 77, 3971-3978.
89. Huang, B.; Wu, H. K.; Kim, S.; Zare, R. N., *Lab Chip* **2005**, 5, 1005-1007.
90. Hu, S. W.; Ren, X. Q.; Bachman, M.; Sims, C. E.; Li, G. P.; Allbritton, N., *Anal. Chem.* **2002**, 74, 4117-4123.
91. Yu, L.; Li, C. M.; Zhou, Q.; Luong, J. H. T., *Bioconjugate Chem.* **2007**, 18, 281-284.
92. Yu, L.; Li, C. M.; Liu, Y. S.; Gao, J.; Wang, W.; Gan, Y., *Lab Chip* **2009**, 9, 1243-1247.
93. Holt, D. B.; Gauger, P. R.; Kusterbeck, A. W.; Ligler, F. S., *Biosens. Bioelectron.* **2002**, 17, 95-103.
94. Rossier, J. S.; Girault, H. H., *Lab Chip* **2001**, 1, 153-157.
95. Tamarit-Lopez, J.; Morais, S.; Banuls, M. J.; Puchades, R.; Maquieira, A., *Anal. Chem.* **2010**, 82, 1954-1963.
96. Bhattacharyya, A.; Klapperich, C. M., *Biomed. Microdevices* **2007**, 9, 245-251.
97. Bui, L. N.; Thompson, M.; McKeown, N. B.; Romaschin, A. D.; Kalman, P. G., *Analyst* **1993**, 118, 463-474.
98. Cheng, J. Y.; Wei, C. W.; Hsu, K. H.; Young, T. H., *Sens. Actuators B* **2004**, 99, 186-196.
99. Gappa-Fahlenkamp, H.; Lewis, R. S., *Biomaterials* **2005**, 26, 3479-3485.
100. Massia, S. P.; Stark, J.; Letbetter, D. S., *Biomaterials* **2000**, 21, 2253-2261.
101. Bai, Y. L.; Huang, W. C.; Yang, S. T., *Biotechnol. Bioeng.* **2007**, 98, 328-339.
102. Wang, H. X.; Meng, S.; Guo, K.; Liu, Y.; Yang, P. Y.; Zhong, W.; Liu, B. H., *Electrochem. Commun.* **2008**, 10, 447-450.
103. Shi, M. H.; Peng, Y. Y.; Yu, S. N.; Liu, B. H.; Kong, J. L., *Electrophoresis* **2007**, 28, 1587-1594.
104. Yu, L.; Liu, Y. S.; Gan, Y.; Li, C. M., *Biosens. Bioelectron.* **2009**, 24, 2997-3002.

105. Hu, W. H.; Liu, Y. S.; Zhu, Z. H.; Yang, H. B.; Li, C. M., *Acs Appl. Mater. Interfaces* **2010**, 2, 1569-1572.
106. Liu, Y. S.; Li, C. M.; Hu, W. H.; Lu, Z. S., *Talanta* **2009**, 77, 1165-1171.
107. Jain, P.; Baker, G. L.; Bruening, M. L., *Ann. Rev. Anal. Chem.* **2009**, 2, 387-408.
108. Dong, H.; Cao, X. D.; Li, C. M.; Hu, W. H., *Biosens. Bioelectron.* **2008**, 23, 1055-1062.
109. Dong, H.; Li, C. M.; Chen, W.; Zhou, Q.; Zeng, Z. X.; Luong, J. H. T., *Anal. Chem.* **2006**, 78, 7424-7431.
110. Schweitzer, B.; Wiltshire, S.; Lambert, J.; O'Malley, S.; Kukanskis, K.; Zhu, Z. R.; Kingsmore, S. F.; Lizardi, P. M.; Ward, D. C., *Proc. Natl. Acad. Sci. U. S. A.* **2000**, 97, 10113-10119.
111. Dongwoo Kim, W. L. D., and Chad A. Mirkin, *Anal. Chem.* **2009**, 81, 9183–9187.
112. Wang, Z. X.; Lee, J.; Cossins, A. R.; Brust, M., *Anal. Chem.* **2005**, 77, 5770-5774.
113. Dorfman, A.; Kumar, N.; Hahm, J., *Adv. Mater.* **2006**, 18, 2685-2690.
114. Xie, F.; Baker, M. S.; Goldys, E. M., *Chem. Mater.* **2008**, 20, 1788-1797.
115. Lang, X. Y.; Guan, P. F.; Zhang, L.; Fujita, T.; Chen, M. W., *Appl. Phys. Lett.* **2010**, 96, 073701.
116. Stranik, O.; McEvoy, H. M.; McDonagh, C.; MacCraith, B. D., *Sensor. Actuat. B-Chem.* **2005**, 107, 148-153.
117. Shtoyko, T.; Matveeva, E. G.; Chang, I. F.; Gryczynski, Z.; Goldys, E.; Gryczynski, I., *Anal. Chem.* **2008**, 80, 1962-1966.
118. Cui, X. Q.; Tawa, K.; Kintaka, K.; Nishii, J., *Adv. Funct. Mater.* **2010**, 20, 945-950.
119. Cui, X. Q.; Tawa, K.; Hori, H.; Nishii, J., *Adv. Funct. Mater.* **2010**, 20, 546-553.
120. Cui, X. Q.; Tawa, K.; Hori, H.; Nishii, J., *Appl. Phys. Lett.* **2009**, 95, 133117.
121. Liu, F. X.; Tang, C. J.; Pan, J.; Cao, Z. S.; Wang, Z. L., *J. Phys. Chem. C* **2010**, 114, 9871-9875.
122. Le Moal, E.; Leveque-Fort, S.; Potier, M. C.; Fort, E., *Nanotechnology* **2009**, 20, 225502.
123. D'Agostino, S.; Della Sala, F., *Acs Nano* **2010**, 4, 4117-4125.

124. Matveeva, E. G.; Gryczynski, I.; Barnett, A.; Leonenko, Z.; Lakowicz, J. R.; Gryczynski, Z., *Anal. Biochem.* **2007**, 363, 239-245.
125. Chi, Y. S.; Byon, H. R.; Lee, B. S.; Kong, B.; Choi, H. C.; Choi, I. S., *Adv. Funct. Mater.* **2008**, 18, 3395-3402.
126. Zhang, W.; Ganesh, N.; Mathias, P. C.; Cunningham, B. T., *Small* **2008**, 4, 2199-2203.
127. Adalsteinsson, V.; Parajuli, O.; Kepics, S.; Gupta, A.; Reeves, W. B.; Hahm, J. I., *Anal. Chem.* **2008**, 80, 6594-6601.
128. Seydack, M., *Biosens. Bioelectron.* **2005**, 20, 2454-2469.
129. Nichkova, M.; Dosev, D.; Gee, S. J.; Hammock, B. D.; Kennedy, I. M., *Anal. Chem.* **2005**, 77, 6864-6873.
130. Wu, H.; Huo, Q. S.; Varnum, S.; Wang, J.; Liu, G. G.; Nie, Z. M.; Liu, J.; Lin, Y. H., *Analyst* **2008**, 133, 1550-1555.
131. Kubitschko, S.; Spinke, J.; Bruckner, T.; Pohl, S.; Oranth, N., *Anal. Biochem.* **1997**, 253, 112-122.
132. Avens, H. J.; Bowman, C. N., *Acta Biomater.* **2010**, 6, 83-89.
133. Hartmann, M.; Toegl, A.; Kirchner, R.; Templin, M. F.; Joos, T. O., *Anal. Chim. Acta* **2006**, 564, 66-73.
134. Situma, C.; Hashimoto, M.; Soper, S. A., *Biomol. Eng.* **2006**, 23, 213-231.
135. Suarez, G.; Jin, Y. H.; Auerswald, J.; Berchtold, S.; Knapp, H. F.; Diserens, J. M.; Leterrier, Y.; Manson, J. A. E.; Voirin, G., *Lab Chip* **2009**, 9, 1625-1630.
136. Dong, H.; Li, C. M.; Zhang, Y. F.; Cao, X. D.; Gan, Y., *Lab Chip* **2007**, 7, 1752-1758.
137. Heyries, K. A.; Loughran, M. G.; Hoffmann, D.; Homsy, A.; Blum, L. J.; Marquette, C. A., *Biosens. Bioelectron.* **2008**, 23, 1812-1818.
138. Wang, Z. H.; Meng, Y. H.; Ying, P. Q.; Qi, C.; Jin, G., *Electrophoresis* **2006**, 27, 4078-4085.
139. Fixe, F.; Dufva, M.; Telleman, P.; Christensen, C. B. V., *Nucleic Acids Res.* **2004**, 32, e9.
140. Fixe, F.; Dufva, M.; Telleman, P.; Christensen, C. B. V., *Lab Chip* **2004**, 4, 191-195.

141. Waddell, E.; Wang, Y.; Stryjewski, W.; McWhorter, S.; Henry, A. C.; Evans, D.; McCarley, R. L.; Soper, S. A., *Anal. Chem.* **2000**, 72, 5907-5917.
142. Li, Y. C.; Wang, Z.; Ou, L. M. L.; Yu, H. Z., *Anal. Chem.* **2007**, 79, 426-433.
143. Olle, E. W.; Messamore, J.; Deogracias, M. P.; McClintock, S. D.; Anderson, T. D.; Johnson, K. J., *Exp. Mol. Pathol.* **2005**, 79, 206-209.
144. Deng, Y.; Zhu, X. Y.; Kienlen, T.; Guo, A., *J. Am. Chem. Soc.* **2006**, 128, 2768-2769.
145. Brogan, K. L.; Shin, J. H.; Schoenfisch, M. H., *Langmuir* **2004**, 20, 9729-9735.
146. Wang, J. S.; Shan, J. X.; Xu, Q.; Ruan, X.; Gong, Y. D.; Kuang, T. Y.; Zhao, N. M., *J. Photochem. Photobiol., B* **2000**, 58, 136-142.
147. Zampieri, S.; Ghirardello, A.; Doria, A.; Tonello, M.; Bendo, R.; Rossini, K.; Gambari, P. F., *J. Immunol. Methods* **2000**, 239, 1-11.
148. Hu, W. H.; Li, C. M.; Cui, X. Q.; Dong, H.; Zhou, Q., *Langmuir* **2007**, 23, 2761-2767.
149. Duan, L. L.; Wang, Y. F.; Li, S. S. C.; Wan, Z. X.; Zhai, J. X., *BMC Infect. Dis.* **2005**, 5, 53.
150. Tsitsilonis, O. E.; Thrasyvoulides, A.; Balafas, A.; Voutsas, J. F.; Papamichail, M.; Lymberi, P., *J. Pharm. Biomed. Anal.* **2004**, 34, 811-822.
151. Zhang, H.; Xu, Y.; Yang, W.; Li, Q. G., *Chem. Mater.* **2007**, 19, 5875-5881.
152. Weinrich, D.; Jonkheijm, P.; Niemeyer, C. M.; Waldmann, H., *Angew. Chem. Int. Ed.* **2009**, 48, 7744-7751.
153. Spisak, S.; Guttman, A., *Curr. Med. Chem.* **2009**, 16, 2806-2815.
154. Rober, M.; Walter, J.; Vlakh, E.; Stahl, F.; Kasper, C.; Tennikova, T., *Anal. Chim. Acta* **2009**, 644, 95-103.
155. Hoebe, A.; Landuyt, B.; Botrus, G.; De Boeck, G.; Guetens, G.; Highly, M.; van Oosterom, A. T.; de Bruijn, E. A., *Anal. Chim. Acta* **2006**, 564, 19-33.
156. Sikes, H. D.; Jenison, R.; Bowman, C. N., *Lab Chip* **2009**, 9, 653-656.
157. Muller, U. R., *Mol. BioSyst.* **2006**, 2, 470-476.
158. Nam, J. M.; Thaxton, C. S.; Mirkin, C. A., *Science* **2003**, 301, 1884-1886.
159. Hu, W. H.; Li, C. M.; Dong, H., *Anal. Chim. Acta* **2008**, 630, 67-74.

160. Hu, W. H.; Lu, Z. S.; Liu, Y. S.; Li, C. M., *Langmuir* **2010**, 26, 8386-8391.
161. Cretich, M.; di Carlo, G.; Longhi, R.; Gotti, C.; Spinella, N.; Coffa, S.; Galati, C.; Renna, L.; Chiari, M., *Anal. Chem.* **2009**, 81, 5197-5203.
162. Jain, P.; Dai, J. H.; Baker, G. L.; Bruening, M. L., *Macromolecules* **2008**, 41, 8413-8417.
163. Orski, S. V.; Fries, K. H.; Sheppard, G. R.; Locklin, J., *Langmuir* **2010**, 26, 2136-2143.
164. Liu, Y. S.; Hu, W. H.; Lu, Z. S.; Li, C. M., *Med. Chem. Commun.* **2010**, 1, 132-135.
165. Hucknall, A.; Kim, D. H.; Rangarajan, S.; Hill, R. T.; Reichert, W. M.; Chilkoti, A., *Adv. Mater.* **2009**, 21, 1968-1971.
166. Xing, Y. J.; Borguet, E., *Langmuir* **2007**, 23, 684-688.
167. Stober, W.; Fink, A.; Bohn, E., *J. Colloid Interface Sci.* **1968**, 26, 62-69.
168. Xu, Y. Y.; Yuan, J. Y.; Muller, A. H. E., *Polymer* **2009**, 50, 5933-5939.
169. Jha, G. S.; Chhabra, P.; Suri, G.; Tyagi, M.; Arora, P.; Seshadri, G.; Khandal, R. K., *E-J. Chem.* **2009**, 6, 399-411.
170. Cheng, Z. P.; Zhu, X. L.; Kang, E. T.; Neoh, K. G., *Langmuir* **2005**, 21, 7180-7185.
171. Aqil, A.; Vasseur, S.; Duguet, E.; Passirani, C.; Benoit, J. P.; Roch, A.; Muller, R.; Jerome, R.; Jerome, C., *Eur. Polym. J.* **2008**, 44, 3191-3199.
172. Kanta, A.; Sedev, R.; Ralston, J., *Langmuir* **2005**, 21, 2400-2407.
173. Nakamura, M.; Ishimura, K., *Langmuir* **2008**, 24, 12228-12234.
174. Duffy, M. J., *Clin. Chem.* **2001**, 47, 624-630.
175. Washburn, A. L.; Gunn, L. C.; Bailey, R. C., *Anal. Chem.* **2009**, 81, 9499-9506.
176. Long, G. L.; Winefordner, J. D., *Anal. Chem.* **1983**, 55, 712-724.
177. Durner, J., *Angew. Chem. Int. Ed.* **2010**, 49, 1026-1051.
178. Haab, B. B., *Mol. Cell. Proteomics* **2005**, 4, 377-383.
179. Kingsmore, S. F., *Nat. Rev. Drug Discov.* **2006**, 5, 310-320.
180. Chen, Y.; Chi, Y. M.; Wen, H. M.; Lu, Z. H., *Anal. Chem.* **2007**, 79, 960-965.
181. Kim, B. C.; Ju, M. K.; Dan-Chin-Yu, A.; Sommer, P., *Anal. Chem.* **2009**, 81, 2388-2393.
182. Engvall, E.; Perlmann, P., *Immunochemistry* **1971**, 8, 871-874.
183. Vanweeme.Bk; Schuurs, A. H. W., *FEBS Lett.* **1971**, 15, 232-236.

184. Sau, T. K.; Rogach, A. L.; Jackel, F.; Klar, T. A.; Feldmann, J., *Adv. Mater.* **2010**, 22, 1805-1825.
185. Wang, Y. S.; Liu, B.; Mikhailovsky, A.; Bazan, G. C., *Adv. Mater.* **2010**, 22, 656-659.
186. Dong, W. J.; Dong, C.; Shuang, S. M.; Choi, M. M. F., *Biosens. Bioelectron.* **2010**, 25, 1043-1048.
187. Hong, B.; Kang, K. A., *Biosens. Bioelectron.* **2006**, 21, 1333-1338.
188. Bras, M.; Dugas, V.; Bessueille, F.; Cloarec, J. P.; Martin, J. R.; Cabrera, M.; Chauvet, J. P.; Souteyard, E.; Garrigues, M., *Biosens. Bioelectron.* **2004**, 20, 797-806.
189. Touahir, L.; Moraillon, A.; Allongue, P.; Chazalviel, J. N.; de Villeneuve, C. H.; Ozanam, F.; Solomon, I.; Gouget-Laemmel, A. C., *Biosens. Bioelectron.* **2009**, 25, 952-955.
190. Kumar, N.; Dorfman, A.; Hahm, J., *Nanotechnology* **2006**, 17, 2875-2881.
191. Kokotov, M.; Biller, A.; Hodes, G., *Chem. Mater.* **2008**, 20, 4542-4544.
192. Kokotov, M.; Hodes, G., *J. Mater. Chem.* **2009**, 19, 3847-3854.
193. Jia, C. P.; Zhong, X. Q.; Hua, B.; Liu, M. Y.; Jing, F. X.; Lou, X. H.; Yao, S. H.; Xiang, J. Q.; Jin, Q. H.; Zhao, J. L., *Biosens. Bioelectron.* **2009**, 24, 2836-2841.
194. Vaisocherova, H.; Faca, V. M.; Taylor, A. D.; Hanash, S.; Jiang, S. Y., *Biosens. Bioelectron.* **2009**, 24, 2143-2148.
195. Guadagni, F.; Ferroni, P.; Carlini, S.; Mariotti, S.; Spila, A.; Aloe, S.; D'Alessandro, R.; Carone, M. D.; Cicchetti, A.; Ricciotti, A.; Venturo, I.; Perri, P.; Di Filippo, F.; Cognetti, F.; Botti, C.; Roselli, M., *Clin. Cancer Res.* **2001**, 7, 2357-2362.
196. Gupta, S.; Bent, S.; Kohlwes, J., *Ann. Intern. Med.* **2003**, 139, 46-50.
197. Liu, J. K.; Chen, C. F.; Chang, C. W.; DeVoe, D. L., *Biosens. Bioelectron.* **2010**, 26, 182-188.
198. Jonsson, C.; Aronsson, M.; Rundstrom, G.; Pettersson, C.; Mendel-Hartvig, I.; Bakker, J.; Martinsson, E.; Liedberg, B.; MacCraith, B.; Ohman, O.; Melin, J., *Lab Chip* **2008**, 8, 1191-1197.

199. Jain, S. R.; Borowska, E.; Davidsson, R.; Tudorache, M.; Ponten, E.; Emneus, J., *Biosens. Bioelectron.* **2004**, 19, 795-803.
200. Fu, Z. F.; Yan, F.; Liu, H.; Yang, Z. J.; Ju, H. X., *Biosens. Bioelectron.* **2008**, 23, 1063-1069.
201. van Lieshout, R. M. L.; van Domburg, T.; Saalmink, M.; Verbeek, R.; Wimberger-Friedl, R.; van Dieijen-Visser, M. P.; Punyadeera, C., *Anal. Chem.* **2009**, 81, 5165-5171.
202. Andersson, O.; Larsson, A.; Ekblad, T.; Liedberg, B., *Biomacromolecules* **2009**, 10, 142-148.
203. Lata, S.; Piehler, J., *Anal. Chem.* **2005**, 77, 1096-1105.
204. Tugulu, S.; Arnold, A.; Sielaff, I.; Johnsson, K.; Klok, H. A., *Biomacromolecules* **2005**, 6, 1602-1607.
205. Schuurman, J.; Perdok, G. J.; Lourens, T. E.; Parren, P.; Chapman, M. D.; Aalberse, R. C., *J. Allergy Clin. Immun.* **1997**, 99, 545-550.
206. Hermanson, G. T., *Bioconjugate Techniques*. Academic Press: 1996.
207. Qian, H.; He, L., *Anal. Chem.* **2009**, 81, 9824-9827.
208. Sun, S.; Yang, M. H.; Kostov, Y.; Rasooly, A., *Lab Chip* **2010**, 10, 2093-2100.

Publication List

1. **Liu, Y. S.**; Hu, W. H.; Lu, Z. S.; Li, C. M., Photografted Poly(methyl methacrylate)-Based High Performance Protein Microarray for Hepatitis B Virus Biomarker Detection in Human Serum. *Med. Chem. Commun.* **2010**, *1*,132-135.
2. Hu, W. H.; **Liu, Y. S.**; Yang, H. B.; Zhou, X. Q.; Li, C. M., ZnO Nanorods-Enhanced Fluorescence for Sensitive Microarray Detection of Cancers in Serum without Additional Reporter-Amplification. *Biosens. Bioelectron.* **2011**, *26*(8), 3683-3687. **(Equal first author)**
3. **Liu, Y. S.**; Guo, C. X.; Hu, W. H.; Lu, Z. S.; Li, C. M., Sensitive protein microarray synergistically amplified by both polymer brush-immobilized probe and reporter. *J. Colloid Interf. Sci.* **2011**, DOI: doi:10.1016/j.jcis.2011.05.030.
4. **Liu, Y. S.**; Wang, W.; Hu, W. H.; Lu Z. S.; Zhou X. Q., Li, C. M., Highly Sensitive Poly[glycidyl methacrylate-co-poly(ethylene glycol) methacrylate] Brush-Based Flow-Through Microarray Immunoassay Device. *Biomed. Microdevices* **2011**, DOI: 10.1007/s10544-011-9547-1.
5. **Liu, Y. S.**; Li, C. M., Advanced Immobilization and Amplification for High Performance Protein Chips. *Anal. Lett.* **2011**, accepted.
6. **Liu, Y. S.**; Li, C. M.; Hu, W. H.; Lu, Z. S., High performance protein microarrays based on glycidyl methacrylate-modified polyethylene terephthalate plastic substrate. *Talanta* **2009**, *77*, 1165-1171.
7. **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*, 3768-3773.
8. Hu, W. H.; **Liu, Y. S.**; Lu, Z. S.; Li, C. M. Poly(oligo(ethylene glycol) methacrylate-co-glycidyl methacrylate) Brush Substrate for Sensitive Surface Plasmon Resonance Imaging Protein Arrays. *Adv. Funct. Mater.* **2010**, *20*, 3497–3503.

9. Hu, W. H.; **Liu, Y. S.**; Zhu, Z. Z.; Yang, H. B.; Li, C. M. Randomly Oriented ZnO Nanorods as Advanced Substrate for High Performance Protein Microarrays. *ACS Appl. Mater. Interfaces* 2010, 2, 1569-1572.
10. Yu, L.; **Liu, Y. S.**; Gan, Y.; Li, C. M., High-performance UV-curable epoxy resin-based microarray and microfluidic immunoassay devices. *Biosens. Bioelectron.* **2009**, 24, 2997-3002.
11. Yu, L., Li, C. M., **Liu, Y. S.**; Gao, J.; Wang, W.; Gan, Y., Flow-through functionalized PDMS microfluidic channels with dextran derivative for ELISAs. *Lab Chip* **2009**, 9, 1243-1247.
12. Wang, W.; Yang, C.; **Liu, Y. S.**; Li, C. M., On-demand droplet release for droplet-based microfluidic system. *Lab Chip* **2010**, 10, 559-562.
13. Hu, W. H.; Lu, Z. S.; **Liu, Y. S.**; Li, C. M. In situ Surface Plasmon Resonance Investigation of Assembly Process of Multiwall Carbon Nanotubes on Alkanethiol Self-assembled Monolayer for Efficient Protein Immobilization and Detection. *Langmuir* **2010**, 26, 8386-8391.
14. Xiao, Y. H.; Li, C. M.; **Liu, Y. S.**, Electrochemical impedance characterization of antibody-antigen interaction with signal amplification based on polypyrrole-streptavidin, *Biosens. Bioelectron.* **2007**, 22, 3161-3166.
15. Yu, L.; Lu, Z. S.; Gan, Y.; **Liu, Y. S.**; Li, C. M., AFM study of adsorption of protein A on a poly(dimethylsiloxane) surface. *Nanotechnology* **2009**, 20, 285101.