DEVELOPMENT OF A HIGH-THROUGHPUT ELECTROKINETICALLY-CONTROLLED HETEROGENEOUS IMMUNOASSAY MICROFLUIDIC CHIP

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Development of a high-throughput electrokinetically-controlled heterogeneous immunoassay microfluidic chip

Doctor of Philosophy, 2008
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Abstract

This thesis was on the development of a high-throughput electrokinetically-controlled heterogeneous immunoassay (EK-IA) microfluidic chip for clinical application. Through a series of experimental studies, a high-throughput EK-IA was developed. This EK-IA was capable of automatically screening multiple analytes from up to 10 samples in parallel, in only 26 min. Flow control in an integrated microfluidic network was realized by numerical simulation of the transport processes. This EK-IA was successfully applied to detect \textit{E. coli} O157:H7 antibody and \textit{H. pylori} antibody from human sera with satisfactory accuracy. Simultaneous screening of both antibodies from human sera was also achieved, demonstrating the potential of this EK-IA for efficiently detecting multiple pathogenic infections in clinical settings. Preliminary work on the application of EK-IA to detect biomarkers of embryo development in embryo culture media also yielded good results. In addition to the experimental studies, the reaction kinetics of this microfluidic EK-IA has also been investigated, using both numerical simulation and a modified Damköhler number. Targeted towards a more sensitive assay, the influences of several important parameters on the reaction kinetics were studied. This EK-IA holds great promise for automated and high-throughput immunoassay in clinical environments.
Acknowledgements

I would like to express my sincere gratitude to my thesis supervisor, Prof. D. Li, for giving me the opportunity to study with him and for his insightful guidance to my research. I am especially grateful for his unwavering support and encouragement, even in the times of difficulty. Also, I want to thank him for setting an example of devotion to his work and responsibleness for his students, which has greatly impacted my life.

I also want to thank my cosupervisor, Prof. Y. Sun, who has given me much help after Dr. Li’s transfer to Vanderbilt University. Discussions with him on my research and career are illuminating.

My deep gratitude also goes to Prof. P. M. Sherman, for his kind and generous support all along. He has offered me training in his lab, helped improve my English writing, and many more. This thesis work would not have been done without his help.

I also want to thank Prof. A. Guenther, for warmly accommodating me in his lab, and for many helpful discussions on my research and career.

I am grateful for Prof. A. N. Sinclair, Prof. P. Sullivan, and the department of MIE, for the financial support they generously granted me after Prof. Li’s transfer.

I want to thank my colleagues for their help and friendship. My special thanks go to Dr. F. Y. H. Lin (for his valuable help with the biology part of the experiments including bacterium culture and serum assays), Dr. G. Hu (for his help on CFD), and Dr. Q. Xiang (for many helpful discussions).

My thanks also go to Prof. R. F. Casper and Dr. A. Jurisicova, for the collaboration in assaying embryo culture media; Prof. M. Bussmann for helpful discussions on CFD; Ms. Katheen Johnson-Henry for giving me training and assistances to work in Prof. Sherman’s lab; and Prof. M. Karmili and Ms. M. Mascarenhas, for their help in providing human serum samples.

The financial support from the Natural Sciences and Engineering Research Council which funded this thesis work and the University of Toronto Open Fellowship is also gratefully acknowledged.

I am indebted to my parents for their unconditional love and acceptance. I also want to express my gratitude for my friends, for their help, encouragement, and prayers.
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<td>IA</td>
<td>Immunoassay</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>dAb</td>
<td>Detection antibody</td>
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<tr>
<td>cAb</td>
<td>Capture antibody</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>EK-</td>
<td>Electrokinetically-</td>
</tr>
<tr>
<td>EK-IA</td>
<td>Electrokinetically-driven/controlled immunoassay</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>µFN</td>
<td>Microfluidic network</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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Table 8.2 The effect of a 10-fold increase of $K$ by varying $k_{on}$ or $k_{off}$ on the coefficients of reaction in Eq. (8.6).
1.1 Immunoassay

1.1.1 Immunological reaction

Immune system protects the body against foreign substances. At the invasion of infectious substances, such as bacteria, viruses or other pathogens, immune responses are stimulated and a specialized class of proteins called antibodies is secreted in blood to neutralize the invading organisms. The substance that stimulates the generation of an antibody is termed antigen. An antibody functions by specifically binding to the corresponding antigen and then either neutralizes it directly or activates further immunological responses, such as cell killing and inflammation. The specific binding reaction between an antibody (Ab) and the corresponding antigen (Ag) is called an immunological reaction.

The specificity of the Ab-Ag interaction comes from the complementary structure of the binding regions on Ab and Ag, named paratope and epitope, respectively. As illustrated in Fig 1.1, antibody is a Y-shaped protein that belongs to the class of immunoglobulin (Ig). While the base part of the molecule is a “constant region” that is the same for all Ig in the same class, the tips of the Y with two paratopes are a “variable region” that is unique for each type of antibody, so that the antibody can “recognize” the specific antigen but not other molecules, like a lock and key configuration.

1.1.2 Immunoassay – concept, application and classification

Exploiting the specific immunological reaction of Ab and Ag, one can identify or quantify substances from a sample \textit{in vitro}. This analytical technique is called immunoassay (IA).
Immunoassay was introduced by Yalow and Berson, and Ekins for analyzing hormones in the late 1950s (Ekins, 1960; Yalow & Berson, 1959). This highly sensitive and selective analytical technique soon led to a revolution in endocrinology and was applied to many other areas. Now, the application of IA has expanded to an extensive spectrum of small and large molecules, such as therapeutic drugs, enzymes, vitamins, tumor markers, antibodies or antigens associated with infectious agents, as well as cells and cellular components. It has become a popular analytical tool not only in clinical diagnosis and biomedical research but also in a range of other fields including veterinary and agricultural research, drug development, environmental studies and food sciences.

Immunoassay can be classified into competitive and noncompetitive ones. In competitive IA the analyte of interest competes with a fixed amount of labeled analytes for a limited number of antibody binding sites. As the analyte concentration increases, more labeled analytes will be displaced, yielding a decrease in signal if the antibody-bound labeled analyte is detected, or an increase in signal if the free labeled analyte is detected. In noncompetitive assays, the signal increases in proportion to the analyte concentration. Usually, a competitive IA is used to detect smaller molecules and a noncompetitive IA is used to detect larger molecules, such as antibody. The microfluidic IA developed in this thesis is a noncompetitive IA.

Immunoassay can also be classified as homogeneous and heterogeneous. In homogeneous IA, the antibody and antigen are free to associate in a homogeneous liquid, with or without a following separation of the bound and the free fractions. Homogeneous IA is fast to conduct but difficult to develop. Also, such assays generally have low
sensitivities and therefore are mainly used in monitoring the levels of therapeutic drugs in blood, which have a relatively high concentration (Christopoulos & Diamandis, 1996).

On the other hand, in heterogeneous IA, the Ab-Ag binding reaction takes place at a liquid-solid interface. The introduction of a solid phase enables simple and efficient separation of the bound and unbound (free) reagents. More importantly, heterogeneous IA allows for preconcentration of antigen or antibody from a dilute solution to a solid substrate, thus achieving a lower limit of detection. The flexible assay design and high sensitivity make heterogeneous IA a more versatile technique than homogeneous IA. The microfluidic IA developed in this thesis is based on a heterogeneous IA system.

1.1.3 Heterogeneous immunoassay

1.1.3.1 Components and formats

There are three basic components of a heterogeneous IA: the capture system, the analyte, and the detection system. Two simplest examples of “direct” IA for antibody and antigen detection are illustrated in Fig. 1.2 (a) and (b), respectively. In Fig. 1.2 (a), the capture molecule, antigen, is immobilized on a solid surface to probe the analyte, antibody molecule, from a sample solution. The antibody is labeled with a signal-generating molecule, such as a radioisotope, a fluorophore, or an enzyme, so that the existence or concentration of the analyte in the sample can be evaluated by detecting the signals. For higher flexibility of the assay, analytes are seldom directly labeled in practice. Instead, a labeled secondary antibody, called detection antibody (dAb), is often invoked for signal generation. Schematics of these so-called “indirect” IA are shown in Fig. 1.2 (c) and (d). In this thesis, an indirect IA for antibody detection as shown in Fig. 1.2 (c) is used predominantly.
In addition to the three major components mentioned above, there are some other operations required in conducting an IA, such as blocking of non-specific binding and washing of excessive unbound molecules. A brief introduction of the stages in a heterogeneous IA is given below.

1.1.3.2 Stages

1) Immobilization of capture molecules to a solid phase

Solid phases used in IAs can be generally grouped into three categories: low-capacity plastic materials such as polystyrene, high-capacity membranous materials such as nitrocellulose and nylon, as well as latex particles (microspheres and beads) (Matson, 2000). By far the most widely used solid phase is the 96-well microtitre plate made of polystyrene, for enzyme-linked immunosorbent assay (ELISA), a very popular heterogeneous IA.

There are three approaches for immobilizing capture molecules (proteins) to solid surfaces – physical adsorption, chemical cross-linking and biomolecular affinity binding (Matson, 2000). Physical adsorption, i.e., “coating”, in spite of some limitations, such as partial denaturation, random orientation and desorption of adsorbed molecules (Butler, 1996), is by far the most popular method, due to its simplicity and satisfactory reproducibility.

2) Immunoreaction and incubation

Interaction between an antibody and the corresponding antigen is the key of an immunoassay. Several non-covalent bonds play a role in this interaction, including hydrophobic forces, electrostatic forces, van der Waals forces and hydrogen bonds.
These forces come into effect only when the two molecules are at a distance of several nanometers or closer.

The time-limiting step in a heterogeneous IA is passive diffusion. Reactants in solution have to diffuse to the solid surface to react with the immobilized probe molecules. The distance to travel is in the order of millimeters in microtitre wells. According to the Einstein-Smoluchowski equation,

$$ d = \sqrt{2D\tau} $$

where $d$ is the diffusion distance, $D$ is the diffusion coefficient and $\tau$ is the required time. The time for an IgG molecule ($D = 4 \times 10^{-7}$ cm$^2$/s) to diffuse 1 mm is approximately 3 hours and 30 minutes. Therefore, incubation time is usually long in conventional IAs, ranging from several hours to two days.

3) Blocking

Besides the specific interaction of Ab-Ag, analytes or impurities in the sample can bind nonspecifically to any unoccupied solid site during an assay, which leads to high background signals. To block this undesirable non-specific binding (NSB), two categories of agents can be used – immunologically inert protein solutions such as bovine serum albumin, casein, skim milk powder and normal serum, and nonionic detergents such as Tween-20 (Butler, 1996). High-concentration protein solutions are either used as a separate blocking step before Ab-Ag reaction, or added into the dilution buffer of analyte or reagent to compete with the nonspecific factors for available binding sites, or a combination of both stages. Nonionic detergents function by preventing the interactions of protein-solid and protein-protein.

4) Washing
Washing steps are to remove free reagents from the reaction chamber after each binding reaction, from the immobilization of capture molecules to the binding of detection antibodies. For IAs on microtitre plates, washing is done by manually emptying the wells of reagents followed by adding buffer into wells. Such a process is repeated at least three times for every well to ensure a thorough removal. These procedures of the conventional IAs make them labor-intensive.

5) Detection

After the reaction of detection antibody and the final washing (for enzyme IA, after stopping the enzymatic reaction of substrate), bound signals on the solid surface can be detected. Depending on the type of the label employed, different detection approaches are used, including radiometric, colorimetric, spectrophotometric, fluorometric, luminometric, electrometric and nephelometric ones.

1.1.3.3 One example

The process of an entire conventional IA is illustrated as an example below – detection of rabbit anti-*Helicobacter pylori* IgG. The analyte is captured by *H. pylori* Ag immobilized on a solid phase and then bound by the dAb, goat anti-rabbit IgG. This model system will be used throughout this thesis. The conventional IA is in an ELISA format, with steps as following:

1) Ag immobilization: overnight, on microtitre plate;
2) Washing: phosphate buffered saline (PBS) with 0.1% Tween, 4 times (PBST×4);
3) Blocking: goat serum, 15-60 min;
4) Washing: PBST×4;
5) Sample incubation: 30-90 min;
6) Washing: PBST × 4;
7) dAb (enzyme-conjugated) incubation: 15-60 min;
8) Washing: PBST × 4;
9) Substrate incubation: 15-30 min.

Due to the long incubation procedures, the entire IA takes 3-6 hours, not including the time for overnight Ag immobilization. Another salient feature is that the IA is a multi-step analysis with many solution operations involved, such as reagent pipetting and well washing. Human errors can be easily introduced in such a labor-intensive task. Although automated IA systems that handle the fluidic operations following a robotic route have been developed (Chan, 1996), the equipments are usually bulky and expensive, restricting their uses mainly to centralized medical laboratories. The following part of the thesis will demonstrate how a revolutionary improvement on the IA technique can be achieved by the technology of microfluidics.

1.2 Microfluidic-based immunoassay

1.2.1 Technology of microfluidics

The technology of microfluidics refers to the manipulation of liquid or gas in microchannels with cross-sectional dimensions on the order of 10-100 µm. Though the technology can be traced back to the development of a miniaturized gas chromatography in Stanford University in the 1970s (Terry et al., 1979), it was not until 1990s that the field started blossoming, owing to the availability of techniques for fabricating microfluidic devices initially from microelectronics, as well as the stimulation from the explosion in biology and biotechnology (Manz et al., 1990; Whitesides, 2006).
The vast interest and fast rising of this field are attributed to the tremendous advantages that the miniaturized devices offers over conventional bench-top analytical instruments, including reduced analysis time, consumption of reagents, and cost of power and material, as well as increased throughput, portability, and flexibility of design.

Based on microfluidics technology, research on miniaturization of analytical procedures, i.e., the so-called lab-on-a-chip, has covered a wide variety of chemical and biological analyses. Many excellent reviews are available (Auroux et al., 2002; Beebe et al., 2002; Dittrich et al., 2006; Erickson & Li, 2004; Sia & Whitesides, 2003; Stone & Kim, 2001; Verpoorte, 2002; Vilkner et al., 2004), in areas including drug discovery (Dittrich & Manz, 2006), DNA analysis (Tegenfeldt et al., 2004), immunoassay (Bange et al., 2005), as well as cell handling and analysis (Huh et al., 2005; Toner & Irimia, 2005).

1.2.2 Microfluidics-based homogeneous IA

Research on miniaturized IAs, including both homogenous and heterogeneous ones, started in the late 1990s and quickly became a very dynamic area. Heterogeneous IA is the focus of this thesis and an extensive review on the microfluidic studies in this area will be given later. Nevertheless, microfluidics-based homogeneous IA appeared earlier than the heterogeneous counterpart and has seen significant achievements. Therefore, this area is briefly reviewed below, with two representative assay types addressed.

Shortly after the remarkable success of microchip-based capillary electrophoresis at the initial stage of the technology of lab-on-a-chip, this technique was applied to miniaturize homogeneous IA (Chiem & Harrison, 1997; Koutny et al., 1996). Research in this area of electrophoresis-based IA has since then been extensive, on which several
reviews are available (Chiem & Harrison, 1997; Schmalzing et al., 2000; Yeung et al., 2003). The assay simply relies on a difference in the electrophoretic mobilities of free antigen or antibody and the Ag-Ab complex to achieve separation of the products of homogeneous IA. Stepwise operations in a homogeneous IA, including mixing of samples and reagents, reaction, separation and detection, can be integrated on one microchip (Chiem & Harrison, 1998). High-throughput assays have also been developed (Bromberg & Mathies, 2004; Dishinger & Kennedy, 2007; Siew Bang et al., 2001). Detection in these devices is achieved primarily with laser-induced fluorescence (LIF), while electrochemical detection strategies have also been developed and shown to render a low limit of detection (LOD) (Wang et al., 2001). Fast reaction and separation (< 10 min in total), system integration and parallel analysis are the main advantages of electrophoresis-based IA. The assays have been applied to the analysis of biological fluids such as serum (Chiem & Harrison, 1997) and saliva (Herr et al., 2007). However, as conventional homogeneous IA, such assays are difficult to develop and to achieve high sensitivity.

Another interesting type of the homogeneous IA is the diffusion IA developed by Yager’s group in University of Washington (Hatch et al., 2001). This system takes advantage of the laminar flow condition in microchannels, under which the transport of molecules or particles across flow lines is limited only by diffusion. A competitive IA is conducted by placing two fluid streams, each containing sample antigen and labeled antigen, and antibody or bead-conjugated antibody, in contact with each other via a T-shaped microchannel network. Components in the two streams will interdiffuse. According to the fact that antigen, a smaller molecule, undergoes a reduction in diffusion
coefficient when binding to a larger molecule, i.e., antibody or bead-conjugated antibody, the fluorescent intensity profile measured across the two streams can be related with antigen concentration. The diffusion IA is extraordinarily rapid (<1 min), as the measurement is taken during the interdiffusion process, eliminating the time for thorough mixing of the reactants. It has been applied to assay biological fluid as complex as whole blood. However, the assay has only been demonstrated in a competitive IA format for a small molecule, phenytoin. Its applicability to detect large molecules, such as antibody, with specificity, remains to be proved.

1.2.3 Microfluidics-based heterogeneous IA

Though appeared later, the microfluidics-based heterogeneous IA has attracted more attention than its homogeneous counterpart. As mentioned above, heterogeneous IA suffers from long incubation times and laborious solution operations. These limitations can be overcome by applying microfluidic techniques which offer fast reaction rates, enhanced integration and automation. A great variety of microdevices of heterogeneous IA have been developed, which will be reviewed in detail here. To make it clear, some representative works have been summarized in Table 1.1.

The work on microfluidic heterogeneous IA will be reviewed below, by addressing specific aspects of the assay, including solid phase, surface modification and Ab/Ag immobilization, delivery of sample/reagent solutions, and detection. This review also allows a glance of the microfluidic technology in general from the window of heterogeneous IA chips.

1.2.3.1 Solid phase
Three major types of solid phase have been used in the studies of microfluidic heterogeneous IA: the walls of a microchannel, beads, and gold film. These solid phases will be reviewed respectively below. Related issues, including microfabrication and the handling of beads in microchannels, as well as experimental procedures using gold film, will also be briefly addressed.

1) Materials of microchannels

Three most common materials for constructing microchannels are silicon, glass and polymer materials. Many of the early microfluidic chips are fabricated in silicon or glass, as the techniques for photolithography, etching and bonding silicon and glass wafers are readily available from microelectronics. Precise patterning can be created on nanometer scale. Silicon and glass are also good support materials for electroosmotic flow, and they possess a negative surface charge and good heat dissipation capability. Another advantage of silicon as the material for microreactors is that the silicon dioxide surface has excellent chemical properties for immobilizing ligands and the relevant techniques are well developed. In addition, silicon can be processed to a porous state to bear a high surface area to enhance surface reaction (Yakovleva et al., 2002). Despite these advantages, silicon has been replaced by glass and polymer in most IA applications. A major reason is that silicon is opaque in the visible/UV spectrum, which makes it not suitable for optical detection (McDonald et al., 2000). Besides, the relatively high cost of silicon wafers and the lengthy etching process also limit the wide use of the material.

Glass is transparent and has outstanding optical properties. But the etching of vertical side wall is even more difficult for glass than for silicon, as glass is amorphous. Nevertheless, glass is extensively used as the flat substrate to form a “hybrid” microchip
with microchannels fabricated in polymer, e.g., poly(dimethylsiloxane), so that many of its merits are exploited, including surface charge, high thermal conductivity, good optical properties and low price. Such a hybrid microchip is also used in this thesis study.

There has been an increasing interest in using polymers to construct microchannels in recent years. Compared to silicon and glass, polymers possess the advantages of lower cost and much simpler manufacturing procedures of molding or embossing, rather than etching. As listed in Table 1.1, microfluidic IA devices have been fabricated using a wide range of polymers, including polyimide, poly (ethylene terephthalate) (PET), polycarbonate, polystyrene (only as flat substrate for hybrid chip), poly(methylmethacrylate) (PMMA), and poly(dimethylsiloxane) (PDMS). Among these polymers, PDMS is the most popular one.

The silicone elastomer of PDMS has been long utilized in the area of health care because of its physiological inertness and non-toxicity (Butler et al., 1997a). It has been extensively used to fabricate disposable lab-on-a-chip devices since the development of the so-called “soft lithography” technique by Whitesides and coworkers (Duffy et al., 1998; Qin et al., 1996; Xia & Whitesides, 1998). The soft lithography technique facilitates rapid prototyping and replica molding, which reduce the time from design to device to less than a day. The features of microdevices can be reproduced with high fidelity (< 0.1 μm). In addition, the fabrication can be done in a normal laboratory, without the need of a clean room. These factors make soft-lithographically-fabricated microdevices a perfect choice for researchers to test their ideas. The stage in fabrication process has been reviewed in detail (McDonald et al., 2000). Also, the PDMS material itself is optically transparent and therefore applicable to a number of detection schemes.
Another important feature of this soft elastomer is that it can be sealed to itself and a group of other surfaces (e.g., glass), either reversibly by forming molecular (van der Waals) conformal contact, or irreversibly after plasma treatment, thereby rendering microchip assembly easy and flexible. In forming an irreversible sealing, silanol groups (Si-OH) are introduced to the PDMS surface at the expense of methyl group (Si-CH$_3$) upon plasma oxidization. The silanol groups then condense with appropriate groups on another surface to form a covalent bonding (Si-O-Si).

In this thesis study, the microchannel was also softlithographically-fabricated in PDMS, and bonded with glass substrate to form a microchip.

2) Beads

As shown in Table 1.1, beads have been employed as the solid phase for immunoreaction in a number of studies. The use of beads, which are normally of diameters one or two orders lower than the size of the microchannels holding them, significantly increases the surface area and thereby enhances the kinetics of the surface binding reaction. Microbeads, fabricated by polymers or silica, are an attractive solid support not only for heterogeneous IA, but also for many other analyses such as DNA hybridization and chromatography. The relevant research has been covered in several recent reviews (Lim & Zhang, 2007; Peterson, 2005; Verpoorte, 2003).

To be used as the solid phase for microfluidic heterogeneous IA, the beads have to be confined in microchannels and not flushed out during flow operations. Several strategies have been adopted to immobilize beads. One approach is creating geometric barrier. Sato et al. fabricated a dam-like structure in their glass channels to hold the beads ($\phi_{\text{beads}} = 25$–$45$ µm) (Sato et al., 2001; Sato et al., 2002). Haes et al. also constructed a
“bead-bed” to be used as the reaction chamber ($\phi_{\text{beads}} = 5 \, \mu\text{m}$) (Haes et al., 2006). Though such physical barriers confine the beads reliably, those structures are relatively difficult to fabricate. This difficulty in fabrication limits the use of smaller beads, i.e., with diameters on micron or submicron scale, which should improve the reaction kinetics even further. In those occasions, immobilization of the microbeads via an externally applied magnetic field appears to be a convenient and flexible approach. The magnetic beads are usually fabricated in polymer, with an iron core. One additional advantage of using magnetic immobilization is that the beads can be flexibly captured and realized, thus allowing the microchannel to be repeatedly used conveniently (Choi et al., 2002). In addition to the two major approaches, a novel method for bead immobilization has been developed to hold beads ($\phi_{\text{beads}} < 6 \, \mu\text{m}$) in the “neck” of a microchannel with recirculating flows, generated by a combination of pressure-driven flow and electroosmotic flow (Lettieri et al., 2003). However, because of the complexity of the system, the experimental conditions have to be carefully tailored for a certain size of beads.

3) Gold film

Gold film is an unusual type of solid phase that has been employed in a couple of studies listed in Table 1.1. In a study on electrochemical IA (Ko et al., 2003), the reaction took place on gold electrodes (200 nm thick), which was deposited on top of a layer of Cr on an embossed PMMA substrate by using an E-beam evaporator. The gold surface was chemically modified to bind probing molecules covalently. After the immunoreaction, the analyte-bound gold electrodes were then subject to enzyme-catalyzed precipitation reaction and voltammetric measurements. Gold support has also been used in another study (Kurita et al., 2006), in which a layer of gold (50 nm) was deposited on top of a
layer of Ti on a glass substrate by using sputtering equipment. The gold surface was chemically modified and used as the solid support for the enzyme-linked immunoreaction in a T-shaped microchannel. Enzymatic reaction product, thiocholine, was then detected on another gold film located downstream by using a surface plasmon resonance system.

1.2.3.2 Surface modification and antibody/antigen immobilization

As summarized in Table 1.1, immobilization on non-polymer surfaces, including silicon, glass, and gold, is usually conducted by covalent bonding after derivatizing the surface. Immobilization on polymer surfaces, in the form of channels, flat substrates or beads, is usually conducted by either direct adsorption or covalent bonding after surface modification. In a number of studies, bioaffinity binding was used to increase the stability and sensitivity of the assay, which was done after either adsorption or covalent bonding. The avidin-biotin system played a primary role for this purpose, while protein A-IgG was also used in a couple of studies (Dodge et al., 2001; Eteshola & Leckband, 2001).

Protocols for derivatizing solid surfaces to allow protein binding have been well documented (Cabral & Kennedy, 1991). Various reagents have been used in the microfluidic IA studies, including carbodimines, succinimidyl esters, glutaraldehyde, and silanes. Except that silanes usually introduces long alkyl groups to a surface to bind proteins noncovalently (Dodge et al., 2001), all the other chemicals derivatize a surface with chemical groups that covalently bind probing molecules. One work on enzyme immunoassay studied different immobilization protocols based on covalent/noncovalent modification of silica surfaces using several different chemicals, followed by adsorption or covalent attachment of the antibody (Yakovleva et al., 2002). Results showed that best stability and sensitivity were achieved when antibodies were covalently attached via a
glutaraldehyde to functionalized silica surfaces.

For non-PDMS polymers, including polystyrene, polyimide, polycarbonate, PET, PMMA, adsorption is used predominantly for protein immobilization, to exploit the hydrophobicity of the plastic surfaces.

PDMS is more hydrophobic than polystyrene, the common material of microtitre plates for conducting conventional ELISA, and therefore has strong avidity for proteins. Though exposure to plasma introduces silanol groups onto the surface, the hydrophilized PDMS is still adsorptive to some proteins. The hydrophobicity of PDMS, on one hand, can be used to adsorb capture molecules. Both native PDMS (Wolf et al., 2004) and its plasma-hydrophilized form (Linder et al., 2002; Linder et al., 2001) have been used for this purpose and will be used in this thesis study as well. On the other hand, the strongly hydrophobic nature of PDMS can induce nonspecific binding of proteins, resulting in the reduction of the sensitivity of assay. Considering the high surface-to-volume ratio of microchannels, the problem of nonspecific binding is more challenging. Therefore, much work has been done on the surface modification of PDMS, as the issue of non-specific protein adsorption is related not only to heterogeneous IAs, but also to many other applications, e.g., electrophoresis-based analysis, where separation can be seriously affected by attachment of proteins on channel walls. Reviews on these works are available (Belder & Ludwig, 2003; Makamba et al., 2003).

Several studies reported that significantly reduced non-specific binding while imparting biospecificity to the PDMS surface of microchannels. In one study (Etshola & Leckband, 2001), the PDMS channel was first coated with 0.5% bovine serum albumin (BSA) solution, then activated with glutaraldehyde to bind protein A covalently. Capture
antibody was then attached to the channel surface via the anchor of protein A. Signal-to-noise ratio increased by 2-3 fold with this method, in comparison with directly adsorbing capture antibody to the channel wall. Linder et al. employed a three-layer coating to passivate the PDMS surface (Linder et al., 2001): the first layer being biotinylated anti-mouse IgG adsorbed to the PDMS channel wall, the second layer being neutravidin, and the third layer being biotinylated dextran in non-reaction region, or biotinylated capture antibody in the reaction region. A high signal-to-noise ratio was obtained with that system.

PDMS surface has also been modified by using supported bilayer membranes (SBM). Vesicle fusion method was used to assemble phospholipid membranes on plasma-oxidized PDMS. The capture molecules for IA reaction were introduced into the bilayer membrane by adding, before the membrane formation, a small concentration of the probing molecule into the membrane-forming phospholipids, either alone (Phillips & Cheng, 2005) or with lipid conjugation (Yang et al., 2001). It has been demonstrated that SBM can reduce the nonspecific adsorption of avidin and BSA on PDMS by 2-3 orders of magnitude, as compared to that on plasma-oxidized surfaces. In addition, the SBM-coated surfaces can maintain the hydrophilicity for months, much longer than plasma-treated or BSA-coated PDMS surfaces (Phillips & Cheng, 2005).

The methods for protein immobilization reviewed above, including direct adsorption and covalent bonding after surface modification, are all passive approaches. Techniques for active protein placement have also been developed, which deliver proteins locally to a solid surface. Examples include ink-jet printing or microarraying with fine metal tips or by electrospray (Morozov & Morozova, 1999), utilized in the
microarray technology. Microcontact printing (μCP) is another noteworthy active patterning technique, which was developed by Whitesides and coworkers (Kumar & Whitesides, 1993; Xia & Whitesides, 1998). The technique employs a PDMS “stamp” bearing geometry patterns to transfer “ink” made up of alkanethiols, as a self-assembled monolayer, to a gold surface. Delamarche and coworkers have successfully applied this technique to the high-throughput patterning of different proteins onto a number of different solid surfaces, including silicon, silicon oxide, glass, polystyrene and gold (Bernard et al., 2000). The μCP technique can transfer proteins from stamps to a substrate within 1 s, with no loss of biological activity. Therefore, it is a promising method for improving the efficiency and throughput of microfluidic IA, although no study has yet reported a microfluidic IA based on this technique so far.

1.2.3.3 Delivery of sample/reagent solutions

The platform for solution transport is an essential component of a microfluidic analytical device. In the heterogeneous IA studies reviewed in Table 1.1, several typical flow systems for microfluidics have been exploited, including pressure-driven flow, capillary flow, centrifugal force-driven flow and electrokinetically-driven flow. Excellent reviews are available on the application of different fluidic platforms in lab-on-a-chip devices (Bruin, 2000; Eijkel & Van Den Berg, 2006; Haeberle & Zengerle, 2007; Stone et al., 2004). A summary on the flow driving and control with these fluidic platforms is briefly given in Table 1.2 and will be addressed below. The electrokinetic-driven flow system, the platform employed for the work of this thesis, will be described in detail.

1) Pressure-driven flow
Pressure-driven flow is by far the most extensively used platform in the studies of microfluidic IA, owing to its merits that the fluid pumping is independent of both the composition of the liquid and the properties of the channel surface, and that the flow rate can be precisely controlled. Pressure-driven flow in a microchannel is similar to the classical Poiseuille flow (incompressible flow in a conduit with constant cross section) except that the flow is retarded to some extent (usually <10%) by the electroviscous effect caused by the streaming potentials generated from an electric double layer (EDL) adjacent to the channel wall (Li, 2001). The pressure difference across a microchannel is mostly exerted by using a syringe pump.

Most pressure-driven microfluidic IAs are based on a straight microchannel with manual operations. The development of integrated and automated IA on a microchannel network necessitates the use of valves for flow control (Choi et al., 2002). Especially noteworthy is the pneumatic PDMS valve developed by Quake’s group, which is based on a multi-layer soft lithography technology (Unger et al., 2000). The basic valving unit is composed of two layers of PDMS, a lower layer containing the fluidic ducts and an upper layer featuring pneumatic control channels, with the control channels across the fluidic channels. The valving function is actuated when a pressure is applied to the control channel, which squeezes the top elastomer into the lower layer and blocks the liquid flow. The small size of the valve, on the order of 100 x 100 \( \mu \text{m}^2 \), enables large scale integration (LSL) of the valves. Based on this technology, a high-throughput multi-antigen IA containing 100 reaction chambers has been developed (Kartalov et al., 2006).

2) Capillary flow
Capillary flow is naturally induced by the surface tensions of liquids (Eijkel & Van Den Berg, 2006). This flow platform is used in a couple of IA studies as listed in Table 1.1, e.g., the so-called “micromosaic immunoassay” developed by Delamarche’s group (Bernard et al., 2001; Cesaro-Tadic et al., 2004b; Wolf et al., 2004). The advantages of capillary flow are that the flow pumping does not involve any energy supply, which makes it a suitable platform for portable, cheap and disposable on-site assay. However, capillary flow is a passive transport method, as the flow direction and velocity are passively controlled by changes in channel geometry and surface wetting properties. Therefore, the time of an assay depends on variations in viscosity and surface tension of the samples, which affects the precision of the IA.

3) Centrifugal force-driven flow

Centrifugal force has also been utilized to move fluids in microfluidic systems, in which microchannels are designed radially on a compact disk (CD)-like platform (Madou et al., 2006). The fluid is driven by centrifugal force under the rotation of the CD. The velocity can be controlled with the rotating speed and the assay can be performed in an automated way. The flow valving function is also passively controlled by surface tension, through changes in channel geometry or surface hydrophobicity, as in the capillary flow. Owing to the rotational symmetry of the disks, a high degree of parallelization can be achieved. A microfluidic ELISA based on this platform includes 24 independent radial assay units on one CD (Lai et al., 2004).

4) Electrokinetically-driven flow

Generally, most solid surfaces obtain surface electrical charges when they are brought into contact with an aqueous solution. These surface charges, in turn, influence
the ion distribution near the solid-liquid interface in the liquid, forming a region near the solid surface where the concentration of counterions is higher than that of coions. The rearrangement of charges on the solid surface and balancing charges in the liquid is called the electrical double layer (EDL) (Hunter, 1981). Electrokinetic transport phenomena arise when the liquid or the solid phase moves relative to the other (Masliyah, 1994). Two kinds of electrokinetic transport phenomena are involved in microfluidic IAs: electroosmosis and electrophoresis.

Electroosmosis refers to the movement of the liquid relative to the solid due to an applied electric field, as illustrated in Fig. 1.3 (a). It has been extensively used for driving fluids in microfluidic devices, known as electroosmotic pumping (Li, 2004). Under ideal conditions, the velocity profile of electroosmotic flow in a microchannel is plug-like, and the velocity is given by: 

$$\bar{u}_{eo} = \frac{\varepsilon \varepsilon_0 \zeta_w}{\eta} \bar{E},$$

where $\varepsilon$ is the relative dielectric constant of the liquid, $\varepsilon_0$ the permittivity of vacuum, $\zeta_w$ the zeta potential of the channel wall, $\eta$ the dynamic viscosity of the liquid, and $E$ the applied electric field strength. The coefficient, $\frac{\varepsilon \varepsilon_0 \zeta_w}{\eta}$, is termed electroosmotic mobility, $\mu_{eo}$.

When solid particles are contained in the liquid, electrophoresis may also be present, which refers to the migration of charged particles relative to the liquid under an applied electric field, as shown in Fig. 1.3 (b). Like the expression for electroosmosis, the electrophoretic velocity is 

$$\bar{u}_{ep} = \frac{\varepsilon \varepsilon_0 \zeta_p}{\eta} \bar{E},$$

where $\zeta_p$ is the zeta potential of the particle surface. The coefficient, $\frac{\varepsilon \varepsilon_0 \zeta_p}{\eta}$, is termed electrophoretic mobility, $\mu_{ep}$. In microfluidic
IAs, transportation of protein-contained solutions under an applied electric field would involve both electroosmosis and electrophoresis, if the protein particles bear surface charges.

Compared with other flow systems, a critical advantage of electroosmotic flow (EOF) is the fluidic pumping and control without moving parts. Because both the flow rate and the flow route is determined by the local electric field, as shown in Table 1.2, a proper electric field can be applied to achieve both flow pumping and flow valving in a microchannel network.

With such an advantage, a high level of automation and system integration can be achieved with a neat and compact electrokinetically (EK)-controlled microfluidic device. Because flow switching in EOF is as easy as changing the applied electric field, sequential steps in a multi-step analysis like immunoassay can be readily integrated and the whole process automated. In the same way, sample and reagent handling could also be integrated to realize a complete lab-on-a-chip. Without the need of external pumps, tubings and valves, microdevices based on EOF can be highly compact and integrated.

The advantages have been demonstrated in the various integrated EK-controlled microfluidic devices (Bruin, 2000), such as microfluidic chips for DNA separation (Chang et al., 2000), enzyme assay (Cohen et al., 1999; Hadd et al., 1997) as well as homogeneous IA as described earlier. A few studies on heterogeneous EK-driven IA have also been reported (Dodge et al., 2001; Haes et al., 2006; Linder et al., 2002), as listed in Table 1.1. But these studies are all based on relatively simple IA configurations involving only one Ab-Ag binding reaction, and are of low throughput. The advantages of EOF for conducting microfluidic IA have not been sufficiently exploited. Therefore, it is the
purpose of this thesis to develop an integrated and high-throughput EK-driven heterogeneous IA.

Still, there are some special considerations with the EK-driven flow. Table 1.3 listed some reported problems associated with the EK-driven platform and the corresponding solutions. It should be noted that the immunoassay appears to be an analysis especially suitable for the EK platform, because some issues do not become problems in the IA. For example, low-salt, low-ionic-strength buffers can be used in IA, thus the effect of Joule heating and electrolysis becomes insignificant. Also, the undesirable electrophoretic separation of compounds due to heterogeneity in surface charges would not be a big problem because the reaction is not of competitive nature (except for competitive IA) (Linder et al., 2002). The potential problems and their solutions have been well considered in this thesis study and will be addressed in detail in later chapters.

1.2.3.4 Detection

Though absorbance methods are predominantly used for detection in conventional IA, especially ELISA, these methods are seldom applied to microfluidic IAs (Sato et al., 2004), mainly because the sensitivity of the assay is limited by the small cross-sectional path length in microchannels.

From Table 1.1, it is clear that fluorescence is the most widely used form of detection, primarily due to its high sensitivity and the ease of integrating a label. The fluorescent detection is usually done by using a fluorescent microscope connected to a charge coupled device (CCD) camera, which is also the detection method used in this
thesis work. A more integrated approach of incorporating a waveguide into the microchip has also been used (Hofmann et al., 2002; Rowe et al., 1999).

Electrochemical detection is the second most commonly used approach of detection, which is also known to provide high sensitivity. In most studies, the measurement is amperometric, i.e., the reaction-induced electric current is monitored while the potential of an electrode is held at a specific value. Advantages of electrochemical detection include that the detection devices are easy to be miniaturized and that the miniaturization provides additional sensitivity to detection.

Other major detection methods employed in the microfluidic IA include chemiluminescence (Yakovleva et al., 2002), surface plasmon resonance (SPR) (Kurita et al., 2006), and thermal lens microscopy (Sato et al., 2001). From the low LOD obtained, these methods have all proved highly sensitive. The SPR-based detection has an additional advantage that the prior labeling of the analytes is not required and therefore it can rapidly monitor dynamic interactions in real-time.

A number of studies have used enzyme IA to improve sensitivity. Enzymes, such as alkaline phosphatase and horseradish peroxidase, catalyze the conversion of substrate into a detectable product. The catalytic turnover by the enzyme amplifies the signal and thereby increases the sensitivity of the assay. This is because the number of detectable molecules can be exponentially higher than the number of analytes. Enzyme IA has been used in combination with many detection methods, including fluorescence (Bernard et al., 2000; Eteshola & Leckband, 2001), electrochemical detection (Ko et al., 2003; Rossier et al., 2000), absorbance (Sato et al., 2004), SPR (Kurita et al., 2006), and chemiluminescence (Yakovleva et al., 2002).
1.2.4 **Objective of this thesis**

It should also be noted that a number of microfluidic IAs are high-throughput ones, which detect multiple analytes from a sample, or test multiple samples simultaneously, or carry both features, as shown in Table 1.1. Those works will be further reviewed in Chapters 4 and 5. These desirable high-throughput features, however, have not been explored in an EOF-driven heterogeneous IA, at the time the thesis work started.

Therefore, the objective of this thesis study is to develop an integrated and high-throughput electrokinetically-controlled heterogeneous IA, for clinical application.

1.3 **Model analytes**

The development of an IA prototype has to employ specific Ab-Ag as a model reaction system. As shown in Table 1.1, the model analytes used in the microfluidic IA cover a variety of different species, many of which are of practical significance, in different areas including clinical diagnostics (Sia et al., 2004; Wolf et al., 2004), environmental science (Rodriguez-Mozaz et al., 2004), food science (Hoegger et al., 2007) and biosecurity (Phillips & Cheng, 2005). This study will mostly focus on two important gastrointestinal microbial pathogens, *Helicobacter pylori* and *Escherichia coli* O157:H7.

1.3.1 **Helicobacter pylori**

The bacterium of *H. pylori* is the causative organism for most of the gastritis and peptic ulcer disease (Sherman et al., 1999). Recently, *H. pylori* has also been identified as a cause for gastric cancer and classified by the World Health Organization as a class 1 carcinogen (Uemura et al., 2001). The burden of illness from *H. pylori* infection is
considerable as it infects at least half of the world’s human population (Sherman et al., 1999).

Noninvasive diagnostic strategies for detecting *H. pylori* include urea breath tests, stool antigen detection (Vaira & Vakil, 2001), and ELISA for detecting *H. pylori*-specific immunoglobulin (Ig) G in serum, whole blood, urine or saliva. The ELISA tests remain popular for use as the breath tests can be challenging for the extremes of age groups and are relatively more expensive (Imrie et al., 2001).

### 1.3.2 *Escherichia coli* O157:H7

*E. coli* O157:H7 belongs to a subgroup of pathogenic *E. coli* causing diarrhea in humans. This pathogen is recognized as a major cause of hemorrhagic colitis (Karmali et al., 1983). The infectious dose for *E. coli* O157:H7 is low, probably less than 300 and even fewer than 100 organisms (Tuttle et al., 1999). There have been numerous outbreaks in the United States, Scotland, and, in Walkerton, Ontario, Canada in the year 2000, when seven people died and over 2,300 were infected from a contaminated city water supply (Ali, 2004). The Center for Disease Control and Prevention (CDCP) estimates that in the United States, *E. coli* O157:H7 causes 73,480 illnesses and 61 deaths each year (Mead et al., 1999). The majority of these cases are attributed to food-borne and water-borne transmission of infection (Verweyen et al., 2000).

ELISA-based methods have played an important role in the detection of *E. coli* O157:H7. ELISA has been applied to assay contaminated water, milk and beef for the bacterium (Daly et al., 2002; Ge et al., 2002). Also, in the diagnosis of the disease, stool antigen test has become an important approach (Beutin et al., 2002; Mackenzie et al., 1998). Serodiagnosis can also prove useful in cases where stool antigen isolation is
negative (Chart & Jenkins, 1999). These two tests are both conducted by using ELISA.

Due to the important role of IA in the testing of the infection status of the two pathogens, it is highly desirable to further improve the performance of the assay by developing miniaturized IA devices, so that the assay can be more rapid, which is especially important for the diagnosis of *E. coli* O157:H7, and cheaper to use. Some microfluidic IAs also have used *H. pylori* (Messina et al., 2005) and *E. coli* O157:H7 (Li & Su, 2006; Varshney et al., 2007) as model analytes.

### 1.4 Overview of this thesis

The content of this thesis includes:

Chapter 1: The motivation and objective of this thesis work are presented and literatures on microfluidic-based immunoassays are reviewed.

Chapter 2: The development of an electrokinetically-driven heterogeneous immunoassay on a straight microchannel will be described.

Chapter 3: The development of an automated electrokinetically-controlled immunoassay on a microchannel network will be demonstrated.

Chapter 4: The improvement of the electrokinetic immunoassay to achieve multi-analyte detection will be described.

Chapter 5: Further improvement of the electrokinetic immunoassay to realize multi-sample detection will be presented.

Chapter 6: The application of the high-throughput electrokinetic immunoassay to testing human serum samples for specific bacterial antibodies will be demonstrated.
Chapter 7: Preliminary work on the application of the electrokinetic immunoassay in testing embryo culture media for specific biomarkers will be described.

Chapter 8: The study on the reaction kinetics of the electrokinetic immunoassay aiming at optimizing assay performance will be presented.

Chapter 9: The main contributions and conclusions of the thesis will be summarized and the perspectives for extending the current work will be proposed.
Figure 1.1 Illustration of the structure of an antibody molecule and the specific binding reaction between an antibody and an antigen.
Figure 1.2 Schematics of different formats of immunoassay. (a) Direct immunoassay for antibody. (b) Direct immunoassay for antigen. (c) Indirect immunoassay for antibody. (d) Indirect immunoassay for antigen.
Figure 1.3 Illustration of electrokinetic transport phenomena. (a) Electroosmosis. (b) Electrophoresis.
Table 1.1 Representative studies on microfluidic heterogeneous immunoassay

<table>
<thead>
<tr>
<th>Flow system</th>
<th>Substrate (channel/substrate)</th>
<th>Immobilization</th>
<th>Detection</th>
<th>Analyte</th>
<th>Time, min</th>
<th>Sample amount</th>
<th>LOD</th>
<th>Throughput</th>
<th>Note</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK</td>
<td>Silicon/PDMS</td>
<td>Covalent bonding (silane)</td>
<td>Fluorescence</td>
<td>rlgG</td>
<td>5</td>
<td>-</td>
<td>50 nM</td>
<td>1</td>
<td>1</td>
<td>(Dodge et al., 2001)</td>
</tr>
<tr>
<td>EK</td>
<td>PDMS/glass</td>
<td>Adsorption/bioaffinity binding(advin)</td>
<td>Fluorescence</td>
<td>IgG</td>
<td>~8</td>
<td>-</td>
<td>5 µg/mL</td>
<td>1</td>
<td>1</td>
<td>Human serum (Linder et al., 2002)</td>
</tr>
<tr>
<td>EK</td>
<td>Glass + silica beads (Φ= 5 µm)</td>
<td>Covalent (carboxymide Hydrochloride)</td>
<td>Fluorescence</td>
<td>Staphylococcal enterotoxin B (SEB)</td>
<td>20</td>
<td>-</td>
<td>1 fM (28.5 fg/mL)</td>
<td>3</td>
<td>1</td>
<td>Field-enrichment (Haes et al., 2006)</td>
</tr>
<tr>
<td>Pressure</td>
<td>Glass + polystyrene beads (Φ= 45 µm)</td>
<td>Adsorption</td>
<td>Colloid gold, Thermal lens microscope</td>
<td>Interferon-γ</td>
<td>50</td>
<td>-</td>
<td>0.01 ng/mL</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td>Glass + polystyrene beads (Φ= 25 µm)</td>
<td>Adsorption</td>
<td>Absorbance, Thermal lens microscope</td>
<td>Interferon-γ</td>
<td>35</td>
<td>0.1 ng/mL</td>
<td>1</td>
<td>1</td>
<td>Enzyme IA (EIA) (Sato et al., 2004)</td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td>PDMS</td>
<td>Covalent (epoxide)</td>
<td>Fluorescence</td>
<td>CRP, ferritin, PSA, VEGF</td>
<td>-</td>
<td>100 nL</td>
<td>10 pM</td>
<td>10</td>
<td>5</td>
<td>(Kartalov et al., 2006)</td>
</tr>
<tr>
<td>Pressure</td>
<td>PDMS/glass(waveguide)</td>
<td>Non-covalent (succinimide ester, silane) + bioaffinity (avidin-biotin)</td>
<td>Fluorescence</td>
<td>SEB, Yersinia pestis F1 antigen, D-dimer</td>
<td>&gt;30</td>
<td>-</td>
<td>1 ng/mL, 25 ng/mL, 50 ng/mL</td>
<td>6</td>
<td>6</td>
<td>Nasal swabs, saliva, urine, serum, plasma, blood (Rowe et al., 1999)</td>
</tr>
<tr>
<td>Pressure</td>
<td>PDMS/glass</td>
<td>Covalent(silane)+ bioaffinity binding</td>
<td>Fluorescence</td>
<td>Deoxynivalenol</td>
<td>~10</td>
<td>0.8 mL</td>
<td>0.2 ng/mL in buffer, 1-50 ng/g in cereals, 4 ng/mL for air sample</td>
<td>10</td>
<td>10</td>
<td>Food (cereals) and indoor air samples tested (Ngundi et al., 2006)</td>
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<tr>
<td>Pressure</td>
<td>PDMS/glass</td>
<td>Covalent(silane)+ bioaffinity binding</td>
<td>Fluorescence</td>
<td>Escherichia coli ATCC 35218, Bacillus subtilis ATCC 49760</td>
<td>15</td>
<td>0.8 mL</td>
<td>E. coli: 10^7 (FIA), 10^2 (IA+PCR) B. subtilis: 10^7 (FIA), 10^2 (IA+PCR) cfu/mL;</td>
<td>6</td>
<td>6</td>
<td>IA + cell culture + PCR (Johnson-White et al., 2007)</td>
</tr>
<tr>
<td>Pressure</td>
<td>Material</td>
<td>Modification</td>
<td>Detection Method</td>
<td>Analyte</td>
<td>Sensitivity</td>
<td>Sample Type</td>
<td>Notes</td>
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<tr>
<td>Pressure</td>
<td>Glass</td>
<td>Covalent binding (silanization)</td>
<td>Fluorescence</td>
<td>Atrazine, Isoproturon, Estrone</td>
<td>15</td>
<td>0.155, 0.046, 0.084 ng/mL</td>
<td>Natural water sample</td>
<td>(Rodriguez-Mozaz et al., 2004)</td>
<td></td>
<td></td>
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<tr>
<td>Pressure</td>
<td>Fused silica (capillary), Glass (channel) + magnetic beads ($\Phi= 1-2 \mu m$)</td>
<td>Covalent bonding (Glutaraldehyde)</td>
<td>Fluorescence</td>
<td>Parathyroid hormone, Interleukin 5</td>
<td>74</td>
<td>$\mu g/mL$</td>
<td>Human plasma tested</td>
<td>(Hayes et al., 2001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td>Porous silicon</td>
<td>Covalent bonding (Glutaraldehyde), adsorption</td>
<td>Chemiluminescence</td>
<td>Atrazine</td>
<td>10</td>
<td>0.8-45 pg/mL (3.7-209 pM)</td>
<td>EIA, different immobilization methods, buffers compared</td>
<td>(Yakovleva et al., 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure</td>
<td>PDMS/glass + phospholipid layer on PDMS</td>
<td>Vesicle fusion method</td>
<td>Fluorescence</td>
<td>Cholera toxin (CT)</td>
<td>&gt;30</td>
<td>37 µL</td>
<td>210 pM</td>
<td>SEM</td>
<td>(Phillips &amp; Cheng, 2005)</td>
<td></td>
</tr>
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<td>Pressure</td>
<td>PDMS/glass with gold film</td>
<td>Covalent (carbodiimide coupling reaction)</td>
<td>Surface plasmon resonance (SPR)</td>
<td>B-type natriuretic peptide (BNP)</td>
<td>-</td>
<td>-</td>
<td>5 pg/mL</td>
<td>EIA; Ultrasensitive; human serum tested</td>
<td>(Kurita et al., 2006)</td>
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<tr>
<td>Pressure</td>
<td>PDMS + polycarbonate membrane</td>
<td>Adsorption</td>
<td>Fluorescence</td>
<td>HIV</td>
<td>&gt;90</td>
<td>180 nL – 14 µL</td>
<td>Dynamic range: 10³</td>
<td>Serially-diluted assay; human serum</td>
<td>(Jiang et al., 2003)</td>
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<tr>
<td>Pressure</td>
<td>PDMS/polystyrene</td>
<td>Adsorption</td>
<td>Light transmission of silver film (self-built detector)</td>
<td>HIV</td>
<td>&gt;30</td>
<td>-</td>
<td>Sensitivity compared with other detection methods</td>
<td>Low-cost and portable; human serum</td>
<td>(Sia et al., 2004)</td>
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<tr>
<td>Pressure</td>
<td>PDMS/polystyrene</td>
<td>Adsorption</td>
<td>Fluorescence</td>
<td>HIV</td>
<td>2</td>
<td>9 µL</td>
<td>~1 nM</td>
<td>Reagent pre-loaded in cartridges; human serum</td>
<td>(Linder et al., 2005)</td>
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<td>Pressure</td>
<td>PDMS/glass + phospholipid on PDMS</td>
<td>Vesicle fusion method</td>
<td>Fluorescence</td>
<td>Dinitrophenyl</td>
<td>-</td>
<td>~2µL</td>
<td>-</td>
<td>Supported bilayer membrane (SEM)</td>
<td>(Yang et al., 2001)</td>
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<tr>
<td>Pressure</td>
<td>Adsorption</td>
<td>Fluorescence</td>
<td>EIA; Infant formula samples</td>
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<tr>
<td>Polyimide Adsorption Electrochemical, gold electrode</td>
<td>Folic acid</td>
<td>Dynamic range: 1.37-87.5 ng/mL</td>
<td>8 1</td>
<td></td>
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<tr>
<td>Polyethylene terephthalate (PET), photoablated Adsorption Electrochemical, carbon electrode</td>
<td>D-Dimer</td>
<td>0.1 nM</td>
<td>1 1</td>
<td></td>
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<tr>
<td>EK+ pressure Glass + polymer (polystyrene, latex) beads (Φ &lt;6 μm) Adsorption Fluorescence Biotin</td>
<td>     </td>
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<tr>
<td>Glass + Cellulose membrane Adsorption Fluorescence</td>
<td>     </td>
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<tr>
<td>Glass+magnetic beads Adsorption Electrochemical, Interdigitated array (IDA) microelectrodes</td>
<td>Mouse IgG</td>
<td>&lt;20 10 μL &lt;100 ng/mL</td>
<td>1 1</td>
<td></td>
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<tr>
<td>PDMS or PDMS/glass Covalent (glutaraldehyde)+bi oaffinity (protein A) Adsorption Fluorescence Sheep IgM</td>
<td>     </td>
<td>     </td>
<td>     </td>
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<tr>
<td>PDMS/PDMA+ gold electrode Covalent (succinimidy ester)</td>
<td>Electrochemical, gold electrode</td>
<td>Ferritin, Biotin</td>
<td>90 100 μL</td>
<td>1 1</td>
<td></td>
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<tr>
<td>Silicone/PDMS Adsorption Fluorescence C-reactive protein(CRP)</td>
<td>     </td>
<td>     </td>
<td>     </td>
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<tr>
<td>Silicone/PDMS Adsorption Fluorescence Tumor necrosis factor α</td>
<td>     </td>
<td>     </td>
<td>     </td>
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<tr>
<td>Silicone/PDMS Adsorption Fluorescence Cell surface receptors</td>
<td>     </td>
<td>     </td>
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<tr>
<td>Poly(methyl methacrylate) Adsorption Fluorescence Rat IgG</td>
<td>     </td>
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### Table 1.2 Comparison of different fluidic platforms

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<thead>
<tr>
<th></th>
<th>Pressure-driven flow</th>
<th>Capillary flow</th>
<th>Centrifugal force-driven flow</th>
<th>EK-driven flow</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow pumping</strong></td>
<td>Pressure difference</td>
<td>Surface tension</td>
<td>Centrifugal force (Rotating of motor plate)</td>
<td>Electric field (high-voltage power supply)</td>
</tr>
<tr>
<td>(exerted by)</td>
<td>(syringe pump)</td>
<td>(spontaneously)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flow rate</strong></td>
<td>Pressure difference</td>
<td>Temperature, conduit geometry, surface properties</td>
<td>Rotating speed</td>
<td>Electric field</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flow route</strong></td>
<td>Valve</td>
<td>Conduit geometry, surface properties</td>
<td>Conduit geometry, surface properties</td>
<td>Electric field</td>
</tr>
<tr>
<td><strong>control</strong></td>
<td></td>
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Table 1.3 Potential problems associated with electrokinetically-driven flow

<table>
<thead>
<tr>
<th>Problems</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joule heating</td>
<td>Use low-salt, low-ionic-strength buffers;</td>
</tr>
<tr>
<td></td>
<td>Lower electric field strength</td>
</tr>
<tr>
<td>Electrolysis at electrodes</td>
<td>Use low-salt, low-ionic strength buffers;</td>
</tr>
<tr>
<td></td>
<td>Lower electric field strength</td>
</tr>
<tr>
<td>Interferences of undesirable pressure-driven flow</td>
<td>Increase flow resistance; Increase the size of wells</td>
</tr>
<tr>
<td>Sensitivity to properties of channel surface (bubble formation)</td>
<td>Optimize condition for protein coating;</td>
</tr>
<tr>
<td></td>
<td>Keep microchannel under wet condition</td>
</tr>
<tr>
<td>Undesirable electrophoretic separation of sample components</td>
<td>Use high-ionic-strength plugs for sample transport</td>
</tr>
</tbody>
</table>

*(Xuan et al., 2004)*  
**(Erickson et al., 2004)**  
†*(Sinton & Li, 2003)*  
††*(Dodge et al., 2001)*  
i*(Bousse et al., 2000)*
Chapter 2 Development of an electrokinetically-driven immunoassay (EK-IA) on a straight-channel *

2.1 Introduction

The task of developing an electrokinetically-controlled immunoassay lab-on-a-chip (EK-IA) can be divided into two parts: the assay part and the control part. The experimental protocol of the IA has to be established first, and then effective flow control can be built in to automate the analysis process. This chapter describes the work on the first part of the task, building the prototype of the EK-IA, using a straight microchannel. Next chapter will then focus on the realization of flow control.

The necessity of investigating experimental protocols lies, first, in the reactor: microchannels are different from conventional IA reactors in diffusion distance, surface-to-volume ratio as well as the solution operation method (flow-through rather than pipetting). Because of these differences in scale and operation, the assay conditions, such as incubation time and reagent concentrations, have to be adjusted accordingly. The dimension of the microchannel itself is also an important factor to consider. Moreover, the electrokinetically-driven flow system raised additional considerations, including the influence of an electric field on buffer solution, and electrokinetic mobilities of the solution and the reactants.

Most of the considerations listed above were investigated experimentally to determine the conditions for conducting the EK-IA. The approaches for such experimental explorations, as well as procedures for conducting the IA, are presented in

the Experimental section. The IA configuration was the same as described in 1.1.3.3, in the form of solid phase – Ag – Ab – dAb. *H. pylori* antigen and antibody were used as model analytes. The solid phase here was a soft-lithographically fabricated microchannel made of PDMS/glass. Anti-*H. pylori* antibody and detection antibody (dAb) (will be referred to as primary antibody and secondary antibody below) were electrokinetically-pumped into the microchannel in sequential stages in the IA.

2.2 Experimental

2.2.1 Antigen preparation and reagents

The procedures for preparing bacterial antigens were reported previously (Lin et al., 2004a). Briefly, *H. pylori* strain ATCC 49503 was cultured on 5% sheep blood agar plates and incubated at 37°C under microaerophilic conditions for 72 hr. Bacteria were then inoculated into Brucella broth with 10% fetal bovine serum and grown overnight with gentle shaking. *Lactobacillus rhamnosus*, strain R011, used as a negative control, was cultured on 5% sheep agar plates at 37°C overnight, and then inoculated into De Mann Ragosa Sharpe broth overnight. The liquid cultures were then centrifuged and the supernatants removed. Bacterial pellets were re-suspended in sterile phosphate-buffered-saline (PBS) and washed for 4 times. The pellets were then re-suspended in a RIPA buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100) containing a mixture of proteinase inhibitors cocktail (Roche Molecular Biochemicals, Mannheim, Germany) for 40 min and lysates were centrifuged and the supernatants then collected as whole bacterial proteins. After assayed for protein concentration, these lysate antigens
were diluted to the final concentrations using a coating buffer (consisting of 0.03M NaHCO$_3$ and 0.02M Na$_2$CO$_3$, pH 9.6).

Polyclonal rabbit anti-\textit{H. pylori} antibody was obtained from DAKO (Glostrup, Denmark). Rhodamine(TRITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) was used as detection antibody. 25 mM Tris-HCl buffer solution was used as the assay buffer. Bovine serum albumin (BSA) was used as blocking reagent.

2.2.2 Microchannel fabrication

The PDMS microchannels were fabricated by using soft lithography and replica molding. A glass microscope slide (75 mm $\times$ 25 mm) cleaned with acetone or NaOH was exposed to plasma oxidization for 5 min. Then, $\sim$2 ml of SU-8 25 negative photoresist (MicroChem Corp., Newton, MA) was poured onto the glass slide and degassed at a deep vacuum. The photoresist was then spin-coated to obtain a uniform film of a certain thickness. Three different film depths, 8, 30 and 60 $\mu$m, were fabricated. Spin-coating recipes were obtained by trial-and-error and the details are given in Table 2.1. The rest of the fabrication process is described below using the conditions for fabricating a 30-$\mu$m film of photoresist as an example. After the film was baked at 65 $^\circ$C for 3 min and at 95 $^\circ$C for 7 min, a photomask, bearing a 2 cm-long and 100$\sim$200-$\mu$m wide rectangular channel geometry, was placed on top of it. A big glass slide (75 mm$\times$50 mm) was put on top of the photomask, with extra weights added on its edges to press the photomask tightly against the photoresist film. Then UV light was exposed to for 7 s. A post-exposure bake was then conducted at 65 $^\circ$C for 1 min and at 95$^\circ$C for 3 min. Masters were developed in 4-hydroxy-4-methyl-2-pentanone for approximately 3 min or until the
non-exposed photoresist was completely rinsed off. The master was put under a heat lamp for one hour to strengthen the bonding. After the master was obtained, a 15:1 (w/w) mixture of PDMS polymer base and curing agent (Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI) was poured over the master, which was placed in a Petri dish. After degassing for 1 hr at –34 kPa (gauge), the mixture was cured at 75°C for 3 hr.

The PDMS replica bearing the pattern of a straight channel was then cut and peeled from the glass master. The thickness of the PDMS slab is about 2 mm. A 4.5 mm-diameter hole was punched at each end of the channel to form a well for solutions.

2.2.3 Microchip assembly and antigen immobilization

The PDMS slab bearing the microchannel pattern, together with a clean glass slide, was plasma-oxidized, for 10 s unless otherwise indicated. Immediately thereafter, the PDMS slab and the glass slide were brought into contact and the two bonded with each other irreversibly with covalent linking.

Five minutes after the channel had been formed, immobilization of antigen was conducted by using passive adsorption. Five microlitres of diluted solution of bacterial lysate antigen was added to one well of the channel and it filled the channel spontaneously due to capillary pressure (Kim et al., 1995). Incubation was conducted under room temperature for 2 hr for antigen to adsorb to the walls of the microchannel. After that, the microchannel was washed with Tris-HCl buffer by aspirating through one reservoir using a syringe. Blocking solution was then introduced and incubated under room temperature for 15 min. The electrokinetically-driven IA was ready to be conducted at this stage.
2.2.4 Electrokinetic mobility measurement

The electroosmotic mobility, $\mu_{eo}$, of 25 mM Tris-HCl buffer solution (buffer of the IA) in a microchannel was measured, by using an established current-monitoring method (Huang et al., 1988; Sze et al., 2003). In brief, a diluted buffer solution (90% of the original concentration, i.e., 25 mM) was electroosmotically pumped through the channel with a length $l$ at a constant applied voltage $\Delta V$. A high voltage power supply (Spellman Corp., Hauppauge, NY) was used to generate the electric field. The current was monitored and allowed to stabilize. An undiluted buffer (25 mM) was then introduced at one reservoir and electroosmotically driven through the same channel to displace the previous buffer. The time required for the current to reach a new plateau was recorded as $t$. The electroosmotic mobility was then calculated as,

$$
\mu_{eo} = \frac{u_{eo}}{E} = \frac{l}{t} = \frac{l}{\Delta V t} \quad (2.1)
$$

Antibody molecules can be regarded as solid particles and would undergo electrophoresis under an electric field if they bore surface charges. As antibody was delivered with the buffer solution, its net movement was a superposition of electroosmosis and electrophoresis. The net electrokinetic mobility was measured with the secondary Ab, TRITC-conjugated donkey IgG. Because the secondary Ab solution contained a mixture of excessive free fluorescent dye (TRITC) and TRITC-conjugated IgG, direct fluorescent visualization of the flow would not necessarily reveal the movement of TRITC-conjugated IgG. Therefore, an approach was taken involving Ab-Ag binding reaction.

A microchannel after antigen coating, blocking and primary antibody incubation was used in the measurement. The two wells were filled with secondary Ab solution and
Tris-HCl buffer solution, respectively. Secondary antibody solution was electrokinetically pumped into a part of the channel during a certain time period (t). The flow was then stopped for 3 min to allow for incubation, during which the secondary Ab reacted with antibodies on the channel wall. At the same time, the channel was observed under a fluorescence microscope, so that any possible pressure-driven flow could be eliminated by adjusting the levels of solutions in the two wells. After incubation, the buffer solution was electrokinetically delivered through the channel to flush out excessive antibodies. Viewing under the fluorescence microscope, the length of the channel yielding a fluorescence signal was taken as l. The net electrokinetic mobility of antibody in 25 mM Tris-HCl buffer was then obtained also by using Eq. (2.1), where the electroosmotic mobility and velocity were replaced by the net electrokinetic mobility and velocity, accordingly. The measurements of the two mobilities, \( \mu_{eo} \) and \( \mu_{net} \), were conducted using the same microchannel. The measurement was repeated for five times using different channels.

2.2.5 Electrokinetically-driven immunoassay

The anti-\( H. pylori \) antibody was diluted to 1:25 (12.8 \( \mu \)g mL\(^{-1} \)) with the blocking buffer, unless otherwise indicated. The secondary antibody, TRITC-conjugated anti-rabbit IgG, was also diluted to 1:25 (60 \( \mu \)g mL\(^{-1} \)) with the blocking buffer.

The electrokinetically-driven IA was composed of six sequential steps of loading, incubation, and washing of the primary antibody, followed by loading, incubation and washing of the secondary antibody. Before each antibody-loading step, 12 \( \mu \)L antibody solution was added in each well and platinum electrodes connected with power supply were placed into the solution to apply the electric field. After each antibody incubation
step, the wells were emptied and washed twice by manually pipetting using Tris-HCl buffer, before the following channel-washing operation was conducted. After the final washing step, bound fluorescence signal on the channel wall was detected by using a fluorescence microscope. All experiments were carried out in duplicate on at least two different occasions.

2.2.6 Detection and image analysis

Fluorescent signal was detected by using a Leica DM-LB fluorescence microscope (Leica Microsystems, Richmond Hill, Ontairo, Canada). A mercury arc lamp was used as the light source. Images were captured using a Retiga 12-bit cooled CCD camera (QImaging corp., Burnaby, BC, Canada) at an exposure time of 2 s.

Fluorescent intensity was analyzed by using Openlab 3.1.5 imaging software (Improvision Inc, Lexington, MA). In order to minimize the influence of non-uniform fluorescence, three fluorescent images were taken for each channel, at ¼, ½ and ¾ length along the channel. For each image, intensity from the channel was determined as the mean intensity of the 100-pixel-wide channel region. Background signal intensity was determined as the averaged mean intensity of the two 60-pixel-wide regions adjacent to the channel from top and bottom. The fluorescent intensity of the image was then calculated as the difference of intensities from the channel and background. Finally, the intensities of the three images were averaged to obtain the fluorescent intensity from reaction.

2.3 Results and discussion

2.3.1 Antigen immobilization

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In this proof-of-concept study, adsorption was employed for antigen immobilization, due to the simplicity of the method and the suitability of the channel material, PDMS, for protein adsorption. PDMS surface is reported to be more hydrophobic than polystyrene, and has a high avidity for proteins (Butler et al., 1997a).

A glass microscope slide was used as the substrate, due to its good heat transfer property (thermal conductivity: 1.4 W m$^{-1}$ K$^{-1}$). As will be demonstrated later, Joule heating is an important consideration to microchips built on the EOF platform. Because of the low thermal conductivity of PDMS (0.18 W m$^{-1}$ K$^{-1}$), the internally generated heat would be difficult to reject with a PDMS/PDMS chip system. Erickson et al. have demonstrated both experimentally and theoretically that under a strong electric field, the temperature increase with a PDMS/PDMS system is almost five times of that from a PDMS/glass system (Erickson et al., 2003).

Before the microchannel was formed, both the original PDMS slab and the glass slide were subject to plasma oxidization to introduce hydrophilic silanol (Si-OH) group on both surfaces. In this way, the two pieces could covalently bond with either other to form an irreversible seal (McDonald et al., 2000). More importantly, the hydrophilicity of microchannel surfaces is a prerequisite for electrokinetic pumping. Otherwise, liquid solution may form droplets and clog the channel (McDonald et al., 2000). This plasma treatment, however, impairs the protein capture capacity of PDMS. To investigate its effect, the duration of plasma treatment was varied from 5 s to 30 s and then TRITC-conjugated secondary antibody was directly coated on channel walls, following the same immobilization protocol as described in 2.2.4. After incubation and rinsing, bound fluorescence signals were detected and quantified, as shown in Fig. 2.1.
Clearly, the fluorescent signal became weaker as the duration of plasma treatment increased, indicating that less protein molecules were adsorbed. Compared to a 5-s treatment, a 10-s treatment impaired the protein capture capability of PDMS by 1/3 and a 30-s treatment significantly reduced the protein capture capability by almost 3/4. A 10-s plasma treatment was used, as a tradeoff between reliable permanent sealing and stable electrokinetic solution transport, and satisfactory protein adsorption. A 5-min interval was left between the chip assembling and antigen immobilization to allow some recovery of the hydrophobicity of PDMS. It is well known that oxidized PDMS surface reverts to being hydrophobic with exposure to air (McDonald et al., 2000; Ren et al., 2001).

Antigen was incubated for 2 hr at room temperature after filled into the channel. This is a condition used in conventional ELISA and also proved to work well here.

2.3.2 Buffer solution test

In conducting the EK-IA, the assay buffer should not only be compatible with the Ab-Ag immunoreaction, but also be stable under an electric field. Specifically, a proper buffer for EK assays should have a low electric conductivity, since high conductivity of a buffer results in a high electric current, which leads to significant Joule heating and electrolysis, thereby changing the temperature and pH of the buffer solution.

Two buffers commonly used for IAs, PBS (100 mM, pH 7.4) and tris-HCl (25 mM, pH 7.5), were tested for their electric conductivities and stability under an electric field. A straight microchannel, 2 cm long, 120 µm wide and 30 µm high, was filled with buffer and an electric field of 250 V cm⁻¹ was applied along the channel for a certain time. During the process, electric current was monitored. The pH of the solutions in the two wells was measured using pH indicator paper afterwards.
The current profiles are shown in Fig 2.2. For PBS, current increased linearly at 1 \( \mu \text{A min}^{-1} \) from 91 \( \mu \text{A} \) to 101 \( \mu \text{A} \) in 10 min. For Tris-HCl, current increased very slowly in the first 15 minutes from 12 \( \mu \text{A} \) to 14 \( \mu \text{A} \), then more rapidly to 20 \( \mu \text{A} \) in another 15 min with an average rate of 0.4 \( \mu \text{A min}^{-1} \). The increase in current was a consequence of the temperature rise of in-channel solution due to Joule heating effect. Electric conductivity increased with temperature and hence, current also increased. Therefore, the higher the conductivity, the greater the Joule heating, and the faster the current increases. That is why current grew more rapidly for PBS.

From the initial current values at room temperature, the conductivities of PBS and 25 mM Tris-HCl were 1.01 S·m\(^{-1}\) and 0.13 S·m\(^{-1}\), respectively, calculated using \( \lambda = \frac{I}{S} \), where I is the electric current, S the cross section area of the microchannel and E the electric field strength. The conductivity of PBS was almost 8 times greater than that of 25 mM Tris-HCl. The corresponding high current load led not only to the significant Joule heating effect, but more notably, to electrolysis. After the 10-min test for PBS, the pH in the wells connected with the anode and cathode changed from 7.4 to 11 and 5, respectively. A pH gradient along the channel was therefore resulted and the change in the local pH would interfere with the pH-dependent immunoreaction. But for 25 mM tris-HCl buffer, even after the 30-min test, the pH changes in the two wells were slight, from 7.5 to 8 and 7, respectively.

At a reduced electric field of 125 V cm\(^{-1}\), no change in pH and current was observed for Tris-HCl buffer. But for PBS, after a 30-min test, the electric current increased from 43 \( \mu \text{A} \) to 52 \( \mu \text{A} \) and the pH in the two wells changed from 7.4 to 11 and 5, respectively.
From the tests, 25 mM tris-HCl demonstrated much better stability than PBS during electrokinetic transport. The problem of high current load for phosphate buffer has also been mentioned in other studies on EK assays (Bromberg & Mathies, 2004; Erickson et al., 2004). 25 mM tris-HCl was therefore employed as the assay buffer. For better stability of buffer solution, a low electric field of 125 V cm\(^{-1}\) was used in this study. It should be noted, however, that although some negative effects exist, a stronger electric field could still be used to conduct IAs. In another study of electrokinetically-driven IA also using 25 mM tris-HCl buffer, electric fields up to ~700 V cm\(^{-1}\) were used (Dodge et al., 2001). Linder et al. employed PBS as the assay buffer and used electric fields up to 300 V cm\(^{-1}\) (Linder et al., 2002). 125 V cm\(^{-1}\) was on the lower, safer side. In later stages in this study, sometimes a moderately higher electric field was applied.

2.3.3 Electrokinetic mobility measurement

In designing the microfluidic IA, the time required for solution dispensing depends on the velocity of antibody transport. Therefore, electrokinetic mobility measurement was conducted first. In addition to the net mobility of antibody, the electroosmotic mobility of 25 mM Tris-HCl buffer, which was used to dilute the antibody, was also measured, in order to understand the respective contributions of electroosmosis and electrophoresis to the electrokinetic transport of antibody molecules.

In measuring the net electrokinetic mobility, the TRITC-conjugated donkey IgG was delivered at 125 V cm\(^{-1}\) for 50 s. After incubation and washing, the channel regions bearing fluorescent signal were 0.76~1.01 cm long. Calculated from Eq. (2.1), the net electrokinetic mobility of TRITC-conjugated anti-rabbit IgG in 25 mM Tris-HCl buffer is 
\[
(1.43 \pm 0.2) \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}.
\]
Under an applied voltage of 250 V, 25 mM Tris-HCl buffer solution was displaced through a 2-cm-long antigen-coated microchannel in 96~125 s. The electroosmotic mobility of the buffer solution was then calculated as \((1.42 \pm 0.16) \times 10^{-8}\) m\(^2\) V\(^{-1}\) s\(^{-1}\), which compares well to electroosmotic mobilities in protein-adsorbed PDMS microchannel reported elsewhere (Dodge et al., 2001; Linder et al., 2002; Linder et al., 2001).

As indicated by the results, the net electrokinetic mobility of antibody was approximately the same as the electroosmotic mobility of the buffer solution. Since the net movement of antibody molecules is a superstition of electroosmosis and electrophoresis, i.e., \(\bar{u}_{\text{net}} = \bar{u}_{\text{eo}} + \bar{u}_{\text{ep}}\) or \(\mu_{\text{net}} \bar{E} = \mu_{\text{eo}} \bar{E} + \mu_{\text{ep}} \bar{E}\), the electrophoresis of antibody molecules in the pH 7.5, 25 mM Tris-HCl should be close to zero.

Under the applied electric field strength, 125 V cm\(^{-1}\), the velocity of antibody movement was \(\sim 179\) \(\mu\)m s\(^{-1}\). It therefore took 112 s for antibody molecules to be transported through a 2-cm microchannel. Solution dispensing steps were set for 3 min to ensure a complete delivery.

### 2.3.4 Determination of the incubation time

Short incubation time is a significant characteristic of microfluidic IAs, owing to the small diffusional distance. In literature on microfluidic IAs, where microchannel height ranged from 10 \(\mu\)m to 100 \(\mu\)m, the incubation time was 3~10 min (Bernard et al., 2001; Rossier & Girault, 2001; Sato et al., 2001). Based on these references, the incubation time in this study was set as 6 min.

It should be noted, however, that this incubation time, although much shorter than hours to days required in conventional IAs, could still be further reduced. The required
time for incubation can be estimated by simply using the Einstein-Smoluchowski equation, $L = \sqrt{2Dt}$, where $L$ is the distance diffused by a molecule, $D$ the diffusion coefficient of the molecule; and $t$ the time of diffusion. The antibody used in this study, IgG, is a large protein with a molecular weight of 150 kDa. It therefore has a low diffusion coefficient of $4 \times 10^{-11}$ m$^2$ s$^{-1}$. The time required for IgG molecules to diffuse 8 µm, 30 µm, 60 µm, or 100 µm was then calculated to be 0.8 s, 11 s, and 45 s, respectively. This result was supported by the experimental studies of Rossier et al. on the Ab-Ag binding kinetics using a 40-µm high microchannel, in which close signal intensities were obtained when incubation time varied in the range of 1~60 min (Rossier et al., 2000).

Therefore, for microchannels with a height of 60 µm or less, an incubation of 1 min is sufficient for all molecules in solution to diffuse to the surface. However, an assumption for the above discussion is that the amount of analytes in the reaction chamber is fixed, i.e., incubation is carried out at a stopped-flow condition. If incubation is conducted at a flow-through mode, analyte in the reaction chamber is continuously replenished and longer incubation can enrich surface binding reaction, as long as equilibrium stage has not been reached. The determination of incubation time should then take into account many factors, including the flow velocity, the concentration of analyte in solution and the surface concentration of capture molecules, as well as the target limit of detection. A detailed analysis of the reaction kinetics under a flow-through condition will be given in Chapter 8.

In this straight-channel IA, incubation was conducted at a stopped-flow condition. In the further developed IA as described in the later chapters, flow-through incubations were applied.
Operation parameters for each step of the IA, including electric field strength and duration, are summarized in Table 2.2. For each loading and washing step, electric potentials of 250 V and 0 V were applied at the two electrodes, respectively.

2.3.5 Determination of the blocking condition

Non-specific binding is an important issue for PDMS microchannels, not only because of the high surface-to-volume ratio of a microchannel reactor, but also due to the strong avidity of PDMS for proteins. It has been reported that conventional blocking approaches cannot suppress non-specific binding effectively and thus lead to decreased sensitivity of immunoassay (Eteshola & Leckband, 2001). Complicated multi-layer surface modifications have been employed to solve this problem (Eteshola & Leckband, 2001; Linder et al., 2001).

In this study, however, it is found that non-specific binding can be greatly relieved by just following conventional blocking approach, with a strong BSA solution. 1% (w/v) BSA has been successfully used as blocking and diluent buffer in previous studies on pressure-driven microfluidic IA in PDMS microchannels (Lin et al., 2005; Lin et al., 2004a). However, as shown in Fig. 2.3, 1% BSA did not effectively inhibit non-specific binding in this study. The fluorescent signal in the no-antigen negative control came from adsorption of primary antibody and/or secondary antibody onto the channel wall.

The difficult-to-quench non-specific binding in this study came from at least two sources. First, the higher surface-to-volume ratio of the microchannel. The microchannel used previously by Lin et al. had a capillary geometry with an internal diameter of 360 µm and a surface-to-volume ratio of $1.1 \times 10^4$ m$^{-1}$. The microchannel in this study had a
rectangular cross section of $30 \times 120 \, \mu m^2$ and the corresponding surface/volume ratio was $8.3 \times 10^4 \, m^{-1}$, approximately 10 folds larger than that of the capillary microchannel. A larger surface-to-volume ratio means there are more binding sites on the surface to cover with a certain volume of the blocking solution. This effect of high surface-to-volume ratio of microchannels on blocking condition has also been reported in other microfluidic IA studies (Rossier & Girault, 2001). Second, the higher concentration of antibody solutions. In the work of Lin et al., the dilution of primary antibody and secondary antibody was both 1:2000, whereas in this study the antibodies were diluted to 1:25. Therefore, a blocking and diluent buffer of the same strength as before was not able to compete with the more concentrated antibody for non-specific binding sites on the channel surface.

Despite these challenges, as the concentration of BSA increased to 5% (w/v), blocking effect improved remarkably, as shown in Fig. 2.3. The very low fluorescent signal from the negative control indicated that non-specific binding of antibodies to the channel walls was effectively inhibited. From the quantified fluorescent signals, the positive to negative signal ratio was 28.7, which was an increase of over 10 times compared to the ratio of 2.4 when blocking with 1% BSA. This improvement was even better than reported results obtained by using surface modification (Eteshola & Leckband, 2001), yet the blocking approach used here was much easier. 5% BSA in Tris-HCl buffer was then used as the blocking and diluent buffer for the rest of the study, unless otherwise indicated.

2.3.6 Determination of the height of microchannel
The height of microchannel is an important factor in IA design. It affects both the IA reaction and the fluid transport. To investigate the influence of channel height on IA reaction, IA experiments were conducted using microchannels with different heights of 8, 30, and 60 µm.

Typical results from the IA experiments are demonstrated in Fig. 2.4. While signals from the no-antigen negative controls almost remained constant for different channels, intensities from positive controls clearly increased with channel height. (Though the microchannels used are of different widths, the moderately varied channel widths should not have significant effect on the IA results.) This increase is simply because higher channels held more reactants – more lysate antigen for adsorption and more antibody solution for immunoreaction. Therefore, the surface binding reaction proceeded to a further extent in higher microchannels. In this sense, a higher channel was preferable for this IA study.

However, this trend was true only in the current stage of the microfluidic IA, where incubation was conducted in a stopped-flow condition. If flow-through incubation is employed, analytes (antibodies) in the reaction chamber can be continuously replenished and the channel height is no longer an important factor in reaction kinetics. Although the effect of channel height on antigen coating still remains in that case, it can be compensated by adjusting the concentration of antigen solution or duration of coating.

Though channel height would not be crucial to the immunoreaction itself with further development of the IA, it still affects some practical aspects of the EK-IA. Pressure-driven flow is one of the major problems for EK-driven microdevices. The pressure difference comes not only from the different liquid levels in different wells
(siphoning effects), but more importantly, from the differential meniscus curvatures in the wells (Laplace pressure) (Sinton et al., 2003). It has been shown that the Laplace pressure-induced flow is 10-20 times greater than that from siphoning effects (Crabtree et al., 2001). Pressure interferences are hard to be completely quenched but can be minimized by increasing flow resistance. As the pressure-driven flow rate is proportional to $h^3$, using shallower channels can effectively reduce undesirable pressure effects. In preliminary testing with EOF in a microfluidic network, strong influence of pressure-driven flow was observed with the 60-µm high microchannel, which caused the flow route to deviate from designed. It was therefore not suitable for use.

Though favorable for minimizing pressure-driven flow, a small channel height can induce another practical problem, channel sagging. PDMS is a soft elastomer and lateral sagging tends to occur in a shallow, long PDMS microchannel (Bietsch & Michel, 2000; Odom et al., 2002). In conducting the IA experiments, channel sagging and collapse was occasionally observed with the 8-µm high microchannel, which ruined the entire assay. This problem of channel collapse could not be solved by making the PDMS stiffer via increasing the ratio of curing agent to PDMS base, to 1:10 or even 1:5.

From the above practical considerations, a medium channel height of 30 µm was determined to be proper. At this channel height, no channel collapse was observed and the effect of pressure-driven flow was at an acceptable level. 30-µm microchannels were then used for the rest of the study.

2.3.7 Immunoassay for antigen

Now that the conditions for the EK-IA were determined, performance of the microfluidic IA was evaluated by testing of its dynamic range for the model analytes.
This test was first conducted against *H. pylori* antigen coated on microchannel walls. Although in most cases, analytes in the liquid phase are the target for a heterogeneous IA, sometimes the IA is designed to recognize solid phase-captured molecules (Lin et al., 2005).

Sample solutions of the assay include dilutions of *H. pylori* lysate with protein concentrations in the range of 0.3-100 µg mL⁻¹, as well as *L. rhamnosus* lysate and coating buffer as negative controls. Results from the assay are shown in Fig. 2.5. The low fluorescent intensities from coating buffer demonstrated effective quenching of non-specific binding. *L. rhamnosus*-coated channels also yielded very low signals. Therefore, little or no cross-reaction occurred between *L. rhamnosus* antigen and *H. pylori* antibody and the IA was specific for *H. pylori* antigen. *H. pylori* antigen was detected in the concentration range of 1–100 µg mL⁻¹. The lower LOD was determined as the concentration yielding a signal exceeding mean ± 3SD of the negative control (coating buffer).

Compared to the previous pressure-driven ELISA (5~500 µg mL⁻¹) or conventional dot-blot ELISA (10~1000 µg mL⁻¹) using the same model analyte (Lin et al., 2004a), the dynamic range of 2 orders of magnitude was the same but the LOD achieved in this study, 1 µg mL⁻¹, was lower. This result is encouraging because due to the almost 10 times larger surface-to-volume ratio of the microchannel in this study than that of Lin et al., as mentioned in 2.3.6, even a same antigen concentration would result in a ~10 times lower surface density of adsorbed antigen. The effective detection of antigen in this study partly owed to the fluorometric detection, which is more sensitive than colorimetric detection (Diamandis & Christopoulos, 1996) used in the study of Lin et al. In addition,
some strategies in the assay design could also contribute to the improvement, such as using a strong BSA solution for better blocking and using more concentrated antibody solutions to enhance the immunoreaction.

2.3.8 Immunoassay for antibody

In the assay for primary antibody in solution, the concentration of *H. pylori* antigen was 40 µg mL⁻¹. Antibody solutions in the concentration range of 0.01~10 µg mL⁻¹ were assayed. A negative control was also included in which the sample solution contained no primary antibody. As shown in Fig. 2.6, primary antibody was reliably detected at concentrations higher than 1 µg mL⁻¹ (6.7 nM). This LOD is comparable to that of some reported microfluidic immunosensors targeting of IgG (Dodge et al., 2001; Lai et al., 2004; Linder et al., 2002), mostly based on idealized reaction systems. However, in the present study, the coating layer was whole cell bacterial lysate, without purification, thus demonstrating the efficiency and potential of this EK-IA. Also, the reproducibility is satisfactory. The variation coefficients of the experimental runs for Fig. 2.5 and Fig. 2.6 were 4~16%.

2.3.9 Shelf life and storage condition of the IA microchip

As the EK-IA can be used to assay for either antigen or antibody, the shelf life of the IA microchip was investigated from two aspects. First, to test the long-term antigen-binding capability of the channel surface, microchannels were filled with coating buffer after formed. Second, to test the long-term affinity of the immobilized antigen for primary antibody, microchannels were immobilized with antigen and filled with the assay buffer, 25 mM Tris-HCl. In both cases, the wells of the microchips were covered with micro cover glass to prevent the evaporation of buffer solution and the chips were stored
at 4 °C. Immunoassay experiments were then performed on different days with the stored chips. It was found that the microchip could be stored for at least 10 days (the longest period tested) without degradation of performance in both antigen adsorption and antibody binding. This finding is in accordance with results from literature, which showed that proteins adsorbed on PDMS could be preserved for days (Bernard et al., 2001) or even two months (Linder et al., 2002) without loss in activity.

It is important to note that microchannels must be stored in a wet condition, i.e. filled with buffer. Exposure of microchannel to the air can result in denature of adsorbed proteins (Cesaro-Tadic et al., 2004b) and change in surface properties of PDMS channels (Ren et al., 2001). Linder et al. found that microchips dry-stored at -20 °C for 2 months could not be employed to perform IA because of the formation of air bubbles when reintroducing buffer solution into the channel. Similar problems had also been encountered in this study, when microchannels were dried accidentally (mostly during antigen coating stage) for longer than ~30 min. The change in surface condition seriously affected electrokinetic pumping, resulting in a much slower flow rate and even obstruction of channel due to bubbles formed. Even if an IA test could be completed with such a channel, the yielded fluorescent signal was seriously heterogeneous over the surface and could not be used, as shown in Fig. 2.7.

### 2.4 Summary

This chapter describes in detail the development of an EK-IA on a straight microchannel, and the performance of this microfluidic IA in screening for either *H. pylori* antigen or antibody. The content is summarized as follows:
Experimental protocol for conducting the EK-IA was established, by means of: 1) Measurement, e.g. of the conductivities of buffer solutions, the electrokinetic mobilities of antibody and buffer; 2) Experimental condition-optimization, e.g. the duration of plasma treatment, the concentration of BSA in the blocking buffer, the channel height; 3) referring to literature and theoretical calculations, e.g. incubation time. Some important experimental conditions are: plasma-treating PDMS slab and glass slide for 10 s before channel formation and antigen coating; Using 25 mM Tris-HCl as assay buffer; Applying an electric field of 125 V cm$^{-1}$ for 3 min to load and wash reagents, for a 2-cm long microchannel; Incubating reagent solution for 6 min at room temperature; Using 5% BSA as blocking and diluent buffer; Using 30-µm high microchannels.

The microfluidic IA detected antigen coated on channel walls with a dynamic range of 1-100 µg mL$^{-1}$. The detection limit of 1 µg mL$^{-1}$ is better than that from earlier pressure-driven ELISA (5 µg mL$^{-1}$) or conventional dot-blot ELISA (10 µg mL$^{-1}$) for the same analyte.

The limit of detection for antibody was 1 µg mL$^{-1}$, comparable to that from some other microfluidic immunosensors.

The shelf life of the IA microchip, for both antigen and antibody assay, was tested to be at least 10 days. Microchips must be stored in a wet condition.

The EK-IA proved a significant improvement over conventional ELISA, in terms of assay time and reagent consumption. Table 2.3 lists some features of the microfluidic IA in comparison with conventional ELISA. Also, the reproducibility of the EK-IA was satisfactory, with a variation coefficient of 4–16%.
Figure 2.1 Effect of duration of plasma treatment on antigen coating. After the microchip was formed, TRITC-conjugated donkey anti-rabbit IgG, at a concentration of 30 µg mL\(^{-1}\), was passively adsorbed onto the channel wall and incubated at room temperature for 2 hr. Detection was performed after rinsing the channel with buffer solution to remove unbound antibody. (a) Fluorescent images taken at the center of the microchannels. The duration of plasma treatment was as indicated under the images; (b) Quantification of the fluorescent intensities in (a). The error bars indicate the standard error of the mean (N=3).
Figure 2.2 The influence of applied electric field on conductivities of buffer solutions. The applied electric field was 250 V cm\(^{-1}\). The microchannel was 2-cm long, 120-µm wide and 30-µm high.
Figure 2.3 The influence of BSA concentration on blocking effect. Negative controls were immunoassays performed with microchannels without antigen coating, (i.e., filled with only coating buffer before the IA experiments) so that the fluorescent signal came only from the non-specific binding of antibody molecules to the channel walls. In the positive control, *H. pylori* antigen was coated at a concentration of 10 µg mL⁻¹. (a) Fluorescent images taken at the center of the microchannels. (b) Quantification of the fluorescent intensities in (a).
Figure 2.4 The effect of microchannel height on the IA results. In positive controls, concentration of antigen solution for coating was 10 µg mL$^{-1}$. Negative controls were IAs without antigen coating. Blocking solution was 1% BSA. (a) Fluorescent images taken from the center of the microchannels. (b) Quantification of the fluorescent intensities in (a).
Figure 2.5 The dependence of signal intensity on the concentration of coating antigen. Rabbit polyclonal anti-*H. pylori* antibody (12.8 µg mL\(^{-1}\)) was used as primary antibody and TRITC-conjugated donkey anti-rabbit IgG (60 µg mL\(^{-1}\)) was employed as secondary antibody. (a) Fluorescent images taken from the center of the microchannels. (b) Quantification of the fluorescent intensities in (a). The dotted line indicates the cut-off value of 271.11, calculated from mean ± 3SD of the negative control (coating buffer). The LOD for *H. pylori* antigen was 1 µg mL\(^{-1}\).
Figure 2.6 The dependence of signal intensity on the concentration of primary antibody. The concentration of coating antigen was 40 µg mL\(^{-1}\). The concentration of secondary antibody, TRITC-conjugated donkey anti-rabbit IgG, was 60 µg mL\(^{-1}\). The dotted line indicates the cut-off value of 131.56, calculated from mean ± 3SD of the negative control (no primary antibody, data point on present on the plot). The LOD of primary antibody was 1 µg mL\(^{-1}\).
Figure 2.7 A typical fluorescent image from an IA experiment which failed due to incidental drying of the microchannel for longer than \(~30\) min during antigen coating stage.
<table>
<thead>
<tr>
<th>Film thickness, µm</th>
<th>Spread cycle</th>
<th>Spin cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ramp time, s</td>
<td>Target speed, rpm</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>1800</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>500</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>500</td>
</tr>
</tbody>
</table>
Table 2.2 Steps in the EK-IA

<table>
<thead>
<tr>
<th>No.</th>
<th>Steps</th>
<th>E (V cm⁻¹)</th>
<th>Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary Ab loading</td>
<td>125</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Primary Ab incubation</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Washing</td>
<td>125</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Secondary Ab loading</td>
<td>125</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Secondary Ab incubation</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>Washing</td>
<td>125</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2.3 Comparing the Characteristics of the EK-IA with conventional ELISA*

<table>
<thead>
<tr>
<th></th>
<th>EK-IA</th>
<th>Conventional ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
<td>6 minutes</td>
<td>1 hour</td>
</tr>
<tr>
<td>Total assay time</td>
<td>~30 minutes</td>
<td>~3 hours</td>
</tr>
<tr>
<td>Sample/Reagent</td>
<td>72 nL (in channel)</td>
<td>24 µL (in wells)</td>
</tr>
<tr>
<td>consumption</td>
<td>24 µL (in wells)</td>
<td>~100 µL**</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>1 µg mL⁻¹</td>
<td>10 µg mL⁻¹</td>
</tr>
<tr>
<td>Reagent dispensing</td>
<td>Flow-through</td>
<td>pipetting</td>
</tr>
<tr>
<td></td>
<td>(electrokinetically-driven)</td>
<td></td>
</tr>
</tbody>
</table>

* Data of conventional ELISA are from dot-blot ELISA in Ref. (Lin et al., 2004a), except for the reagent consumption.
** Typical value for ELISA conducted with a 96-well microtitre plate.
Chapter 3 Development of an automated EK-IA on a microfluidic network*

3.1 Introduction

In the last chapter, the establishment of the experimental protocol for an EK-IA was described, which was the first stage towards developing an electrokinetically-controlled IA. Despite the merits of the IA prototype, such as short assay time, small reagent consumption and low limit of detection, many solution operations in the multi-step analysis were still manually conducted – the wells of the microchip had to be emptied, washed and refilled, each time a different reagent was used. The purpose of using electrokinetic pumping in this thesis project is to automate the whole IA process. This chapter will then focus on the realization of an automated EK-IA.

The task can be further split into two parts. The first part is assay design, including the design of a microfluidic network ($\mu$FN) as well as the flow route of reagent solutions for each step in the assay, such as antibody loading or washing. The second part of the task, then, is to realize the fluidic control of the EOF to carry out the designed solution operations.

Flow control is a crucial component in the automated EK-IA. Lack of flow control leads to deviation of the flow route from designed and thereby incomplete solution operation or cross-contamination of the reagents (Erickson et al., 2004). In some reported studies on EK-driven IA, the flow control in each step were realized

straightforwardly by applying one of the three conditions – a high electric potential, ground or “floating” – at the different ports of a $\mu$FN (Chiem & Harrison, 1998; Dodge et al., 2001). However, to switch between different steps, not only the applied potentials had to be adjusted, but the electrodes also needed to be manually connected/disconnected with the chip, in presence of a “floating” condition. The sequential operations thus cannot be integrated. Automated control of the entire IA process has to depend on proper applied electric fields over a $\mu$FN to achieve flow valving.

The effort in seeking the controlling parameters, i.e., applied electric potentials, increases dramatically with the complexity of the $\mu$FN, i.e., the number of channels and wells. It is therefore difficult to accomplish the task merely by experimental optimization. To solve the problem, computational fluid dynamics (CFD) was employed to simulate the microfluidic processes to help determine the parameters. CFD is a powerful tool to build virtual prototypes and to simulate the performance of proposed designs in many engineering fields. Built on the information from computational simulations, the number of experimental testing can be significantly reduced and the efficiency of scientific research greatly enhanced. CFD has already been extensively used in microfluidic studies (Erickson & Li, 2003; Ermakov et al., 1998; Griffiths & Nilson, 2001; Patankar & Hu, 1998).

In this study, the applied electric potentials, after estimated by using Kirchhoff’s rules, were optimized with CFD by trial-and-error. To check the effectiveness of the obtained electric fields, experimental flow visualization was conducted using fluorescent imaging.
To test the performance of this automated EK-IA microchip, IA experiments were briefly conducted. The model analyte was still *H. pylori* antigen. More experimental demonstrations with the automated EK-IA will be given in the next chapter.

### 3.2 Chip and assay design

In designing the µFN for the EK-IA, the following factors were considered:

1. The µFN should include at least one well and one supply channel for each reagent. In addition, there should be one well for the waste solution, to avoid cross-contamination. Therefore, for the immunoassay in this study, four wells would be required, each for the primary Ab, secondary Ab, washing buffer or waste solution.

2. The channels should be as short as possible to minimize transport distance, but long enough to avoid the cross-contamination of different reagents due to diffusion or slight side flows.

3. The overall size of the µFN should fit onto a commonly used 75 × 25 mm$^2$ glass microscope slide.

4. While the reagent consumption is kept reasonably low, the size of wells should be made as large as possible to minimize the change of liquid levels and menisus shape in wells, which could cause undesired flows driven by hydrostatic pressure difference.

A simple design adopted in this study is presented in Fig. 3.1. Figure 3.1 (a) provides a schematic illustration of the H-shaped µFN and Fig. 3.1 (b) is a picture of the microchip. All the channels are 120 µm wide. The overall dimension of the µFN was 24 × 10 mm$^2$. The horizontal arm of the “H” connecting the four supply channel sections acted as a buffering zone during primary Ab dispensing, to avoid contamination of the secondary Ab and buffer solution.
The flow paths for the six steps in this IA – loading, incubation and washing of the primary Ab, and the same operations of the secondary Ab – are illustrated in Fig. 3.2. The flow patterns during incubations were the same as those in the preceding loading step. Flow valving took place at the two junctions of the “H”. Since antigen was still immobilized by adsorption and thus was coated on the walls of the entire µFN, immunoreaction took place on the overlapping flow path for primary Ab and secondary Ab, which was the left arm of the “H”.

3.3 Flow control establishment

3.3.1 Physical model and numerical simulation

Steady electroosmotic flow (EOF) and transient sample transportation were considered in modeling. The steady-state simplification is justified because EOF reaches steady state instantaneously (in several milliseconds) once an electric field is applied (Patankar & Hu, 1998). Another simplification was to decrease the dimensionality of the problem from 3-D to 2-D because all the channels had a constant depth and the surface properties of the channels were assumed uniform.

According to the theory of electrostatics, the applied electrical potential, Φ, is described by the Laplace’s equation,

$$\nabla^2 \Phi = 0$$  \hspace{1cm} (3.1)

The local electric strength can then be calculated by $E = -\nabla \Phi$. EOF generated by an applied electric field is described by the steady incompressible Naiver-Stokes equations and the continuum equation,
\[ \vec{u}_{eo} \cdot \nabla \vec{u}_{eo} = -\frac{1}{\rho_f} \nabla P + \nu \nabla^2 \vec{u}_{eo} + \frac{\rho_e}{\rho_f} \vec{E} \]  
\[ \nabla \cdot \vec{u}_{eo} = 0 \]

where \( \vec{u}_{eo} \) is the bulk electroosmotic flow field, \( P \) the pressure, \( \nu \) the kinematic viscosity of the fluid, \( \rho_f \) the density of the fluid and \( \rho_e \) the electrical charge density.

However, there exists a similitude between the velocity field and electric gradient field under certain conditions, including a steady electric field, uniform fluid and electric properties, a thin Debye layer (Cummings et al., 2000), which were satisfied in the case of this study. Therefore, the velocity field can be directly obtained from the electric field using the relationship

\[ \vec{u}_{eo} = \mu_{eo} \vec{E} = -\mu_{eo} \nabla \Phi \]  

This significant simplification eliminated the necessity of solving the nonlinear Navier-Stokes equations and the continuum equation, Eq. (3.2)-(3.3).

The concentration field of reactant is described by the transient mass transport equation,

\[ \frac{\partial C_i}{\partial t} + (\vec{u}_{eo} + \vec{u}_{ep}) \cdot \nabla C_i = D_i \nabla^2 C_i \]

where \( C_i \) is the concentration of the \( i \)-th sample, \( \vec{u}_{eo} \) the electroosmotic velocity obtained from Eq.(3.4), \( \vec{u}_{ep} \) the electrophoretic velocity of the protein molecules calculated by

\[ \vec{u}_{ep} = \mu_{ep} \vec{E} = -\mu_{ep} \nabla \Phi \]  

\( D_i \) the diffusion coefficient of the \( i \)-th sample, e.g. primary antibody or secondary antibody.

Boundary conditions of Eq. (3.1) and (3.5) are,

at inlets, \( \Phi = \Phi_i \), \( C_i = C_{i0} \);
at outlets, \( \Phi = \Phi_i, \quad \vec{n} \cdot (D \nabla C_i) = 0 \);

at walls, \( \vec{n} \cdot \nabla \Phi = 0, \quad \vec{n} \cdot [D \nabla C_i - C_i (\vec{u}_{eo} + \vec{u}_{ep})] = 0 \).

where \( \Phi_i \) is the electrical potential applied at each well, \( \vec{n} \) the unit normal vector to the surface.

The above differential equations were solved by using a commercial software package, COMSOL Multiphysics 3.2 (COMSOL AB, Stockholm, Sweden), which is based on finite element method.

### 3.3.2 Optimization process

For each step in the EK-IA, according to the flow pattern and aimed electric strength, the applied electric potentials were first estimated by according to the Kirchhoff’s rules (Hadd et al., 1997; Qiu & Harrison, 2001), by taking the \( \mu FN \) as a network of electric resistors. The potential values were then applied to the numerical simulation of the flow field and were optimized following a trial-and-error scheme. The check condition in the optimization process was whether the flow valving at the junctions was effectively realized. Once the applied electric field for a certain step was determined, the duration of the step was decided by simulating the transient mass transport. Theoretically, a loading/washing process was complete when the reactant was delivered into/out of the whole reaction region (left arm of the “H”). Then, mass transport during reagent loading and incubation was simulated. If obvious mass leakage occurred from the valving point, e.g. primary antibody traveled from the left junction to the right junction of the “H”, or secondary antibody diffused from the right junction to the buffer well, it was necessary adjust the applied electric field and restart the simulation. Detailed illustrations of this optimization process will be given in Chapter 5 on the design of a parallel IA.
3.4 Experimental

3.4.1 Flow visualization

The microchannel network with the “H”-shaped design was fabricated and the microchip assembled in the same way as described in Chapter 2. After the chip was formed, 25 mM sodium carbonate/bicarbonate buffer (SCB) was filled into the channel.

The two wells for primary Ab and secondary Ab (P and S in Fig. 3.1) were both filled with 20 µL of 10 µM fluorescein diluted with 25 mM SCB. The other two wells were filled with 20 µL of 25 mM SCB.

The experimental setup is shown in Fig. 3.3. HVS 448 High-Voltage Sequencer (Labsmith, Livermore, CA) was used as the power supply. The sequencer has eight independent outputs and four of them were used here. The controlling parameters shown in Table 3.1, i.e., the applied electric potentials and duration for each step, were pre-set with the Sequence software (Labsmith, Livermore, CA). The controlling sequence was then transferred to the high voltage sequencer to program the outputs. The output channels of the sequencer were connected with the microchip through platinum electrodes placed in the wells of the chip. After the sequencer was triggered on, all sequential operations were automatically conducted. The two junctions of the H-shaped µFN were successively viewed on-line under a fluorescence microscope and images were automatically taken at the interval of one second.

3.4.2 Electrokinetically-controlled immunoassay

The reagents used and procedures for antigen coating and blocking were the same as described in Chapter 2. After the blocking stage, each well was filled with 12 µL of the corresponding reagent solution except that the waste well was filled with assay buffer.
The EK-IA was then ready to run. The experimental setup and operations were same as just described for flow visualization. The off-line fluorescent signal detection was conducted the same way as described in Chapter 2.

### 3.5 Results and discussion

#### 3.5.1 Controlling parameters

In the simulations, the electroosmotic mobility and electrophoretic mobility used, $\mu_{eo}$ and $\mu_{ep}$, were $1.42 \times 10^{-8} \text{ m}^2\text{ V}^{-1}\text{ s}^{-1}$ and 0, respectively, as measured in Chapter 2. The diffusion coefficient of antibody was $4.0 \times 10^{-7} \text{ cm}^2\text{ s}^{-1}$.

The controlling parameters for the EK-IA obtained by computational optimization were listed in Table 3.1. For simplicity, the ports for primary Ab, secondary Ab, buffer solution and waste are donated by P, S, B and W, respectively. The reagent loading/washing time was set to be $\sim$30-40% longer than that appeared adequate in numerical simulation, to ensure the operation was complete. The electric field was generally kept below 200 V cm$^{-1}$, with the maximum being 305 V cm$^{-1}$, in the final washing step. This was higher than 125 V cm$^{-1}$ used in the straight-channel study, but still appeared appropriate. The total time for the analysis cycle was 26 min and 20 s.

Incubation was conducted under a flow-through mode. During incubations, the flow route remained the same as the preceding reagent-loading steps, but the electric field strength was lowered by half to reduce the current load. As mentioned in the last chapter, the flow-through mode is superior to a stopped-flow mode for incubation. As the antibody molecules are continuously replenished in the reaction region, the antigen-antibody binding reaction is enhanced. Another advantage of the flow-through incubation
to this study is that the influence of undesirable pressure-driven flow was suppressed in presence of a much stronger EOF. Proper incubation time depends on many factors, such as flow velocity, antibody concentration and the surface concentration antigen. From most literatures on microfluidic IAs with flow-through incubation (Dodge et al., 2001; Linder et al., 2002; Wolf et al., 2004), the duration of 5-min appeared sufficient and was thus used here.

### 3.5.2 Flow visualization

The electroosmotic mobility of SCB buffer and the electrophoretic mobility of fluorescein dye were measured previously as $5.9 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ and $-3.3 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, respectively (Biddiss et al., 2004). That follows a net mobility of $2.6 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ for the fluorescein dye, about 80% higher than that of the antibody. Therefore, the loading/washing time used for dispensing antibody, as listed in Table 3.1, were sufficient for the transport of the dye.

Figures 3.4 and 3.5 show the images taken at the two junctions. Since the washing operation was much easier to control, only images from reagent-loading processes (step 1 and step 4) are shown. Images in each figure were taken at two different moments when the local reagent dispensing was incomplete and complete, respectively. Simulation results at the corresponding moment are also presented for comparison.

The experimental results displayed a very good agreement with the numerical predictions, on both the timing and concentration distribution. From Fig. 3.4, the leakage of sample into the horizontal arm was very small. The case is the same in Fig. 3.5, where sample from the “S” well was precisely delivered into the horizontal arm as designed. During the second 5-min incubation, sample diffusion into the buffer channel was
observed, but the distance traveled was short, less than 2 mm. Therefore, cross-contamination between reagents would not occur during the EK-IA.

3.5.3 Electrokinetically-controlled immunoassay

Figure 3.6 shows the fluorescent images from several different locations of the \( \mu FN \), in testing coated \( H. pylori \) antigen at a concentration of 100 \( \mu g \) m\( L^{-1} \). The uniform fluorescent intensity in different images on the left arm in Fig. 3.6 (a) indicated that the immunoreaction occurred to a comparable extent along the whole reaction region. Clearly from the images at the left junction of the “H” in Fig. 3.6 (a) and the larger view in Fig. 3.6 (b), fluorescent signal intensity at the horizontal arm of the “H” decreases sharply from left to right, indicating the transition from reaction to non-reaction. This result further confirmed that the primary antibody solution was dispensed strictly following the designed path (shown in Fig. 3.4) and that sample diffusion during incubation was not a problem. The difference in signal intensity from the reaction region and non-reaction region on the horizontal arm and the right junction was visually significant. The very low signal intensity from the non-reaction region again proved the effective inhibition of non-specific binding, by using 5% (w/v) BSA for blocking.

Different concentrations of antigen were tested and the fluorescent intensities at the left and right junctions were quantified and plotted in Fig. 3.7. The limit of detection (LOD) cannot be directly obtained from the figure, as no-antigen negative control was not performed. Nevertheless, the figure still provides meaningful information, when compared with the results from the last chapter, Fig 2.6. Obviously, the dynamic range shows an overall shift to the lower side in this study. Saturation is reached at 10 \( \mu g \) m\( L^{-1} \). But in the straight-channel IA, saturation is not met at this concentration. Also, the
intensity at 1 µg mL⁻¹ is higher than that in the straight-channel IA. Therefore, though not tested, this automated EK-IA can most probably yield a lower LOD than the EK-IA with the straight channel. While the incubation time was a bit reduced here, this improvement was attributed to the enhanced reaction kinetics with flow-through incubation.

Based on this work, a portable microfluidic IA system has been developed, which employed a laser-induced fiber optical fluorescence module for signal detection (Xiang et al., 2006).

### 3.6 Summary

This chapter describes the realization of an automated electrokinetically-controlled immunoassay, conducted using an H-shaped microfluidic network.

The controlling parameters, i.e., applied electric potentials and duration for each step, were determined by using CFD. Flow visualization experiment proved that the flow route was controlled as designed.

The electrokinetically-controlled IA was successfully applied to testing *H. pylori* antigen coated on channel wall. Using a programmable high voltage sequencer, the EK-IA was automatically conducted. Incubation time for each antibody was 5 min and the total time of the EK-IA was 26 min and 20 s.

Fluorescent images from the EK-IA experiments demonstrated that immunoreaction occurred uniformly in the reaction region. Coated antigen could be detected at 1 µg mL⁻¹, or even lower (not tested). The dynamic range was shifted towards the lower side compared to the straight-channel IA in Chapter 2, an improvement owing to the flow-through incubation.
Figure 3.1 (a) Schematic illustration the μFN used in the EK-IA (not proportional to actual size). All the channels are 120 µm wide and 30 µm deep. Overall dimension of the chip is 24 mm × 10 mm. The diameter of all the wells is 5 mm. (b) A picture of the immunoassay microchip (The channel is filled with rhodamine B to illustrate the geometry).
Figure 3.2 The sequential steps in the EK-IA. Solution delivery occurred in the dark colored channels whereas in the light colored channels, the solution is almost stagnant. The arrows indicate the flow direction. (a) Loading and incubation of primary antibody; (b) Washing of primary antibody; (c) Loading and incubation of secondary antibody; (d) Washing of secondary antibody.
Figure 3.3 Schematic of the experimental setup.
Figure 3.4: The first reagent-loading process (step 1) – experimentally and numerically obtained concentration field at the left junction of the “H”. $t_1$ and $t_2$ indicate the lapse time after step 1 started.
Figure 3.5: The second reagent-loading process (step 4) – experimentally and numerically obtained concentration field at the right junction of the “H”. $t_1$ and $t_2$ indicate the lapse time after step 4 started.
Figure 3.6 Results from a experimental run of the EK-IA. Concentration of *H. Pylori* antigen for coating was 100 µg mL⁻¹. The primary antibody, rabbit polyclonal anti-*H. pylori* antibodies, was at a concentration of 12.8 µg mL⁻¹ and the secondary antibody, TRITC-conjugated donkey anti-rabbit IgG, was at a concentration 60 µg mL⁻¹. 5% (w/v) BSA was used as the blocking and diluent buffer. (a) Fluorescent images from different locations of the reaction region and the non-reaction region, taken using a 32X objective. (b) A larger view of the reaction and non-reaction region around the left junction of the “H”, taken using a 10X objective.
Figure 3.7 Comparison of the signal intensities at different antigen concentrations. The intensities for the positive reaction and negative control were from the left junction and right junction of the “H”-shaped channel, respectively.
Table 3.1 Controlling parameters in the EK-IA

<table>
<thead>
<tr>
<th>Step</th>
<th>Operation</th>
<th>Applied potentials at wells (V)</th>
<th>Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\Phi_w$</td>
<td>$\Phi_n$</td>
</tr>
<tr>
<td>1</td>
<td>Primary Ab loading</td>
<td>0</td>
<td>95</td>
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<tr>
<td>2</td>
<td>Primary Ab incubation</td>
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<td>4</td>
<td>Secondary Ab loading</td>
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<td>6</td>
<td>Washing</td>
<td>0</td>
<td>500</td>
</tr>
</tbody>
</table>
Chapter 4 Improvement of the EK-IA: multiplexed detection*

4.1 Introduction

Through the work described in the last two chapters, an automated EK-IA lab-on-a-chip was developed. Yet the potential of a miniaturized electrokinetic immunoassay was not fully exploited. In the next two chapters, it will be demonstrated how the experimental protocol and the assay design were modified to significantly improve the throughput of the IA lab-on-a-chip. The focuses of this and the next chapter are the realization of multi-analyte (multiplexed) assay and multi-sample (parallel) assay, respectively, both being highly desirable features for an IA.

4.1.1 Microfluidic multiplexed IA: motivation and approaches

A multiplexed IA measures multiple analytes from the same sample simultaneously in a single test. The development of multiplexed IA is driven by the growing requirements of profiling various substances from one sample, both in clinical diagnostics and biomedical studies. Different diseases can display similar symptoms. For example, the infection with *Escherichia coli* O157:H7, *Salmonella*, or *Listeria monocytogenes* all leads to diarrhea. For accurate diagnosis, clinical samples need to be screened against different analytes of relevance. Also, in biomedical research, it is often necessary to measure the cell expression levels of multiple substances in one sample, such as cytokines (Carson & Vignali, 1999). Using conventional techniques, the above assays would necessitate dividing the original sample, such as patient serum, into multiple portions. Thus multiple tests can be conducted for each analyte of interest, with

associated time and reagent cost for each test. The large amount of original sample required for the multiple tests can be a big challenge, making the assay difficult in some situations, such as for pediatric patients.

Therefore, it is highly desirable to develop multiplexed assays to reduce the sample and reagent consumption while greatly enhancing the assay efficiency. The feasibility of multianalyte IA has been proposed and demonstrated almost two decades ago (Ekins, 1989). With the increasingly acute demand to quantify multiple analytes and the booming of miniaturized analytical systems, the development of multiplexed IA has become a dynamic area of research in the last ten years (Joos et al., 2002).

According to the strategy of distinguishing the multiple antibody-antigen (probe-target) complexes, miniaturized multiplexed assays can be divided into two categories – those based on dye labels and those based on spatial resolution. The former category of assays use microparticle-based flow cytometric technology (Bellisario et al., 2001; Carson & Vignali, 1999; Fulton et al., 1997), in which the solid phase for each analyte is a microparticle with a distinct ratio of red/orange fluorescent labels. An advantage of this type of assay is that the washing step can be eliminated, because signals from immunosorbent assays are at the surface of the microparticles. In the second category of assays, the solid phase is a planar surface spotted with an array probing molecules for each analyte. Immunosorbent assay then yields an array or matrix of signals for detection. Probe immobilization follows different approaches, usually with the aid of a microfluidic network (μFN), as will be addressed below. It should be noted that the flow cytometry technology has evolved to a relatively mature stage and most of the studies in that area have employed commercial systems. This study will focus on the microfluidic-based
spatial resolution approach for the realization of multiplexing. This method possesses some advantages over flow cytometry, such as the short assay time, simple operations, and more importantly, easy combination with the multi-sample assay, as will be demonstrated in the next chapter.

4.1.2 Microfluidic multiplexed IA based on spatial resolution: literature review

For microfluidic-based IA, the key to introducing multiplexing is to immobilize multiple probing molecules in the reaction region, so as to localize the different Ab-Ag reactions. As illustrated in Fig. 4.1 with the “H-shaped” microchannel, the assay is against one single analyte with one antigen coated over the entire µFN, as demonstrated in the last chapter. But if antigens are patterned on discrete spots as shown in Fig. 4.1 (b), multiple antibodies in a sample can be identified in one assay. To realize the multiple probe patterning, the simplest approach is to pipette dots of capture molecules onto the substrate (Erickson et al., 2004). However, in most studies on microfluidic multiplexed IA, a µFN was employed to confine the antigen solutions to achieve fine, precise and reproducible probe pattern, as reviewed below.

One of the earliest work in this area is the development of an “array immunosensor” (Rowe et al., 1999). The assay is composed of two steps: first, biotin-labeled capture antibodies are patterned onto a neutravidin-coated waveguide by using six vertical channels; subsequently, samples and detection antibodies are pumped through six horizontal channels. Localized immunoreactions at the intersections generate a 6 x 6 matrix of signals. Delamarche and co-workers (Bernard et al., 2001; Cesaro-Tadic et al., 2004a; Wolf et al., 2004) developed a “micromosaic immunoassay” using a similar two-step approach - lines of capture molecules are passively adsorbed onto a
poly(dimethylsiloxane) (PDMS) substrate by using a \(\mu\)FN fabricated in silicon; solutions to be analyzed are then delivered through a second \(\mu\)FN put across the immobilized capture molecules. Fluid flow in the 20-\(\mu\)m wide microchannels is driven by capillary forces. Whitesides and coworkers also used two-step approaches in developing a serially diluted IA (Jiang et al., 2003) and a so-called “POCKET” (portable and cost-effective) IA (Sia et al., 2004), in which lines of capture molecules are adsorbed to polycarbonate membrane and polystyrene substrate, respectively, by using PDMS microchannels. Kartalov et al. recently developed a high-throughput, multi-antigen microfluidic IA system, in which capture antibodies are covalently bond to the epoxide floor of the microchannels (Kartalov et al., 2006). Different from the two-step approach, the entire assay, from capture antibody bonding to later immunoreactions, is conducted on one complex microchip, with built-in pneumatic valves for flow control.

Most of the studies reviewed above realize a multiplexed microfluidic assay by using a two-step approach: capture molecule immobilization followed by immunoreactions, with a different \(\mu\)FN employed in each step. This approach is easy and flexible for building working prototypes.

4.1.3 Overview of this chapter

In the aforementioned studies, the reagents are delivered by either pressure-driven flow or capillary flow. This study, on the other hand, is on the realization of multiplexed IA with an EK-driven flow system. A two-step approach was employed here, which allowed a straightforward modification from the existing EK-IA described in the last chapter, that is, adding an antigen-patterning step while keeping the \(\mu\)FN design and immunoreaction part unchanged.
Adsorption continued to be employed for antigen immobilization, due to its simplicity and the satisfactory sensitivity achieved so far. However, glass substrate is not adsorptive for proteins. To solve this problem, a thin film of PDMS was spin-coated on top of the glass slide. In this way, we take advantage of both the good heat dissipation property of glass and the avidity of PDMS for proteins.

The IA configuration was the same as before, in the form of solid phase – Ag – Ab – dAb. Both *E. coli* O157:H7 and *H. pylori* were employed as model analytes, with *E. coli* O157:H7 used in most tests. The EK-IA was first applied to test multiple samples of coated antigen, then used to screen for two different antibodies in a sample.

### 4.2 Experimental

#### 4.2.1 Microchip design and preparation

A thin film of PDMS was coated onto a 75×25 mm² glass microscope slide by using spin-coating. Briefly, PDMS polymer base and curing agent were mixed at a ratio of 15:1 (w/w). One milliliter of the mixture was deposited onto a clean glass slide and degassed for one hour at −34 kPa (gauge). After that, the PDMS was spin-coated onto the glass slide into a film of ~10 µm. The PDMS film was then cured at 75°C for 2 hr.

Two different µFNs were used. One was consisted of five independent channels and used for antigen immobilization, as shown in Fig. 4.2 (a). The five channels were parallel in the center region to generate an array of antigen pattern, and radially aligned at the two ends to leave room for wells. The channels were 60-µm high and 100-µm wide, with a gap of 80~100 µm between the channels. The other µFN was the same H-shaped
network used in the Chapter 3 (30 \( \mu \)m high and 120 \( \mu \)m wide), for conducting the EK-IA. The procedures for microchannel fabrication were the same as described in Chapter 2.

4.2.2 Antigen preparation and reagents

The procedures for preparing bacterial antigens of *H. pylori* and *L. rhamnosus* were as described in Chapter 2. The protocol for preparing bacterial antigen of *E. coli* O157:H7 was as follows. *Escherichia coli* O157:H7, strain CL-56 was cultured on 5% sheep blood agar plates at 37 °C overnight, and stored at 4 °C after that. Bacteria were then cultured in static, nonaerated Penassay broth overnight at 37 °C. The rest of the procedures for culture solution centrifugation, bacterial pellets washing and lysing, and bacterial protein collection and concentration measurement were the same as those for *H. pylori* and *L. rhamnosus*. The lysate antigen of *E. coli* O157:H7 were diluted to final concentrations using the same pH 9.6 carbonate buffer as for the other two antigens.

The assay buffer was still 25 mM Tris-HCl buffer (pH 7.5) and blocking buffer was still 5% (w/v) BSA in Tris-HCl. Polyclonal goat anti-*E. coli* antibody was obtained from KPL (Gaithersburg, ML). The detection antibody for *E. coli* was TRITC-conjugated donkey anti-goat IgG purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The bacterial antibody and detection antibody for *H. pylori* were the same as used before, polyclonal rabbit anti- *H. pylori* antibody and TRITC-conjugated donkey anti-rabbit IgG, respectively. The anti-*E. coli* antibody and anti-*H. pylori* antibody were diluted to 1:25 and 1:8 with the blocking buffer solution, respectively, to a same final concentration of 40 \( \mu \)g mL\(^{-1}\). Both secondary antibodies were diluted to 1:25 with the blocking buffer to a final concentration of 60 \( \mu \)g mL\(^{-1}\).

4.2.3 Electrokinetically-controlled immunoassay
Process for the first stage of the immunoassay, antigen immobilization, was illustrated in Fig. 4.2. The PDMS slab bearing the \( \mu \text{FN} \) of parallel microchannels was plasma-treated for one minute and brought into conformal contact with a PDMS-coated glass slide, as shown in Fig. 4.2 (a). Then, 1.5 \( \mu \text{L} \) of lysate antigen (1 \( \mu \text{g mL}^{-1} \) to 30 \( \mu \text{g mL}^{-1} \)) was added to one well of each channel. After the channels were wetted spontaneously, the same amount of lysate antigen was added to the wells at the other end. The wells were then covered with a piece of micro cover glass to prevent the evaporation of solutions.

After 15 minutes of incubation at room temperature for antigen-coating, the micro cover glass was removed and the microchip put under a deep vacuum (-20 inchHg) for 6 minutes to quickly evaporate the solutions. The position of the \( \mu \text{FN} \) was marked from the reverse side of the glass slide and the PDMS slab was peeled from the substrate. The substrate was then rinsed with 1 mL assay buffer and 3 mL double distilled water, successively, to remove any unbound antigens deposited on the surface. A monolayer of antigen was patterned on the substrate at this stage, as illustrated in Fig. 4.2 (b).

The PDMS slab bearing the H-shaped \( \mu \text{FN} \) was plasma-treated for one minute and put into conformal contact with the antigen-patterned substrate, with the left arm of the “H” crossing the antigen pattern perpendicularly, as shown in Fig. 4.2 (c). Special care was taken during this process to keep a constant relative position between the H-shaped \( \mu \text{FN} \) and the antigen pattern each time. The intersections of the parallel lines of antigen pattern and the H-shaped \( \mu \text{FN} \), in the shape of an array of squares as shown in the enlargement in Fig. 4.2 (c), became the reaction regions in the EK-IA experiments, when antibody molecules were delivered through the channel.
Once the microchip was assembled, blocking buffer, 5% BSA in 25mM Tris-HCl, was delivered into the \( \mu \)FN and incubated for 10 minutes. After the blocking stage, each well was filled with 12 \( \mu \)L of the corresponding reagent solution as indicated in Fig. 4.1 except that the waste well was filled with Tris-HCl buffer.

The EK-IA was conducted in the same way as described in Chapter 3, with the steps and controlling parameters all unchanged.

4.2.4 Detection and image analysis

The equipments for fluorescent signal detection were the same as used in the last two chapters. Fluorescent images were captured at an exposure time of 5 s unless indicated otherwise. The array of square-shaped reaction regions was captured in one frame.

Fluorescent intensities from both the square signal regions and their neighboring background regions were quantified by using Openlab 3.1.5 imaging software. Then, the signal for each reaction region was determined as the difference between its own intensity and the average intensity of its two neighboring regions, to reduce the influence of background fluorescence.

4.3 Results and discussion

4.3.1 Composition of channel surfaces and measurement of electroosmotic mobility

In the microchip assembly described in the last two chapters, both the glass slide and the PDMS slab bearing the channel pattern were subjected to plasma treatment to introduce silanol groups (Si-OH) on both surfaces, so that they can bond with each other
covalently and irreversibly. However, in this study, the bottom substrate was patterned with antigen for which plasma treatment would be destructive. Therefore, only the top PDMS slab was plasma-treated and the sealing between the two pieces was reversible. This assembly proved to work well in the EK-IA, as the device was not subjected to external pressures. Plasma treatment hydrophilized the top PDMS surface, so that after the chip was formed, blocking solutions could be driven by capillary forces to fill the network. BSA molecules in the blocking buffer adsorbed to both the top and the bottom PDMS surface, imparting hydrophilicity the bottom PDMS surface (Wolf et al., 2004).

The surface composition of the microchannels used here was different from that used earlier. As illustrated in Fig. 4.3, the microchannel used in Chapter 2 and 3 was composed of antigen/BSA coated PDMS walls and a wall of glass. The glass surface should be mostly bare since it is not adsorptive for proteins. Whereas in this study, the microchannel was composed of plasma-treated PDMS and native PDMS walls, all coated with BSA (except for the reaction region where the bottom surface was coated with antigen/BSA). Since EOF is directly dependent on the properties of the solid surface, the electroosmotic mobility was re-measured in this study.

The electroosmotic mobility was measured using the same current-monitoring method as described in Chapter 2, with a straight microchannel (w = 120 µm, h = 30 µm). The straight-channel microchip was made following the fabrication protocol in this chapter and was subjected to BSA coating before mobility measurement. The electroosmotic mobility of the 25 mM Tris-HCl buffer was measured to be $1.6 \times 10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$, a bit higher than that in Chapter 2, $1.42 \times 10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$. The increase is probably due to the deeper plasma-hydrophilization of the top PDMS slab (1 min Vs. 10 s), which
introduced more silanol groups on the surface. As there was no significant change in the
mobility and thus the flow velocity, the durations of the loading and washing steps were
kept unchanged from previous settings. The EK-IA was still conducted using the
parameters in Table 3.1.

4.3.2 Parallel immunoassay for antigen

Aside from detecting multiple antibodies from one sample as discussed earlier,
this EK-IA can also be applied to test multiple antigen samples in parallel. The IA for
antigen was conducted first, the result of which would help determining the optimal
antigen concentration to use in later assay for antibody.

An important consideration in performing the parallel antigen assay is whether the
reaction from each site can be evaluated independently. This concern was raised because
antibodies were delivered to the array of reaction sites in a sequential order, causing
slight variations in the reaction kinetics on different sites. To check if this affected the
parallel assay for antigen, an EK-IA test was conducted with the same concentration of E.
coli O157:H7 lysate antigen, 10 µg mL⁻¹, applied to four coating channels. The
fluorescent image obtained is shown in Fig. 4.4. The exposure time for image capture
was reduced to 3 s, to avoid signal saturation. From the image, fluorescent intensities at
the four sites were clearly at comparable levels. Variation coefficient of quantified
intensities was only 14.5%. This result proved that the immunoreaction occurred almost
to the same extent on different sites. Therefore, the array of reaction sites could be
regarded as independent reactors. This conclusion will be further evidenced by the
theoretical studies in Chapter 8.
The dynamic range of the EK-IA in detecting *E. coli* antigen was then tested. Different concentrations of *E. coli* lysate antigen, in the range of 1~20 µg mL⁻¹, were applied to the five coating channels. The result is shown in Fig. 4.5, in which (b) is a plot of quantified data fitted with a sigmoidal function. *E. coli* O157:H7 antigen was reliably detected in the concentration range of 3~10 µg mL⁻¹. The detection limit of 3 µg mL⁻¹ is comparable to the 4 µg mL⁻¹ obtained with both the conventional dot-blot immunoassay and a hand-held immunosensor developed earlier (Lin et al., 2004b). But the efficiency of the IA was greatly enhanced here, in that with the multiple-antigen coating ability of the microchip, the dose-response curve was obtained from a single experiment.

It is noteworthy that the dynamic range obtained was highly dependent on the duration of antigen coating. When the EK-IA was conducted at a coating time of 30 min, the intensity peaked at 5 µg mL⁻¹ with a value of ~2100, while the intensities for 10 and 20 µg mL⁻¹ were only ~1000. These results implied that antigen coating was a rapid process which easily led to too dense an antigen monolayer. The dense layer of antigen was detrimental for the Ab-Ag interaction, most probably due to steric effects. This phenomenon of the strong dependence of the assay performance on coating time will be demonstrated with more data in the next chapter.

The rapid coating process, together with the narrow dynamic range of only one order of magnitude (two orders of magnitude was obtained in Chapter 2), suggested that the protein adsorption properties of native PDMS was distinct from that of the hydrophilized PDMS used in Chapter 2 and 3. With the hydrophilized PDMS, the antigen-coating process was observed to be far less sensitive to time than with the native PDMS. From the experimental results in Chapter 2, antigen coating on the hydrophilized
PDMS for 2 hr seemed to reach a stable stage with proper antigen density for binding reaction. Recollect the demonstration in Section 2.3.1 that plasma-hydrophilization impaired the protein-adsorption ability of PDMS, the results here suggested that this sacrifice in hydrophobicity could be advantageous. The plasma-treatment process might be optimized to modify the high avidity of PDMS for proteins, to achieve a proper density of antigen monolayer at the equilibrium stage of antigen adsorption, though the adsorption rate would be slower.

**4.3.3 Multiplexed immunoassay for antibody**

The EK-IA was then tested as a multiplexed assay in the simultaneous detection of both anti-*H. pylori* antibody and anti-*E. coli* antibody from one sample. Antigen coating was applied as illustrated in Fig. 4.6 (a). In addition to the antigens of *H. pylori* (30 µg mL⁻¹) and *E. coli* O157:H7 (10 µg mL⁻¹), *L. rhamnosus* antigen (10 µg mL⁻¹) was also used as a negative control. Immunoassay experiments were carried out under the conditions when the primary antibody solution contained only anti-*E. coli* antibody, or only anti-*H. pylori* antibody, or both anti-*E. coli* O157:H7 and anti-*H. pylori* antibody. The secondary antibody solution contained the detection antibodies both analytes, TRITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-goat IgG, in all runs.

Fluorescent images and intensity profiles are shown in Fig. 4.6 (b)-(g). Whenever a certain primary antibody was present, the corresponding antigen-coating site gave a detectable fluorescent signal. By comparing the profiles in Fig. 4.6 (c) and (e) accordingly with Fig. 4.6 (g), the signal intensities were consistent, whether a certain primary antibody existed alone or in an antibody mixture, thus proving the feasibility of
multiplexed assay with the EK-IA. The negative control site coated with *L. rhamnosus* antigen did not produce a visible signal in any of the runs.

However, from Fig. 4.6 (d) and (e), the *E. coli*-coating site yielded a very weak signal when only anti-*H. pylori* antibody was present, indicating that a slight cross-reactivity existed between the *E. coli* antigen and polyclonal anti-*H. pylori* antibody. This result suggests that the multiplexed EK-IA was a fast approach to discover the cross-reactivity of a substance with different ligands. On the other hand, the result also implies that multiplexed screening of real samples is only applicable when there is no cross-reaction between the different antibodies and antigens. Slight cross-reactivity between the non-specific reactants is sometimes acceptable when there is no high demand on the sensitivity of the assay. However, if low- and high- abundance proteins are measured in parallel, even such weak cross-reactions may cause false conclusions (Dupuy et al., 2005).

### 4.4 Summary

Based on the work of last two chapters, this chapter describes the further improvement of the EK-IA lab-on-a-chip to realize multiple-analyte detection. This was done by adding a separate antigen-coating step, during which lines of different antigens were patterned to a PDMS-coated glass slide by using a µFN of independent straight channels. The microchip was assembled from the antigen-patterned substrate and the same H-shaped µFN used in the last chapter. The left arm of the “H” crossed the lines of antigen pattern perpendicularly to form an array of square reaction regions.

With antigen-coating protocol being 15 min at room temperature, the dynamic range of the EK-IA for detecting *E. coli* O157:H7 antigen was 3-10 µg mL⁻¹.
With different species of probing antigen, multiplexed assays were successfully conducted to simultaneously detect both anti-\textit{E. coli} O157:H7 and anti-\textit{H. pylori} antibodies. The results implied that a prerequisite for performing multiplexed assay of real samples is the absence of cross-reactivity between the different antibodies and antigens.
Figure 4.1 Comparison of single-analyte and multi-analyte microfluidic immunoassay. (a) A single-analyte IA. Only one species of antigen is immobilized in the region reaction, as was the case of the EK-IA presented in Chapter 3. (b) A multi-analyte IA. Several different species of antigen are patterned on discrete sites in the reaction region, as will be investigated in this chapter.
Figure 4.2 The preparation process of the multiplexed EK-IA (schematics are not proportional to actual size). (a) Antigen immobilization using a $\mu$FN with independent channels; (b) Antigen molecules patterned on the substrate after 15-minute incubation; (c) H-shaped $\mu$FN put across the antigen pattern. The intersections of the parallel regions of antigen coating and the H-shaped $\mu$FN became reaction regions in the EK-IA.
Figure 4.3 Illustration of the surface composition of the microchannels (cross-section) (a)

The microchannel used in Chapters 2 and 3. (b) The microchannel used here.
Figure 4.4 Evaluating the performance of the EK-IA in detecting multiple antigen samples in parallel. Concentration of *E. coli* O157:H7 lysate antigen for coating was 10 \( \mu g \, mL^{-1} \) for all sites. Concentrations of primary antibody and secondary antibody were 40 and 60 \( \mu g \, mL^{-1} \), respectively.
Figure 4.5 Dependence of fluorescent signal intensity on the concentration of antigen. (a) Fluorescent image of one immunoassay test. Concentrations of coating *E. coli* lysate antigen were as indicated below each reaction site, with the unit being $\mu$g mL$^{-1}$. (b) The quantified intensity-concentration curve. Data were from four independent experimental runs. Error bars represent the standard error of the mean (SEM).
Figure 4.6 Demonstration of the multi-analyte screening ability of the EK-IA. (a) Three reaction sites were coated with different species of antigen (from left to right): *H. pylori* (30 µg mL⁻¹), *L. rhamnosus* (10 µg mL⁻¹), *E. coli* O157:H7 (10 µg mL⁻¹). (b), (d) and (f) are fluorescent images from the assay. (c), (e) and (g) are corresponding fluorescent intensity profiles obtained using MATLAB. (b) and (c) - the primary antibody solution contained only anti-*E. coli* antibody; (d) and (e) - the primary antibody solution contained only anti-*H. pylori* antibody; (f) and (g) - the primary antibody solution contained both anti-*H. pylori* and anti-*E. coli* antibodies. The secondary antibody solution contained both TRITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-goat IgG in all runs.
Chapter 5 Improvement of the EK-IA: parallel assay*

5.1 Introduction

The last chapter demonstrates how the EK-IA can be adapted to achieve the important function of multi-analyte screening. However, so far, only one antibody sample can be tested at a time. This chapter will then focus on further improvement of the EK-IA to realize a multi-sample (parallel) IA prototype.

The parallel analysis of multiple samples is apparently highly desirable, provided that samples for immunoassay are usually in large quantities – tens or even hundreds, in both biochemical studies and clinical environments. Simultaneous testing of many samples can substantially reduce analysis time. Conventional IA is usually conducted in a parallel manner. Take the assay on a 96-well microtitre plate as an example, after sample or reagent solutions are added to each well, incubation, the most time-consuming operation, is simultaneously applied to all the wells on the plate.

Unlike conventional IA, microfluidic IAs are usually not static, but based on a flow system. Conducting such assays in a high-throughput, parallel manner then means driving the flow in multiple reaction chambers simultaneously. All of the multiplexed microfluidic IAs reviewed in Section 4.1.2 are, at the same time, multi-sample IAs. The “reaction chambers” in those studies are discrete probe-coated sites in a row of independent channels. The flows in such channels are driven by either pressure difference (Linder et al., 2002; Rowe et al., 1999; Sapsford et al., 2002; Sia et al., 2004), or by capillary forces (Bernard et al., 2001; Cesaro-Tadic et al., 2004a; Wolf et al., 2004).

Aside from the above-mentioned assays that test both multiple samples and multiple analytes simultaneously, multi-sample IA has also been explored in other studies. Lai et al. designed a compact disk-like microfluidic device for ELISA, with 24 independent radial assay units symmetrically arrayed on one circular disk (Lai et al., 2004). The multiple assays were driven simultaneously by centrifuge forces when the disk rotated. Sato et al. constructed a sandwich IA microchip with branching channels to process four samples simultaneously with only one microsyringe pump (Sato et al., 2002). Bromberg et al. developed a high-throughput homogeneous IA in which electrophoretic separation of 48 homogeneous IA products was conducted on a half-circular microchip (Bromberg & Mathies, 2004). In the latter two works, parallel analysis is realized not only in a sense that multiple IAs are simultaneously driven, but the multiple assay units are also partially integrated. In the study of Sato et al., the four assays shared one antibody inlet. In the study of Bromberg et al., the 48 assays shared one common cathode well and adjacent pairs of assays were grouped into doublets that shared common anode and waste wells. Such integration reduced the numbers of channels and wells, thus making the microfluidic network (µFN) more compact and simplifying the operations.

Heterogeneous microfluidic IA has not been investigated with an EOF system in a parallel format. In this study, such a lab-on-a-chip prototype was built. Compared with most other parallel microfluidic IAs, the advantages with this high-throughput EK-IA are not only that the assay is automated, but also, the µFN is integrated, which brings about reduced consumption of reagents, minimized manual work, as well as small footprint of the device. The chip design process will be illustrated in detail. The new EK-IA was applied to the testing of both *E. coli* O157:H7 and *H. pylori* antibody samples.
5.2 Experimental

5.2.1 Chip preparation

The assay was conducted using a two-step approach as described in the last chapter. Namely, antigen was first patterned on a substrate, followed by the assembly of the microchip. The \( \mu \)FN used for antigen patterning was the same as used in the last chapter, which consisted of five independent channels (100-\( \mu \)m wide, 60-\( \mu \)m high). The other \( \mu \)FN used directly for the EK-IA was composed of ten channels each connected with a sample well (S), one channel connected with detection antibody well (A), one with buffer well (B) and one with waste solution well (W), as shown in Fig. 5.1 (b). The parallel part of the ten sample channels was designed as the reaction region. The width of the sample channels was 50 \( \mu \)m each, while other channels in this network were all 500 \( \mu \)m wide. To minimize pressure effects, the height of the \( \mu \)FN was only 8 \( \mu \)m. The overall dimension of the \( \mu \)FN was 16 \( \times \) 24 mm\(^2\). The microchannels were fabricated by using soft lithography, as described in Chapter 2.

5.2.2 Antigen immobilization

The antigen, antibody and blocking reagents used were the same as described in the previous chapters. Antigen immobilization procedures were mostly the same as that used in Chapter 4. Briefly, the PDMS replica for antigen patterning was plasma-retreated and brought into conformal contact with a PDMS-coated glass microscope slide, as shown in Fig. 5.1 (a). Immediately thereafter, 0.5 \( \mu \)L of antigen solution was added to one end of each channel and the channels were wetted spontaneously. The chip was then covered with a piece of micro cover glass and incubated at room temperature (22~25 \(^\circ\)C) for 15 minutes. After incubation, the chip was placed under a deep vacuum (-20 inchHg)
to evaporate the solvent. Then, the PDMS slab was peeled off, and the substrate was rinsed with water and dried under a gentle blow of nitrogen. A monolayer of antigen was patterned onto the surface at this stage.

The PDMS replica for the EK-IA was plasma-treated and put into conformal contact with this antigen-patterned substrate, with the parallel part of the sample channels crossing the lines of antigen pattern perpendicularly, as shown in Fig. 5.1 (b). The matrix of rectangular intersections became reaction regions when the EK-IA was conducted, as illustrated in the enlargement. After the chip was formed, blocking solution was loaded in and incubated for 15 min at room temperature.

5.2.3 Electrokinetically-controlled immunoassay

Sample solutions used in the experiments were either *E. coli* O157:H7 antibody or *H. pylori* antibody, diluted in 5% BSA-containing Tris-HCl buffer. Detection antibodies were also diluted using 5% BSA solution.

After the blocking step, 8 µL of each sample, 30 µL of detection antibody, 30 µL of diluent buffer, and 50 µL of 25 mM Tris-HCl buffer were added to the wells for sample, antibody, buffer and waste, respectively, which had been punched at different sizes (φₜₕ = 2.5 mm, φₜₐ = φₜₜ = 4.8 mm, φₜₜ₃ = 6.3 mm). Using a big waste well helped reduce pressure effects. Platinum electrodes were then placed into the wells and the assay ready to run. A picture of the chip at this stage is presented in Fig. 5.1 (c).

The EK-IA was conducted at room temperature by using a HVS 448 high-voltage sequencer. The process was composed of seven steps – loading, incubation, and two steps of washing of sample solution, followed by loading, incubation and washing of detection antibody. Incubation time for sample and antibody was 10 min and 5 min, respectively.
The flow paths of solutions in each step were designed as shown in Fig. 5.2. Flow routes during incubations were kept unchanged from the preceding loading step. In addition to the major functional flow to deliver the reactant, sometimes a minor auxiliary flow was added. For example, in Fig. 5.2 (b) and Fig. 5.2 (d), a minor flow towards the waste well countered possible pressure-driven flow from the waste well to the reaction region. Also, in Fig. 5.2 (c), minor flows from the sample wells and buffer well served to confine the fluid towards the waste well only.

Based on the flow routes, the applied electric potentials in each step were determined using the same approach as before, i.e., estimation according to Kirchhoff’s rules, followed by adjustments through numerical simulation of the transportation processes. Flow visualization was also employed for verification and necessary fine-tuning.

The physical modeling and numerical method were the same as described in Section 3.3.1. The optimization process by using numerical simulation will be illustrated in detail below.

### 5.2.4 Signal acquisition

Fluorescent signals were detected by using a Leica DM-LB fluorescence microscope. Images were captured by using a Retiga 12-bit cooled CCD camera at an exposure time of 2 s.

Uneven illumination was compensated for by applying shading correction to captured images. This was performed by standardizing pixel intensities against those from a reference image of a homogeneous fluorescent area, according to

\[
D(x,y) = S(x,y) \times \frac{\bar{R}}{R(x,y)}
\]  

(5.1)
where $D$ is the destination image and $S$ the source image, $R$ the reference image and $ar{R}$ the mean grayscale of the reference image.

Fluorescent intensities were analyzed using ImageJ software (NIH, Bethesda, MD). Mean intensities from both the rectangular reaction regions and neighboring background regions were obtained. Signal intensity of each reaction site was then determined as the difference between its own intensity and the average intensity of two neighboring dark regions.

5.3 Results and discussion

5.3.1 Assay design

In Chapter 3, the rules in designing an EK-IA lab-on-a-chip have been described and the process of obtaining the electrokinetic controlling parameters briefly explained. The design and control of an integrated multi-sample IA is a more challenging task, and also a good example for illustrating the assay design. The designing process is depicted in Fig. 5.3 using a flow chart. The procedures will be described step by step as follows.

5.3.1.1 Design of $\mu$FN and flow paths

The first two steps are the design of the $\mu$FN and the flow paths. Unlike that in many other parallel microfluidic immunoassays where the parallel assay units are independent from one another (Cesaro-Tadie et al., 2004a; Lai et al., 2004; Rowe et al., 1999), the $\mu$FN in the parallel EK-IA is a highly integrated one, with one well for detection antibody, one well for buffer and one well for waste solution shared among all the samples. With this compact microdevice, the consumption of reagent solution is significantly reduced and manual solution operations in preparing the assay are
minimized. Designing the integrated parallel assay chip, consequently, involved special considerations.

An important consideration is that different samples should not be cross-contaminated, so as to make sure that the reaction within each channel is from the corresponding sample solution only. This rule is embodied in the following design elements: First, the reaction region was upstream of the intersection of the sample channels during sample loading and incubation, so that each sample solution passed the reaction site independently before mixing with others. Second, the ten samples channels intersected with the vertical channel independently. If the sample channels were united first and intersected with the vertical channel, as shown in Fig. 5.4, mixed sample solutions that enter the united region would be flushed back to the reaction regions in sample-washing step and contaminate the reaction in each channel.

The ten sample channels were each 50 \( \mu m \) wide, so that when connected in parallel they had an equal flow resistance per unit length with the other channels (500 \( \mu m \) wide). This assured an equal flow rate at any section of the \( \mu FN \).

It should also be noted that the flow paths shown in Fig 5.2 were final ones obtained after the design cycle. At the initial design, the auxiliary flows, as well as the second sample-washing step, were not included. They were added during chip optimization, as will be shown below.

5.3.1.2 Estimation of the applied electric potentials

After the \( \mu FN \) and flow route were designed, the next step was to derive the applied electric potentials using Kirchhoff’s rules. The microchannels filled with conductive fluid were viewed as electrical resistors and the \( \mu FN \), a circuit. The circuit
equivalent to the \( \mu \)FN used in the EK-IA is presented in Fig. 5.5, where \( R \) denotes the relative electric resistance of each channel. Because all the channels have the same width, including the 10 sample channels connected in parallel, and that the channels were filled with the same buffer solution, the electric resistance is directly proportional to the channel length. With this circuit and targeted electric field strength of \( \sim 150 \text{ V cm}^{-1} \), the applied electric potential at each port can be derived by using Ohm’s law and Kirchhoff’s rules, according to designed flow routes. For example, for the sample-loading step, when the waste well is grounded, the electric potential applied at the sample wells is calculated as \( V_s = V_w + \Delta V = 0 + 150 \text{ V cm}^{-1} \times (1.4 + 0.8) \text{ cm} = 330 \text{ V} \). Since the flow is directed towards the waste well only and there is no flow in the antibody and buffer channels, the electric potential applied at the antibody and buffer well can be obtained by \( V_A = V_B = V_J = 150 \text{ V cm}^{-1} \times 0.8 \text{ cm} = 120 \text{ V} \), where \( V_J \) is the potential at the junction. The applied electric potentials for other loading and washing steps were obtained in the same way and are listed in the upper half of Table 5.1.

During incubations, electric field was reduced to half of that for the preceding loading step. For the antibody-washing step, the potentials were only roughly estimated, since the flow does not need to be valved or precisely partitioned at the junction. The duration for sample incubation was set to be 10 min, as preliminary experiments showed the binding reaction proceeded furthered and thus the LOD was lower under this condition, compared to that of 5-min sample incubation. The durations of other steps were to be determined via numerical simulation.

5.3.1.3 Numerical simulation and assay optimization
With the applied electric potentials obtained above, the microfluidic transport during the IA could be numerically simulated step by step. In the computation, the value of $\mu_{eo}$ and $\mu_{ep}$ were $1.6 \times 10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$ and 0, respectively, which were measured as described in Chapter 2 and 4. The diffusion coefficient of antibody was $4.0 \times 10^{-7}$ cm$^2$ s$^{-1}$. In the computation of concentration field, the initial condition was zero everywhere for the first step. Then, for later steps, the initial condition was the concentration field at the end of the previous step.

For each step, the simulated flow field and the reactant concentration field were checked to see if the operation was effectively realized. Several controlling conditions for checking include: sufficient reagent loading/washing, effective flow valving at the junction and no cross-contaminations among reagents, as well as equal reaction conditions in each channel. For a multi-sample IA, it is important that the reaction conditions in different reaction chambers are equally controlled so that the multiple assays are comparable. Controlling parameters for an IA normally include the reagent concentration, incubation time, as well as the velocity of reagent transport. If any relevant controlling condition was not met, it was necessary to go back to adjust the design. This adjustment can be made at three levels, the applied electric potentials, the flow route, or the chip design, as shown in Fig. 5.3. Examples of each of the adjustments are presented below.

1) Adjusting the applied electric potentials

Adjusting the applied electric potentials by trial-and-error is the most direct and frequently applied approach of optimization. The antibody-loading step will be illustrated
below as an example, because this is associated with the important checking condition of equalizing the reaction conditions in the multiple channels.

Figure 5.6 (a) demonstrates the antibody concentration field at the junction region 40 s after antibody-loading started, computed using the parameters estimated from Kirchhoff’s rules \((\Phi_s = 0, \Phi_A = 435 \text{ V}, \Phi_B = 210 \text{ V}, \Phi_w = 210 \text{ V})\). Clearly, antibody concentration in the ten channels gradually decreased from top to bottom. Taking the inflow concentration as unity, antibody concentration was 0.9–0.8 in the top seven channels, then 0.77, 0.65 and 0.37 in the bottom three channels. The lower concentration in the bottom channels is attributed to the mixing of antibody with a slight flow from the buffer well, as well as reduced concentration at flow front due to diffusion. The bottom channel was at an especially disadvantageous position. The concentration at the reaction region (~3 mm right to the junction region) in this channel was 0.81 even after 120 s of loading, while the concentration in the top channel reached 0.95 in only 58 s. This unequal reagent concentration called for adjustments in the flow field.

The situation was relieved by reducing the potential at the buffer port to allow a slight flow to the buffer well, so as to eliminate the antibody dilution by incoming buffer flow. The upgraded antibody concentration field after 40-s loading is presented in Fig. 5.6 (b), computed using the electric potentials from trial-and-error optimization \((\Phi_s = 0, \Phi_A = 435 \text{ V}, \Phi_B = 182 \text{ V}, \Phi_w = 195 \text{ V})\). The concentration in the bottom three channels now increased to 0.84, 0.79 and 0.66, respectively. Due to the dispersion of antibody during loading, the concentration difference among the channels cannot be completely inhibited. Though further decreasing \(\Phi_B\) would improve the situation here, simulation results showed antibody would then approach the buffer well during the 5-min incubation,
inducing the risk of contaminating the buffer solution. Now, the concentration at the reaction region in the bottom channel reached 0.95 in 82 s, only 26 s later than that in the first channel (56 s). Considering that the concentration of antibody was high, the 26-s difference was negligible, for a total incubation time of more than 300 s.

Once the applied electric field was finalized, the duration of the step can be determined from the simulation of the mass transport process. Theoretically, a loading/washing operation was completed when the reactant was delivered into/out of the reaction region. In this case of antibody loading, the operation was considered completed at 82 s, when antibody concentration reached 0.95 in the bottom channel. Nevertheless, the durations were extended to 120 s in practice, to ensure complete solution delivery.

2) Modifying flow route design

Sometimes it was necessary to modify the flow route design, such as adding auxiliary flows or an additional step. The example shown below is on the introduction of the second sample-washing step.

As illustrated by the results from numerical simulation in Fig. 5.7 (a) and (b), during the first sample-washing step, the mixed sample solution accumulated at the intersection during sample loading and incubation was inevitably pushed into the antibody channel. If the antibody-loading step followed immediately afterwards, this mixed sample solution would re-enter the multiple reaction regions and contaminate the reaction. To prevent this contamination, a second sample-washing step was added. As shown in Fig. 5.7 (c), the mixed sample solution was flushed into the waste well before antibody loading.

3) Modifying the design of microfluidic network
Modification at the \( \mu \text{FN} \) level is necessitated when adjustments on the applied electric field and/or flow route design have reached their limits while the fluidic control is still not satisfactory. The modification of \( \mu \text{FN} \) design then provides a thorough solution. The power of numerical simulation is also further demonstrated: it can be exploited not only to obtain applied electrical fields, but also to optimize the microchip design.

The operation of antibody loading, discussed earlier on electric field optimization, will be employed again here. If after correcting the electric field, the result was still not acceptable, i.e., the 26-s difference in incubation time considered too big, then the \( \mu \text{FN} \) should be modified. Three solutions to this problem are briefly listed below.

A straightforward approach is to extend the length of the buffer channel, so that antibodies can travel further into the buffer channel during incubation, without contaminating the buffer solution. The adjustment will allow lower potential at the buffer well and thus more uniform concentrations among the multiple sample channels.

Another solution is to apply a higher flow velocity at the bottom channels by reducing their lengths, to partially compensate for their weaker reaction kinetics of shorter incubation time/lower reactant concentration. Higher velocity improves the reaction kinetics by transporting more reactant molecules to the reaction site per unit time. The influence of flow velocity on reaction kinetics will be illustrated in detail in Chapter 8. Here, by reducing the length of the bottom channel by 0.5 mm or 1 mm (from 14 mm to 13.5 or 13 mm), the flow velocity increases from 225.9 \( \mu \text{m s}^{-1} \) to 234.2 or 243.2 \( \mu \text{m s}^{-1} \), respectively, which is equal to or 4.0 \% higher than the velocity in the top channel. The lengths of other lower channels can be tuned similarly.
The third approach is to restructure the μFN by switching the position of antibody channel and waste channel. Comparing with dispensing from one side of the multiple channels, dispensing antibody solution from the middle of the channels reduces the concentration gradient among the channels. This is because the surface of sample channels inlets (here in the vertical direction) better matches the concentration contour. As demonstrated in Fig. 5.8, in the original design, the concentration contour lines are almost perpendicular to the face of sample inlets, while for the new design, the contour line and the inlet line are close to parallel in the middle region and diverge from each other at the sides. The incubation time, therefore, is still shorter for the side channels. From simulation, antibody concentration reached 0.95 at the reaction region in the top and bottom channels 10 s later than in the middle channels, which is much reduced from the 26-s difference with the original design.

Illustrated above are just some basic strategies for μFN modification. When being applied, the approaches can still be fine-tuned to yield better result. For example, for the third approach, if the face of channel inlets is designed to better parallel the concentration contour, the concentration gradient among the channels could be further reduced.

From the optimization process demonstrated above, evenly distributing solution from one reservoir to different channels is a major challenge with parallel assay on an integrated μFN. Similar difficulties have also been reported for a pressure-driven IA (Sato et al., 2002). But with numerical simulation as a design tool, the difficulties can be predicted and minimized.

The μFN design modified using the third approach, with the antibody channel laid horizontally, yields more equal reaction conditions in the multiple channels. It can thus
be used for any continuing studies on this subject. But in the experimental studies presented below, the µFN used was still the original one, with the bottom channel ~0.5 mm shorter than others, as a compensation for its weaker reaction kinetics. The optimized controlling parameters used in the experiments are listed in the lower half of Table 5.1.

5.3.2 Optimization of antigen-coating condition

Optimal performance of an immunoassay depends primarily on an appropriate surface density of capture molecules. Therefore, the condition for antigen coating was optimized at the first step. This was done by fixing the antigen-coating duration at 15 min and seeking the optimal antigen concentration. EK-IA experiments were conducted with varied antigen concentrations applied in the five coating channels. Three different concentrations of antibodies, each in a duplicate of three, plus one negative control (diluent buffer), were used as the ten samples.

Test results are presented in Fig. 5.9, in which (a) and (b) are the fluorescent image and quantified data plot for *E. coli* O157:H7, and (c) and (d) are the results for *H. pylori*. The curves show a similar trend for both bacterial pathogens – signal intensity came to a peak value at a certain antigen concentration, meaning that the optimal surface density of antigen was reached. At higher antigen concentration, the signal intensity dropped. This trend will be discussed more below. The optimal antigen concentration corresponded to the peak intensity was 30 µg mL\(^{-1}\) and 200 µg mL\(^{-1}\) for *E. coli* O157:H7 and *H. pylori*, respectively. These conditions were used for the rest of the study.

To examine if the immunoreactions in different channels were comparable with each other, data from each of the three duplicates were statistically examined: variation coefficients were ~13% for stronger signals (intensity >5) and ~25% for weaker signals
(intensity <5). One source of the variation was the heterogeneity in the fluorescent signals, as was especially clear in the middle column of signals in Fig. 5.9 (a), which suggested heterogeneity in antigen coating, a limitation of the antigen-immobilization protocol. Other possible contributors to the variations included uncompensated non-uniform illumination, as well as variations in reaction conditions among the channels. Nevertheless, for the purposes of this proof-of-concept study, the precision was considered adequate.

The optimal antigen concentration of 30 µg mL⁻¹ for *E. coli* O157:H7 and 200 µg mL⁻¹ for *H. pylori* was valid only under the current coating protocol with a coating time of 15 min at room temperature. The optimal concentration range shifted from 100-500 µg mL⁻¹, as shown in Fig. 5.9 (b), to 50-200 µg mL⁻¹, as shown in Fig. 5.10, when coating time was extended from 15 min to 20 min. This result confirmed the suggestion in Section 4.3.2 that the optimal surface density was an unstable stage during the continuous adsorption of antigen to native PDMS.

Antigen adsorption from solution to the channel wall is a diffusion-controlled process. Therefore, according to Fick’s second law, $J = -D \nabla C$ (where $J$ is the diffusion flux, $D$ the diffusion coefficient and $C$ the concentration), the higher the antigen concentration, the faster the diffusion, the sooner the optimal surface density was reached, as suggested by Fig. 5.9 and Fig. 5.10. Continuing adsorption of antigen afterwards impaired the Ag-Ab binding reaction more and more seriously, possibly due to steric effects (Butler, 1996), desorption of antigen packed in multilayers (Crowther, 1995) or denature of adsorbed antigen (Butler, 1996).
As concluded in Chapter 4, the adsorption of antigen on native PDMS is rapid, but not stable, which affects the reproducibility of EK-IA experiment. This limitation of the current IA can be improved by using low concentrations of antigen for which the equilibrium of adsorption corresponds to a proper surface density, or using hydrophilized PDMS as proposed in Chapter 4, or employing non-adsorptive immobilization methods.

5.3.3 Parallel immunoassay for antibody

EK-IA experiments were then conducted to test the dynamic range of the bacterial antibodies. Five coating channels were all used for duplicate assays. The concentration ranges of sample solutions were 0.02-10 µg mL⁻¹ for *E. coli* O157:H7 antibody and 0.1-50 µg mL⁻¹ for *H. pylori* antibody. Using the parallel IA, the range of concentrations was tested in a single assay. The results are shown in Fig. 5.11, in which (b) and (d) are quantified data fitted with sigmoidal curves. From these curves, concentration-response dependence covered the full range of tested concentrations for both analytes, with lower LOD determined as the concentration yielding an average intensity above mean ± 3SD of the negative control. Shapes of the curves suggested that the dynamic ranges could be still broader, from both the lower and higher limits of testing. Nevertheless, the demonstrated dynamic range with 2-3 orders of magnitude is considered wide enough, because it is typical for most ELISA in practical use (Kemeny, 1991). The LOD of 0.02 µg mL⁻¹ (130 pM) and 0.1 µg mL⁻¹ (670 pM) for *E. coli* O157:H7 and *H. pylori* antibodies are close to the LOD of fluorescence immunoassays (~100 pM) (Edwards, 1985). Table 5.2 shows the LOD from other reported microfluidic heterogeneous immunoassays for antibody detection. Clearly, the LODs achieved in this study are at a comparable or better level as compared to others, owing to the optimized experimental
conditions. Especially, considering that the capture antigens employed in this study were whole cell bacterial lysates, without purification, the LODs reached were quite satisfactory.

Data from the five duplicates were statistically examined: variation coefficients were ~18% for stronger signals (intensity >20, i.e., $C \geq 1 \mu g mL^{-1}$ for *E. coli* O157:H7 antibody and $C \geq 5 \mu g mL^{-1}$ for *H. pylori* antibody), and ~33% for weaker signals (intensity <20). For the stronger or weaker signals, the major cause of signal variation was different. To illustrate this point, profiles of signal intensities from the five reaction sites were plotted in Fig. 5.12, using quantified data of Fig. 5.11 (a). Data from seven out of the nine concentrations (that is, the highest three and lowest four) are presented.

The three data curves of high analyte concentrations revealed different extents of reactions on the five sites, with the reaction on the middle site being the strongest. This variation is attributed mainly to variability of antigen coating and to uncompensated inhomogeneous illumination. This type of error can be partially compensated for by fixing one horizontal channel as a reference and normalizing the intensity of each signal against the signal intensities in the reference channel (Rowe et al., 1999).

While the effect of coating and illumination variation remained as analyte concentration decreased, in the four low-concentration curves, an overall intensity decline along the flow direction of sample solution was apparent, which became a major contributor to the large variation coefficients. This decline occurred as a result of different reaction conditions for the five reaction sites. As the solution flowed over the antigen-coated sites, the concentration of analyte present in solution decreased, because of the reactions between analytes and surface-bound antigens. Therefore, for a transport-
controlled reaction, the reaction kinetics associated with upstream binding sites is always better than downstream. However, this difference in reaction kinetics has no effect on the assay outcome when the concentration of analyte is high enough to allow the immunoreactions on the downstream binding sites to develop to the equilibrium stage during incubation. By contrast, at decreased analyte concentrations, the effect of reaction kinetics becomes more pronounced, resulting in stronger reactions on the upper stream binding sites than on the downstream ones, as Fig. 5.12 shows. The analyte-depletion phenomenon is common for microchannels, conduits with high surface/volume ratios (Delamarche et al., 1998; Fosser & Nuzzo, 2003). This phenomenon will be investigated by using numerical simulation in Chapter 8. The signal variation induced by reaction kinetics is not considered a problem of the present study, as the multiple reaction sites were present in excess. In practice, if duplicate reaction sites are desired, the analyte-depletion can be readily alleviated by either increasing flow rate or extending incubation time.

5.4 Summary

Based on the work of last three chapters, this chapter presents further improvement of the EK-IA to a parallel assay, using an integrated microchip. The improved EK-IA analyzed ten samples in parallel in 22 minutes.

The assay design and optimization process was summarized using a flow chart and illustrated step by step in detail. It was demonstrated that numerical simulation was a useful tool not only in refining the controlling parameters, but also in optimizing the chip design. Two important considerations in designing a parallel IA are prevention of cross-
contamination among different samples and the equalization of reaction conditions in the multiple channels.

The optimal surface density of antigen for immunoreaction was an unstable stage during the continuous adsorption of antigen to native PDMS. More stable antigen immobilization approaches should be used in the future for better reproducibility of the assay, such as using low concentrations of coating antigen, using hydrophilized PDMS, or employing non-adsorptive immobilization methods.

At optimized antigen-coating conditions, *E. coli* O157:H7 antibody and *H. pylori* antibody were detected in concentration ranges of 0.02-10 µg mL\(^{-1}\) and 0.1-50 µg mL\(^{-1}\), respectively. The limits of detection were comparable to those from other microfluidic immunosensors.
Figure 5.1 Preparation process of the EK-IA (Schematics are not proportional to actual size). (a) Antigen was immobilized onto a PDMS-coated glass microscope slide by using a $\mu$FN; (b) The $\mu$FN for EK-IA was placed over the patterned antigen (in gray). The matrix of rectangular intersections became reaction regions in the assay. (c) A picture of the microchip being connected by electrodes with the output cables of a high-voltage sequencer. The wells of the chip were filled with dye to illustrate the geometry.
Figure 5.2 Steps in the EK-IA (Schematics are proportional to actual size). The undistinguished parallel region of the ten sample channels is a result of low-resolution printing. Arrows indicate flow directions. Solid arrows stand for major flows, and dashed arrows, minor flows. (a) Loading and incubation of samples. Sample solutions were dispensed from the sample wells to the reaction region and discharged into the waste well. (b) Washing of samples. Buffer solution flushed sample solutions from the reaction region back into the sample wells. (c) Second washing of samples. Sample solutions having entered the antibody channel during the previous three steps were flushed into the waste well. (d) Loading and incubation of detection antibody; (e) Washing of detection antibody.
Figure 5.3 Flow chart of the assay design process.
Figure 5.4 Example of a $\mu$FN design that causes contaminated reactions from the different samples, because mixed sample solutions at the united region would be flushed back to the reaction regions in the sample-washing step.
Figure 5.5 Circuit equivalent of the $\mu$FN used in the EK-IA. The electrical resistance $R$ is denoted in a relative value, which is the channel length in millimeter.
Figure 5.6 The simulated antibody concentration fields at the junction region after 40 s of loading. Arrows in the figures denote the flow field. (a) The applied electric potentials were estimated using Kirchhoff’s rules. The potentials at the sample well, antibody well, buffer well and waste wells are: \( \Phi_s = 0, \Phi_A = 435 \text{ V}, \Phi_B = 210 \text{ V}, \Phi_w = 210 \text{ V} \). (b) The applied electric potentials were obtained from trial-and-error optimization using numerical simulation. The potentials are: \( \Phi_s = 0, \Phi_A = 435 \text{ V}, \Phi_B = 182 \text{ V}, \Phi_w = 195 \text{ V} \).
Figure 5.7 The simulated sample concentration fields at the junction region. Arrows in the figures denote the flow field. $t$ is the time after the step begins. The reduced local concentration in (c) resulted from sample dispersion.
Figure 5.8 The simulated antibody concentration fields at the junction region during antibody loading ($t = 30$ s), with contour lines. (a) From the original $\mu$FN design in which antibody channel is on the vertical direction (up side). (b) From an improved $\mu$FN design in which antibody channel is switched to the horizontal direction (left side).
Figure 5.9 Signal intensity as a function of antigen concentration. (a) Fluorescent image for *E. coli* O157:H7 test. Concentrations of coating lysate antigen are indicated at the bottom of the image, and concentrations of *E. coli* antibody on the right hand side of the image. The negative control (4th channel from top) was diluent buffer containing no antibody. (b) Quantified results of each three duplicates. Error bars represent standard error of the mean (SEM) for the three duplicates at each antibody concentration. (c),(d) Fluorescent image and quantified results for the *H. pylori* test.
Figure 5.10 Coating effect of *H. pylori* antigen at the coating duration of 20 min, as illustrated by EK-IA experiment.
Figure 5.11 Signal intensity as a function of antibody concentration. (a) *E. coli* O157:H7 antibody concentration test. Concentrations of *E. coli* antibody were as indicated on the right hand side of the image. (b) Quantified data from (a) fitted with sigmoidal function. Error bars represent the SEM for the five duplicated reaction sites. (c),(d) Results from *H. pylori* antibody concentration test.
Figure 5.12 Intensity profile of the fluorescent signals from the five duplicates in Fig. 5.11 (a). The presented data were from antibody concentrations of 10 (■), 5 (●), 2(▲), 0.2(▼), 0.1(◆), 0.05(○) and 0.02 (△) µg mL⁻¹.
Table 5.1 Controlling parameters in the EK-IA

<table>
<thead>
<tr>
<th>Step</th>
<th>Estimated using Kirchhoff's rules</th>
<th>Optimized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Applied electric voltage, V</td>
<td>Duration, min</td>
</tr>
<tr>
<td></td>
<td>$\phi_S$</td>
<td>$\phi_A$</td>
</tr>
<tr>
<td>1. Sample loading</td>
<td>330</td>
<td>120</td>
</tr>
<tr>
<td>2. Sample incubation</td>
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<td>60</td>
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<td>3. Sample washing</td>
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<tr>
<td>4. Antibody loading</td>
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<td>435</td>
</tr>
<tr>
<td>5. Antibody incubation</td>
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<td>217</td>
</tr>
<tr>
<td>6. Antibody washing</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. Sample loading
2. Sample incubation
3. Sample washing
4. Antibody loading
5. Antibody incubation
6. Antibody washing

Estimated

Optimized
Table 5.2 Comparison of the LOD for antibody detection from various heterogeneous immunoassay microfluidic chips

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Flow system</th>
<th>Detection</th>
<th>LOD</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Rabbit IgG</td>
<td>EK</td>
<td>Fluorescence</td>
<td>7.5 µg mL(^{-1})</td>
<td>(Dodge et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(50 nM)</td>
<td></td>
</tr>
<tr>
<td>Human IgG</td>
<td>EK</td>
<td>Fluorescence</td>
<td>5 µg mL(^{-1})</td>
<td>(Linder et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(33 nM)</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Pressure</td>
<td>Electrochemical</td>
<td>0.1 µg mL(^{-1})</td>
<td>(Choi et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(670 pM)</td>
<td></td>
</tr>
<tr>
<td>Rat IgG</td>
<td>centrifugal</td>
<td>Fluorescence</td>
<td>4.7 µg mL(^{-1})</td>
<td>(Lai et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(31 nM)</td>
<td></td>
</tr>
<tr>
<td>Anti-guinea pig IgG</td>
<td>capillary</td>
<td>Fluorescence</td>
<td>6 ng mL(^{-1})</td>
<td>(Bernard et al., 2001)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(40 pM)</td>
<td></td>
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<tr>
<td>Rabbit IgG</td>
<td>Bench-top instrument</td>
<td>Fluorescence</td>
<td>0.025 µg mL(^{-1})</td>
<td>(Sia et al., 2004)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(163 pM)</td>
<td></td>
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Chapter 6 Application of the EK-IA to testing clinical fluids I: assay of human serum

6.1 Introduction

From the studies described in earlier chapters, a high-throughput EK-IA has been developed and successfully used to detect *E. coli* O157:H7 antibody and *H. pylori* antibody from synthetic samples. Because a target application of the EK-IA is clinical setting, it is important to conduct further studies to verify the feasibility of applying the EK-IA to test clinical samples.

Clinical samples, such as serum and urine, differ from synthetic samples in their complex matrix components, which denote everything present in the sample besides the analyte. While synthetic samples contain assay buffer spiked with the analyte and immunologically inert BSA only, clinical samples, e.g., human sera, consist of a range of different proteins and other substances. The complex matrix of clinical samples can interfere with the immunoassay in several ways. The first one is the so-called matrix effects, which refers to a change in the reactivity of an analyte caused by differences in its environment in a sample. This is due to the fact that antigen-antibody binding reactions are often quite sensitive to variations in protein concentration, lipid concentration, pH and ionic strength (Miller & Levinson, 1996). The second interference is cross-reaction, which refers to the binding of molecules different from, yet with structurally similar or identical epitopes/paratopes to the analyte, to the probing antibody/antigen. Cross-reactions impair the sensitivity and selectivity of the

immunoassay. Different from synthetic samples where only the target antibody is present, clinical samples like human sera contain a pool of different IgG and thus bring in chances for cross-reaction. For miniaturized IAs, clinical sample testing induces additional challenges under some circumstances, such as the interference of blood cells (the size of which are comparable to the dimension of microchannels) for whole blood assay, and the adsorption of serum proteins to the highly hydrophobic PDMS.

For these reasons, increasingly studies on microfluidic IA use clinical fluids as assay samples. The testing of a variety of clinical samples has proved feasible with microfluidic IAs, such as blood (Hatch et al., 2001; Moorthy et al., 2004; Mulvaney et al., 2007), serum (Caulum et al., 2007; Koutny et al., 1996; Sato et al., 2001; Schmalzing et al., 1997), plasma (Wolf et al., 2004), urine (Chan & Herold, 2006), saliva (Christodoulides et al., 2005; Herr et al., 2007), and nasal swabs (Rowe et al., 1999). From these assays, a wide range of analytes have been studied, including carcinoembryonic antigen (Sato et al., 2001), C-reactive protein and other cardiac markers (Christodoulides et al., 2005; Wolf et al., 2004), *H. pylori* antibody (Lin et al., 2004a; Messina et al., 2005), HIV antibody (Linder et al., 2005; Sia et al., 2004), or whole IgG (Linder et al., 2002). Platform for those studies covers pressure-driven flow, capillary force-driven flow, as well as electrokinetically-driven flow.

However, most studies on EK-driven IA use homogeneous IA based on electrophoretic separation (Koutny et al., 1996; Schmalzing et al., 1997). Only one study has been conducted on using EK-driven heterogeneous IA for testing a clinical sample (Linder et al., 2002). In that study, Linder et al. reported strong matrix effects when human serum was tested for IgG. The authors suggested that the interactions of serum
components with PDMS channel walls could interfere with electrokinetic delivery of analytes to the reaction site. Therefore, the work to be described in this chapter is an important further investigation as to whether an EK-driven flow system is an appropriate platform of heterogeneous IA for clinical diagnostic purposes.

In this chapter, the focus will be the assay of human serum samples for anti-\textit{E. coli} O157:H7 and anti-\textit{H. pylori} antibodies, so as to prove the clinical applicability of the EK-IA. The serological test is clinically important for the diagnosis of both \textit{H. pylori} and \textit{E. coli} O157:H7 infections.

In the diagnosis of \textit{H. pylori} infection, though the combination of culture and histological examination of gastric biopsy specimens has been considered the “gold standard” (Uemura et al., 2001), and urea breath test is also extensively used, both methods have problems applying to young children, due to difficulties in operation (Best et al., 1994), and lost in accuracy (Imrie et al., 2001), respectively. Serological testing does not have these limitations, and is inexpensive and convenient. Moreover, serology has been shown in many investigations to have a sensitivity and specificity of at least 90\% in untreated patient (Anderson et al., 1997). These merits have rendered serological tests a popular approach for the diagnosis of \textit{H. pylori}.

In the diagnosis of \textit{E. coli} O157:H7, stool antigen test is one of the major approaches, yet serological tests can prove useful in cases where stool antigen isolation is negative (Chart & Jenkins, 1999). In early stages of infection, there may be very high numbers of \textit{E. coli} O157:H7 bacteria in feces. However, as the disease progresses, the number of pathogenic bacteria drops dramatically in fecal samples. Diarrhea may no longer be present two weeks after the onset of intestinal symptoms and the diagnosis then
can be especially problematic (Paton & Paton, 2003). On the other hand, antibodies developed in response to exposure of *E. coli* O157:H7 are long-lasting (Karmali et al., 2003). Therefore, serology, complementary to stool antigen test, can be a useful tool for the diagnosis of *E. coli* O157:H7 at relatively late stages of infection.

Conventionally, the serological tests were conducted by using ELISA on the 96-well microtitre plate. In this study, the human sera were tested using the high-throughput parallel EK-IA, as described in the last chapter. The EK-IA was first applied to assay human sera for the infection status of either of the pathogens. Then, simultaneous screening for both *E. coli* O157:H7 antibody and *H. pylori* antibody was conducted, as a demonstration of multiplexed assay of serum samples.

### 6.2 Experimental

The samples of human serum post *H. pylori* infection, as well as control samples, were collected as part of a previous study involving both sick children and their parents and siblings (Best et al., 1994), from the Hospital for Sick Children (Toronto, Ontario, Canada). The serum samples used in this study were obtained from parents and siblings of children undergoing gastroscopy for evaluation of upper abdominal symptoms. The *H. pylori* status of the samples has been determined by a validated ELISA (Best et al., 1994). The samples of human serum post *E. coli* O157:H7 infection were collected in a previous study from patients who were culture positive for *E. coli* O157:H7 (Karmali et al., 2003), from the Hospital for Sick Children (Toronto, Ontario, Canada). The IgG antibody level in the sera was tested by using validated immunoblot assay (Karmali et al., 2003).
The human sera were tested by using the high-throughput EK-IA. The experimental protocol was mostly the same as described in the last chapter, except that the detection antibody was changed to TRITC-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) and the BSA concentration in the blocking and diluent buffer was increased from 5% to 10% (w/v), unless otherwise indicated. In all the tests, detection antibody was diluted to a final concentration of 60 µg mL⁻¹. Serum samples were diluted to 1:100, unless otherwise indicated.

6.3 Results and discussion

6.3.1 Determination of experimental conditions

In assaying samples of human serum, while the experimental protocol of the EK-IA remained unchanged, the dilution of serum needed to be determined. Serum contains a variety of proteins, including different species of immunoglobulin. If not diluted properly, high concentrations of serum proteins can adsorb to the PDMS channel wall and IgG will bind to the anti-human detection antibody to generate background noise.

Figure 6.1 shows the result from an EK-IA experiment testing different dilutions of a *H. pylori*-positive and a *H. pylori*-negative serum samples, using 5% (w/v) BSA as the blocking and diluent buffer. Apparently, for both the positive and the negative samples, the higher the serum concentration, the stronger the background noise. Signal intensity from the reaction site also increased with serum concentration, due to the corresponding higher concentration of analyte. However, as illustrated by the quantified result in Fig. 6.1 (b), the signal intensity dropped as serum dilution changed from 1:50 to 1:20, probably because too much non-specific binding of other proteins at the reaction
site interfere with the Ag-Ab interaction. The optimal serum dilution should be the one generating the greatest distinction between the \textit{H. pylori}-positive and \textit{H. pylori}-negative samples, which was 1:50 in this case.

Aside from optimizing serum dilution alone, the non-specific binding of serum proteins can also be relieved by using a stronger diluent buffer, i.e. adding more immunologically inert molecules to compete with serum proteins. After testing different types and concentrations of blocking reagents, 10\% (w/v) BSA solution was chosen as the blocking/diluent buffer, as it suppressed the non-specific binding still better than 5\% BSA. Other blocking reagents tested, such as 5\% BSA + 10\% normal donkey serum, or 5\% BSA + 10\% normal donkey serum + 0.1\% Tween 20, while produced even lower background signals than 10\% BSA, also impaired antigen-antibody binding reactions, and, thereby, lower the signal-to-noise ratio.

After changing the blocking solution and re-testing the serum dilution, serum dilution of 1:100 and BSA concentration of 10\% in the diluent and blocking buffer were chosen to be used for the rest of the study. But 5\% BSA was still used to dilute detection antibody.

\textbf{6.3.2 Assay of human serum}

EK-IA experiments were then conducted to test the serum samples with known infection status. Both \textit{E. coli O157:H7} and \textit{H. pylori} were tested, in separate experimental runs. Results from the assays are shown in Fig. 6.2. The five coating channels were all used to provide duplicates. For both \textit{E. coli O157:H7} and \textit{H. pylori}, positive and negative samples can be clearly distinguished from the fluorescent intensities. Using mean ± 3SD of the signal intensity from negative sera as cut-off values (shown as the dotted line),
there was neither false-positive nor false-negative result. With one-way analysis of variance (ANOVA), statistically significant distinction (P<0.05) existed between all \textit{H. pylori}-positive and \textit{H. pylori}-negative samples, and between the first four \textit{E. coli}-positive samples and all \textit{E. coli}-negative samples. Although the fifth \textit{E. coli}-positive sample could be distinguished from only two out of the four negative samples with statistical significance, it was a weakly positive sample, with the microtitre ELISA reading closest to the cut-off value compared to the other four positive samples. The overall accuracy was thus satisfactory in using the EK-IA to screen for either \textit{E. coli} O157:H7 or \textit{H. pylori} antibody in human serum.

The close-to-zero signal intensities of the negative sera implied that very little cross-reaction occurred between serum proteins and immobilized antigens. Also, the low background fluorescence in the images demonstrated effective inhibition of non-specific binding of serum proteins to channel walls.

6.3.3 Multiplexed assay of human serum

Simultaneous screening of both \textit{E. coli} O157:H7 antibody and \textit{H. pylori} antibody from synthetic samples has been briefly demonstrated in Chapter 4. Here, we verified the applicability of the multiplexed assay in testing human serum, with the improved parallel EK-IA. Figure 6.3 shows the fluorescent image from the experiment. Duplicate antigen coatings of \textit{H. pylori} and \textit{E. coli} O157:H7 were applied alternately. Samples included positive and negative serum samples for each pathogen, as well as mixed samples containing both antibodies. The results demonstrated good specificity for the assay: an antigen-coating site yielded a strong fluorescent signal only when the corresponding antibody was present in the sample. This finding indicated that no cross-reactions
occurred between different antibodies and antigens. Also, different samples yielded consistent fluorescent intensities for the same analyte, by comparing signals of *H. pylori* from S1 and S8, S2 and S9, or signals of *E. coli* O157:H7 from S4 and S8, S5 and S9.

Though simultaneous detection of the two pathogen of *E. coli* O157:H7 and *H. pylori* might not be of clinical importance, this result has nonetheless demonstrated the capability of this EK-IA for simultaneous, accurate screening of the infection status of multiple pathogens from human serum. As noted in Chapter 4, a prerequisite for such multiplexed test is the absence of cross-reaction between the different antibodies and antigens.

### 6.4 Summary

In this chapter, the application of the EK-IA to the testing of human serum, an example of clinical samples, was successfully demonstrated.

In the 18 serum samples, *E. coli* O157:H7-positive or *H. pylori*-positive sera were accurately distinguished from the corresponding negative controls, mostly with statistical significance. There was neither false-positive nor false-negative result.

Simultaneous screening of both *E. coli* O157:H7 and *H. pylori* antibody from human serum was achieved, demonstrating the potential of the EK-IA for efficiently detecting multiple pathogenic infections in clinical environments.

With a serum dilution of 1:100 and diluent buffer of 10% (w/v) BSA, the assay results showed no evidence of adsorption of serum proteins to channel walls and consequent disturbance to electrokinetic transport. These results prove the feasibility of EOF as a platform for heterogeneous immunoassay in clinical environments.
Fig. 6.1 Influence of the dilution of human serum on the performance of the EK-IA for *H. pylori*. Concentration of probing *H. pylori* antigen was 200 µg mL⁻¹. Detection antibody was anti-human IgG at a concentration of 60 µg mL⁻¹. The diluent buffer used in this test was 5% BSA (w/v) in 25 mM Tris-HCl. (a) Fluorescent image from the IA experiment. (b) Quantified data from (a).
Figure 6.2 Detection of bacterial-specific antibodies from human serum. All samples of serum were diluted to 1:100 using buffer solution containing 10% (w/v) BSA. (a) Assay for *E. coli* O157:H7 antibody. (b) Quantified data from (a). The dotted line represents the cut-off value, calculated as mean ± 3SD of the signals from *E. coli* O157:H7-negative samples. (c),(d) Assay for *H. pylori* antibody. The detection antibody was donkey anti-human IgG diluted using 5% (w/v) BSA for both assays.
Figure 6.3 Simultaneous detection of both *H. pylori* and *E. coli* O157:H7 antibodies from human serum. Antigens of *H. pylori* and *E. coli* O157:H7 were coated alternately, as indicated at the bottom of the image. Samples were labeled from S1 to S10 and the contents of each sample are indicated on the right side of the image. Capital “P” or “N” denotes a positive or negative sample, respectively. For S1 to S7, the dilution of serum was 1:100. S8 and S9 were mixed samples of *H. pylori*-positive and *E. coli* O157:H7-positive serum. The overall serum dilution was 1:50 for S8 and S9, in order to match the concentration of each antibody to that in the corresponding unmixed serum. For example, the concentration of *H. pylori* antibody in S1 and S8 were equivalent.
Chapter 7 Application of the EK-IA to testing clinical fluids II: assay of embryo culture media

7.1 Introduction

Different from previous chapters focusing on the detection of bacterial antibodies for the purpose of clinical diagnostics, this chapter will address preliminary explorations on applying the EK-IA to detect biomarkers of embryo development. This work is in the area of *in vitro* fertilization (IVF), and was conducted in collaboration with Dr. Casper in the Mount Sinai Hospital, Toronto, ON. The motivation for this collaborative study is that the features of the EK-IA, including short assay time, low sample consumption, multiplexed detection and parallel assay, suit particular well with the requirements in the application area, as described below.

In IVF practice, usually 15-20 oocytes are retrieved from one woman. After fertilized and cultured for 3-6 days, the “best” embryo(s) are chosen based on morphological criteria and transferred to the woman. However, the morphological criteria are severely limited in their ability to provide sturdy evidence for the subsequent normal development of the embryos. A significant number of embryos (≥ 70%) fail to implant, and only 14% of transferred embryos give rise to a full-term infant (Noci et al., 2005). This fact has led many researchers to seek potential biomarkers indicative of embryo competency, in order to provide additional independent criteria for embryo selection (Gardner & Leese, 1993; Hansis & Edwards, 2003; O'Neill et al., 1987).

Two major potential markers are soluble human leukocyte antigen-G (sHLA-G) and pregnancy specific β-1glycoprotein (SP-1). sHLA-G is expressed on the placental

* Based on this chapter, the manuscript for a journal paper is currently in preparation.
cytotrophoblast cells at the maternal-fetal interface and may play an important role in maternal-fetal immune tolerance (Jurisicova et al., 1996). sHLA-G was reported to be a mandatory prerequisite for the development of pregnancy (Fuzzi et al., 2002). The pregnancy rate from embryos with sHLA-G was significantly higher than that of those without (48.4% vs. 17.1%) (Yie et al., 2005). SP-1 is a placental hormone secreted by syncytiotrophoblast. SP-1 is also believed to play a role in protecting the fetus from the recognition and rejection of maternal immune system (Jurisicova et al., 1999).

Though the retrospective studies have shown much evidence on the relevance of IVF success rate with the level of the potential biomarkers, especially sHLA-G, these results have not found routine clinical application yet. One barrier to both the research and clinical application of the biomarker criterion could be the conventional ELISA method itself, which has been used to measure the level potential biomarkers from embryo culture media, as addressed below.

First, conventional ELISA requires at least 50~100 µL of sample. The embryo culture media is generally no more than 100 µL, which is merely enough for one ELISA test. In some IVF programs, such as the one in Mount Sinai Hospital we collaborated with, only ~15 µL of medium is available. Since the concentration of secreted substances is too low to allow dilution of the media, it is hard to conduct even one ELISA test and impossible to measure several different molecules from one medium sample, which greatly limits potential research work on correlation studies.

Second, conventional ELISA takes a long time. Aside from the overnight coating of capture antibody, each test takes from 3~5 hours (Noci et al., 2005; Sher et al., 2004) to 1~2 days (Jurisicova et al., 1999). Although such a time-consuming procedure is
acceptable in retrospective studies, it would become a problem in the clinical practice, where “up-to-date” status of the embryo culture media is required for embryo selection.

The EK-IA can solve the above conflicts, as it has been shown to require only ~10 µL of sample and less than 30 min of assay time, and to be capable of detecting multiple analytes in one test. Moreover, with its capability of parallel assay, the EK-IA can be used to assay multiple samples of embryo culture medium from one patient in a single run.

The work presented below is the preliminary investigation on using EK-IA to assay samples of embryo culture medium. The configuration of the IA is illustrated in Fig. 7.1, which is a sandwich-type IA for antigen. For sHLA-G, both capture antibody (cAb) and detection antibody (dAb) are monoclonal antibodies (mAb). The biotin-avidin system was employed for signal amplification.

Because the available amount of embryo culture medium was too small (~15 µL), this preliminary work on establishing the experimental protocol was mostly conducted using solutions of positive and negative control. Assays were first conducted using the conventional ELISA on a 96-well microtitre plate, to set up a gold standard. Then, assays were carried out with EK-IA on a straight microchannel. A few samples of embryo culture medium were also tested with the EK-IA. Experimental procedures and some brief experimental results are presented in the next two sections. Then, in a separate section, some challenges confronted in developing the EK-IA with the new model systems will be discussed.

7.2 Experimental
7.2.1 Reagents

The cAb and dAb for sHLA-G were mAb MEM-G9 (MCA2044, Serotec, Raleigh, NC) and biotin conjugated mAb w6/32 (MCA81B, Serotec), respectively. The cAb and dAb for SP-1 were rabbit polyclonal anti-SP1 immunoglobulin G (IgG) (Dako, Copenhagen, Denmark) and biotinylated anti-SP1 antibody (Dako), respectively. Streptavidin-horseradish peroxidase (HRP) was obtained from Calbiochem (La Jolla, CA). Streptavidin-DTAF (DTAF is a fluorophore with same excitation wavelength to FITC) was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Blocking buffer and diluent buffer were 5% and 2% skimmed milk powder in PBS, respectively, for conventional ELISA, and 5% BSA and 2% BSA in tris-HCl, respectively, for the EK-IA.

Placental cytotrophoblast cell lysate and placental supernatants were both tested as positive controls for sHLA-G. Pure placental culture medium was used as negative control for sHLA-G. Pooled cord blood serum was used as a positive control for SP-1. The solutions for positive and negative controls were all provided by Dr. Casper’s lab.

7.2.2 Conventional ELISA

Experiments of conventional ELISA for both sHLA-G and SP-1 were conducted with polystyrene 96-well microtiter plates. The procedures are shown in Table 7.1 and explained below using sHLA-G as an example. First, the microtitre plate was coated with 50 µL capture antibody diluted to 1:50 with 0.1 M carbonate buffer (pH 9.6). The plate was then covered with parafilm and refrigerated overnight at 4 °C. On the following day, the coating solutions in the plates were emptied and the plate was washed twice using 200 µL PBS buffer. For all the washings, the wells were soaked with PBS for 5 min.
under gentle shaking condition to enable a thorough separation of unbound molecules. Blocking solution was then added and incubated at room temperature for 1 hr. 75 µL of sample solution (positive or negative control) was then added to the wells and incubated at 37 °C for 2 hours. After that, the plate was washed four times using PBS, followed by incubation with biotinylated detection antibody at a 1:20 dilution for 1 hr at 37 °C. The plate was again washed four times with PBS and incubated with HRP-conjugated streptavidin for 35 min at 37 °C. Then, after a thorough washing of five times using PBS, 3,3,5,5-tetramethylbenzidine (TMB) substrate was introduced to the plate. The enzymatic reaction was allowed to develop for 30 min, before it was stopped by 0.5 M H₂SO₄. After the stopping reaction, the colorimetric signals were measured by absorbance at 450 nm using a microtitre plate reader.

### 7.2.3 Electrokinetically-driven immunoassay

The EK-Ia experiments were conducted using a straight microchannel that was 1.5-cm long, 30-µm high and 100-µm wide. The assay follows a two-step “coating + assay” procedure same to that used in Chapter 4 and 5. The EK-Ia has mainly been used to assay sHLA-G so far. Capture antibody of sHLA-G was diluted to 1:20 using carbonate buffer and immobilized onto a PDMS-coated glass slide by using a microfluidic network (µFN) of parallel microchannels, in the same way as the antigen immobilization conducted before. The straight microchannel was then put across the lines of patterned cAb perpendicularly.

Steps in the EK-Ia, as well as the operation parameters used, are given in Table 7.2. 10 µL of solution was added in each well for every step. The two wells were washed twice after each incubation step, before buffer solution was added to flush the channel.
The assay buffer was still 25 mM Tris-HCl buffer. The dAb for sHLA-G was used directly without dilution. DTAF-streptavidin was diluted to 1:5 before use. After the experiment, fluorescent signals were detected by using a fluorescent microscope, as described earlier.

7.3 Results and discussion

It should first be noted that the results presented in this section are those obtained after optimizing the experimental conditions, including the concentration and incubation time of the control solutions and reagents. Results of sHLA-G detection using conventional ELISA and EK-IA are shown in Fig. 7.2 (a) and (b), respectively. From these results, the EK-IA clearly yielded a lower LOD for sHLA-G than conventional ELISA. Using mean ± 3SD of the intensities from negative control as the cut-off value, conventional ELISA and EK-IA detected sHLA-G in cytotrophoblast cell lysate down to 1:10 and 1:50, respectively. The EK-IA thus achieved a better sensitivity at much reduced (~2/3 less) assay time and economized (~90% less) sample consumption than conventional ELISA. The improvement in sensitivity can be attributed mainly to the flow-through sample incubation and the fluorometric detection.

The EK-IA was then applied to assay a few samples of embryo culture medium and the negative controls. The results are shown in Fig. 7.3. The precision of these results can hardly be evaluated, as conventional ELISA cannot be performed with these small media sample. Neither can the results from this small batch of samples be related to pregnancy outcome. Nevertheless, some important information can be retrieved from these results. First, the oocyte culture medium yielded a weak signal comparable to that
of the negative control medium. This is a correct result, since oocytes do not secret sHLA-G. Moreover, two samples of embryo culture medium (the two day_6 media) produced intensities as low as that of the negative control, while the other two samples (the two day_3 media) yielded significantly stronger signals. These brief results thus demonstrated that the EK-IA was sensitive enough to distinguish different levels of sHLA-G in the embryo culture media. This capability preliminarily confirmed the suitability of the EK-IA for the application area, since the discrimination of relatively “positive” and “negative” media is all that is required for embryo selection. The absolute concentration of the analyte is not of concern.

Though the assay for SP-1 has not been investigated systematically using the EK-IA, the gold standard of conventional ELISA has been established. As shown in Fig. 7.4 (a), SP-1 could be detected from pooled cord blood serum in the dilution down to 1:100 using the ELISA method. Preliminary EK-IA testing of a positive (pooled cord blood serum diluted to 1:10) and a negative media, using an experimental protocol different from the one described in Section 7.2.3, also yielded promising results. As shown in Fig. 7.4 (b) and (c), the signals from the positive control were significantly stronger than those from the negative one.

7.4 Challenges

The above results on EK-IA were obtained from experiments on a straight microchannel, the first step of IA development. Experimental trials on automated IA on a microchannel network (µFN) and on multiplexed IA have also been conducted, but yielded no good results yet. The challenges on IA development are summarized below.
The major difficulty in developing an automated IA with a microchannel network lies in the long incubation time. The incubation time of sample solution was 60 min. Preliminary experiments showed that the signal intensity dropped significantly when the incubation was shortened to 30 min, even when the experiment was conducted at an elevated electric field.

Long incubation affected the IA from two aspects. First, there exists a risk of cross-contamination of reagents caused by the dispersion of sample solutions due to the long-time diffusion and slight flow (electrokinetic or pressure-driven) to other reagent-delivery channels and wells. Therefore, the microchannels have to be designed as low as possible to minimized the flow and long enough to avoid cross-contamination. However, as PDMS is a soft elastomer, long and slit-shaped channels tend to sag and collapse, which ruins the assay. Channel collapse was frequently observed in EK-IA experiments with a µFN, with the longest straight channel in the µFN being 36 mm and the cross section of all channels being 100 x 8 µm² (w x h). This problem could not be solved by using hard (PDMS base and curing agent ratio: 5:1) and thick (~4 mm) PDMS slab. But experiments showed that using a wider microchannel should relieve this problem. Another consequence of long incubation time was the evaporation of reagents, since all the reagents, in amounts of 10-20 µL, were added to the wells before the 2-hr assay started. But this problem could be relieved by conducting the EK-IA experiment in a closed chamber with humidity control.

Despite the strategies discussed above, a thorough solution to the undesirable consequences is to reduce the required long incubation time. While the reagent concentrations had been optimized, the long incubation required for the flow-through
EK-IA, which was only ½ shorter than that required in conventional ELISA, was very probably due to the loss in the activity of cAb upon coating. In Chapter 5 it has been mentioned that the adsorption of proteins to native PDMS results in denaturation. While the results are acceptable for antigen coating in this study, for the coating of mAb the denaturation is known to be especially pronounced (Butler et al., 1986). The loss of specificity due to denaturation of cAb had been observed in the EK-IA experiments for sHLA-G. When the dilution of cAb changed from 1:20 (currently used) to 1:5, signals from the positive and negative controls became comparably strong, indicating that substantial cross-reactions occurred between the substances in the negative control and the cAb.

Therefore, the improvement on cAb immobilization might be a thorough solution to the problems discussed above. In addition to the methods mentioned in the previous chapters, such as using hydrophilized PDMS as the solid phase or using covalent attachment, an approach fits particularly to the immobilization of antibody is to introduce an intermediate anchor layer of protein A or protein G between the cAb and the channel wall. In this way, direct contact of the antibody molecules and the hydrophobic PDMS surface can be prevented and the indirectly bound IgG is significantly more detectable than the directly adsorbed (Butler et al., 1997b). Moreover, protein A and protein G bind to the Fc region of IgG and thus the antibody will be in the correct orientation to receive the antigen.

Difficulties were also confronted when the two analytes, sHLA-G and SP-1, were assayed simultaneously in a multiplexed IA. The cAb-coating sites of both sHLA-G and SP-1 yielded a positive signal, when only the positive controls for sHLA-G were assayed.
The cause of the problem may lie in the reagents. Since there was no commercially available purified sHLA-G, the positive controls used were placental cytotrophoblast cell lysate and placental supernatants, in which the SP-1 status is unknown. Therefore, sHLA-G and SP-1 should first be removed from the control solutions for each other, before checking the cross-reactivity of the different Ab and Ag using conventional ELISA. Multiplexed EK-IA can be developed when there is no cross-reactivity present.

7.5 Summary

This chapter describes the potential application of the EK-IA to assay embryo culture media for two potential biomarkers for embryo development, sHLA-G and SP-1, for the long-term goal of embryo selection in IVF practice.

EK-IA with a straight microchannel achieved a lower LOD than conventional ELISA in detecting sHLA-G from control solutions. The assay of several samples of embryo culture media demonstrated that the LOD of the EK-IA was low enough to distinguish between different levels of sHLA-G in the embryo culture media, thus preliminarily confirming the capability of the EK-IA for the application area.

Experimental studies on using EK-IA to detect SP-1 were still at the initial stage, but the gold standard of conventional ELISA has been established.

The long incubation required is a major challenge on developing the EK-IA to an automated one using a µFN. Improving cAb immobilization might be a solution to this problem.

The feasibility of multiplexed EK-IA depends on the verification of the cross-reactivity of the antibodies and antigens of the two analytes.
Figure 7.1 Schematic of the sandwich immunoassay. This configuration was used for the assay of both sHLA-G and SP-1. Streptavidin-HRP (horseradish peroxidase) was used in conventiona ELISA and streptavidin-DTAF was used in the EK-IA.
Figure 7.2 Detection of sHLA-G from control solutions. (a) Using conventional ELISA. (b) Using EK-IA.
Figure 7.3 Detection of sHLA-G from samples of embryo culture medium or oocyte culture medium using EK-IA.
Figure 7.4 Detection of SP-1. (a) Assay of different dilutions of positive control solution using conventional ELISA. (b), (c) Assay of a positive control (1:10 pooled cord blood serum) and a negative control (pure embryo culture medium), respectively. In the EK-IA experiments, the electric field strength for sample loading and incubation were 136 and 68 V cm\(^{-1}\), and the durations for the two steps were 4 min and 25 min, respectively.
Table 7.1 Procedures of the conventional ELISA

<table>
<thead>
<tr>
<th>Steps</th>
<th>sHLA-G</th>
<th>SP-1</th>
</tr>
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<tbody>
<tr>
<td>1. cAb coating</td>
<td>1:50 in carbonate buffer, 50 µL, overnight at 4°C</td>
<td>1:500 in PBS, 100 µL, overnight at 4°C.</td>
</tr>
<tr>
<td>2. Washing</td>
<td>200 µL, 2xPBS (wash twice)</td>
<td>200 µL, 2xPBS</td>
</tr>
<tr>
<td>3. Blocking</td>
<td>1 hr at room temperature (RT)</td>
<td>1 hr at RT</td>
</tr>
<tr>
<td>4. Sample incubation</td>
<td>75 µL, 2 hr at 37 °C</td>
<td>100 µL, overnight at RT</td>
</tr>
<tr>
<td>5. Washing</td>
<td>200 µL, 4xPBS</td>
<td>200 µL, 4xPBS</td>
</tr>
<tr>
<td>6. dAb incubation</td>
<td>1:20, 100µL, 1 hr at 37 °C</td>
<td>1:100, 100 µL, 4 hr at RT</td>
</tr>
<tr>
<td>7. Washing</td>
<td>200 µL, 4xPBS</td>
<td>200 µL, 4xPBS</td>
</tr>
<tr>
<td>8. Streptavidin-HRP incubation</td>
<td>1:100, 100 µL, 35 min at 37 °C</td>
<td>1:100, 100 µL, 1.5 hr at RT</td>
</tr>
<tr>
<td>9. Washing</td>
<td>200 µL, 5xPBS</td>
<td>200 µL, 5xPBS</td>
</tr>
<tr>
<td>10. TMB substrate incubation</td>
<td>100 µL, 30 min</td>
<td>100 µL, 10 min</td>
</tr>
<tr>
<td>11. Stopping reaction</td>
<td>100 µL, 0.5 M H₂SO₄</td>
<td>100 µL, 0.5 M H₂SO₄</td>
</tr>
<tr>
<td>Total duration (not including cAb coating and blocking)</td>
<td>5 hr and 10 min</td>
<td>~1.5 days</td>
</tr>
</tbody>
</table>
Table 7.2 Procedures of the EK-I A

<table>
<thead>
<tr>
<th>Steps</th>
<th>E (V cm⁻¹)</th>
<th>Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sample loading</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>2. Sample incubation</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>3. Washing</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>4. dAbloading</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>5. dAb incubation</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>6. Washing</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>7. Streptavidin-DTAF loading</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>8. Streptavidin-DTAF incubation</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>9. Washing</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Total duration</td>
<td></td>
<td>113</td>
</tr>
</tbody>
</table>
Chapter 8 Reaction kinetics of the microfluidic EK-IA*

8.1 Introduction

From the experimental studies presented in earlier chapters, it is clear that the performance of the EK-IA depends on many factors, including the surface concentration of probing antigen, the incubation time and the flow velocity. Understanding the role of the different parameters and how they work together to determine the kinetics of Ab-Ag binding reaction is essential for the optimization of the EK-IA in the future, towards either a low limit of detection (LOD), or rapid detection, or reduced sample or reagent consumption. This requirement is the motivation of this chapter.

There are a number of good references on the reaction kinetics study of heterogeneous biosensors. Though analytical solutions for specific reaction regimes have been explored (Gervais & Jensen, 2006), numerical simulation is the most widely used approach in the study of this convection/diffusion/reaction problem (Glaser, 1993; Hu et al., 2007; Zimmermann et al., 2005). Most of the studies in this area focused on the recovery of intrinsic reaction rate constants from transport-limited binding data. The experimental data in those studies were obtained from real-time monitoring of the Ab-Ag binding by using different techniques including surface plasmon resonance (SPR) (Myszka et al., 1998; Myszka et al., 1997), resonance sensor mirror (Schuck et al., 1998), and fluorescence-based waveguide (Vijayendran et al., 1999).

Much fewer studies have reported on reaction kinetics of an immunosensor from an application perspective of optimizing reaction conditions. A study recently reported modeling of a microfluidic-based heterogeneous IA and its optimization mainly towards

* Based on this chapter, the manuscript for a journal paper is currently in preparation.
low sample consumption (Zimmermann et al., 2005). In spite of the comprehensive numerical investigations conducted, there seems to be lack of a picture of the fundamental relationship among the reaction parameters.

This work proposed to optimize the reaction conditions of the EK-IA based on understanding of the relationship of different parameters to the reaction kinetics. For this purpose, a modified Damköhler number was proposed to relate the reaction and transport parameters and to indicate extent of transport-limitation to the reaction kinetics. While the Damköhler number provided a big picture, numerical simulation of the transport and reaction processes was also conducted, by using the complete model of convection-diffusion equation coupled with surface binding reaction (Gervais & Jensen, 2006; Sigurdson et al., 2005; Zimmermann et al., 2005).

Targeted towards a more sensitive assay, the influence of several important reaction parameters was investigated here, including both intrinsic parameters such as reaction rate constants and surface concentration of antigen, and operational parameters such as velocity and reaction time. Different from other reported studies, the investigation here was conducted in a way closely associated to the operation and optimization of the EK-IA. Issues from earlier experimental studies, such as the depletion effects of analyte, will also be investigated.

### 8.2 Mathematical model and numerical method

Figure 8.1 schematically illustrates the geometry for modeling and computation, a segment of a microchannel. The mathematical model is based upon binding of antibody from the EOF-transported bulk solution to surface-immobilized antigen. It is sufficient to
use the 2-D geometry, as the width of a microchannel is usually much greater than the height, and thus variation of concentrations across the width can be neglected.

8.2.1 Bulk analyte transport

The analyte solution is electrokinetically driven into the microchannel from the left inlet with a concentration of $C_0$, as shown in Fig. 8.1. The transport of antibody in the bulk phase is described using the traditional convection-diffusion equation,

$$\frac{\partial C}{\partial t} + \vec{u} \cdot \nabla C = D \nabla^2 C$$  \hspace{1cm} (8.1)

where $C$ is the concentration of antibody in bulk phase, $D$ is the diffusion coefficient of antibody, and $\vec{u}$ is the electroosmotic flow velocity. $\vec{u}$ is assigned a constant value because EOF has a plug-like velocity profile across the cross-section. Electrophoretic velocity is not included in the equation as it has been measured to be close to zero, in Chapter 2.

8.2.2 Surface binding kinetics

Antigen, as the probing molecule, is immobilized on the bottom surface of the channel. The binding between antibody and the coated antigen can be described by,

$$C + R \xrightarrow{k_{on}} B \xrightarrow{k_{off}} C$$  \hspace{1cm} (8.2)

where $R$ is the concentration of the free binding sites on the surface, $B$ the concentration of bound antibody on the surface, and $k_{on}$ and $k_{off}$ the association rate constant and dissociation rate constant, respectively.

The time evolution of antibody binding, taking into account the surface diffusion of antigen-antibody complexes, is governed by the following equation,
\[ \frac{\partial B}{\partial t} = k_{on} CR - k_{off} B + D_S \nabla^2 B \]  
(8.3)

where \( D_S \) is the coefficient of surface diffusion of complexes. The first term on right-hand side of Eq. (3) is the source term accounting for the binding of antibody while the second term accounts for the dissociation of bound antibody from the surface. Because the number of the free binding sites, \( R \), is the difference between the total binding sites, \( R_t \), and the sites already occupied, Eq. (8.3) can be changed to

\[ \frac{\partial B}{\partial t} = k_{on} C(R_t - B) - k_{off} B + D_S \nabla^2 B \]  
(8.4)

Eq. (8.4) includes the bulk concentration of antibody, \( C \), which must be solved in combination with its transport in the microchannel. The coupling between the mass balance in the bulk (2-D Eq. (8.1)) and at the surface (1-D Eq. (8.4)) is realized by imposing a boundary condition in Eq. (8.1). Boundary condition at the reaction region, in terms of mass flux, is,

\[ \vec{n} \cdot D \nabla C = -k_{on} C(R_t - B) + k_{off} B \]  
(8.5)

where \( \vec{n} \) is the unit normal vector to the surface.

Other boundary conditions for Eq. (8.1) are as follows,

\( C = C_0 \) at the inlet;

\( \vec{n} \cdot (D \nabla C) = 0 \) at the outlet;

\( \vec{n} \cdot (C \vec{u} - D \nabla C) = 0 \) at the non-reaction regions.

Eq. (8.4) can also be solved analytically, when assuming a constant analyte concentration, \( C \), and neglecting the surface diffusion of bound complex. The solution is,

\[ B = \frac{k_{on} R_t C}{k_{on} C + k_{off}} (1 - e^{-(k_{on} C + k_{off}) t}) \]  
(8.6)
8.2.3 Numerical solver

Still, COMSOL Multiphysics 3.2 software package was used for the computation. The coupled Eqs. (8.1) and (8.4) were simultaneously solved by the Multiphysics model. The 1-D surface diffusion-reaction equation was added to the 2-D convection-diffusion equation by using the Boundary Weak Form feature of the software.

8.3 Results and discussion

The computational model was verified and the results agreed very well with those from other studies (Hu et al., 2007; Zimmermann et al., 2005). Unless otherwise indicated, the following parameters were used in the computation: the association rate constant \( k_{\text{on}} = 10^6 \text{M}^{-1}\text{s}^{-1} \), the dissociation rate constant \( k_{\text{off}} = 10^3 \text{s}^{-1} \), which were chosen within the range of \( 10^5 \sim 10^7 \text{M}^{-1}\text{s}^{-1} \) for \( k_{\text{on}} \) and \( 10^{-4} \sim 10^{-2} \text{s}^{-1} \) for \( k_{\text{off}} \), from the data of various heterogeneous immunosensors (Myszka et al., 1997; Vijayendran et al., 1999; Wolf et al., 2004). The equilibrium constant \( K = \frac{k_{\text{on}}}{k_{\text{off}}} = 10^9 \text{L mol}^{-1} \), also fell in the middle of the typical range for IA systems, \( 10^7 \sim 10^{11} \text{L mol}^{-1} \) (Edwards, 1985). The surface concentration of probing antigen was chosen at \( R_s = 1 \times 10^{-8} \text{mol} \cdot \text{m}^{-2} \). That is because the maximum surface concentration for capture protein in a heterogeneous IA is in the order of \( 10^{-7} \text{mol} \cdot \text{m}^{-2} \) (Amzel & Poljak, 1979; Rossier et al., 2000). But as mentioned before, protein undergoes conformational change upon adsorption to a solid surface. The loss in capture antibody activity resulted from denaturation as well as mis-orientation can exceed 90% (Butler, 1996). The denaturation of capture antigen has also been experimentally proved (Hsu et al., 1981). Therefore, here an assumption was made that 10% of adsorbed capture antigens were preserved for capturing antibody, following a
surface concentration of $1 \times 10^{-8}$ mol·m$^{-2}$. The diffusion coefficient of antibody in solution was $D = 4 \times 10^{-11}$ m$^2$s$^{-1}$ (Rossier et al., 2000) and the surface diffusion coefficient of antigen was $D_S = 2 \times 10^{-13}$ m$^2$s$^{-1}$ (Tilton et al., 1990). The flow velocity was $u = 100$ µm s$^{-1}$, which was close to the velocity used in the in the EK-IA experiments presented in earlier chapters (flow velocities in incubation steps were $\sim 120$ µm s$^{-1}$).

8.3.1 The effect of flow velocity on binding kinetics

It is of primary importance to investigate the role of flow velocity in the binding reaction, since the operation under a flow-through mode is a major difference between microfluidic-based IA and conventional IA. Also, velocity of EOF is perhaps the easiest parameter to vary in optimizing the EK-IA.

Figure 8.2 (a) and (b) show the binding kinetics curve under different velocities, at antibody concentration of 100 nM and 1 nM, respectively. 1 nM corresponds to the lower LOD of a relatively sensitive IA in clinical applications. A LOD at this level (0.67 nM, i.e., 0.1 µg mL$^{-1}$) has been achieved by the EK-IA in detecting $H. pylori$ antibody, as demonstrated in Chapter 5. The higher concentration, 100 nM, then corresponds to the upper limit of the IA, as the dynamic range of most IA in practical use is 2-3 orders of magnitude. The velocities examined here were in the range of 30–1000 µm s$^{-1}$. A velocity of $\sim 100$ µm s$^{-1}$ was used predominantly in sample or antibody incubation, in Chapters 3-6. In Chapter 7, a reduced velocity of $\sim 30$ µm s$^{-1}$ was used in the long time incubation of the low-concentration sample solution. 1000 µm s$^{-1}$ represents the usual upper limit of EOF in microfluidic devices (Stone et al., 2004). The velocities of 1-10 µm s$^{-1}$ are also included in Fig. 8.2 (b), to demonstrate further transport limitation, as will be discussed later.
As manifested by the curves of both high and low antibody concentrations, the reaction kinetics was greatly improved by the flow-through mode, as compared to the no-flow case in which antibody at the reaction surface is replenished only by diffusion. The transport-limitation in the binding kinetics is apparent, by comparing the curves at different flow velocities with the one from Eq. (8.6), representing the case of only reaction-limited kinetics. Nevertheless, the transport limitation is not strong. Figure 8.2 (c) shows the inversely proportional relationship between the velocity and the time to reach equilibrium, using the data from Fig. 8.2 (a), at antibody concentration of 100 nM. When the velocity is increased from 100 £ìm s\(^{-1}\) to 1000 £ìm s\(^{-1}\), the time to reach equilibrium is shortened just from 60 s to 50 s.

At a much lower concentration of antibody, 1 nM, the reaction rate is much slower because of the much smaller \((k_{on}C + k_{off})\), the coefficient of time in Eq. (8.6). As it takes a long time for the reaction to reach equilibrium in this situation, in practical applications, the reaction can be stopped at any convenient time. Figure 8.2 (d) illustrates the dependence of surface concentration of bound antibody, \(B\), on velocity, under two different reaction times. The data points are fitted with sigmoidal curves. The plateau and the lower limit, though not covered by the referred velocity range, should correspond to the solely reaction-limited case and the no-flow, diffusion-only case, respectively. Clearly, the enhancement of increasing flow velocity on reaction kinetics would be the most effective in the central linear region. When the velocity increases from 100 £ìm s\(^{-1}\) to 300 or 1000 £ìm s\(^{-1}\), \(B\) increases by 24% or 43%, respectively, at a reaction time of 600 s; but when reaction time is extended to 1200 s, the corresponding increases of \(B\) drop to
14% and 18%, respectively, as the curve has obviously grown out of the linear region and is approaching the plateau.

The latter case discussed above, i.e., the influence of velocity at low antibody concentration, is practically more important, as the lower LOD is a general concern for an IA. From the above discussions, in the velocity range of 30–1000 $\mu$m s$^{-1}$, increasing the velocity can improve the reaction kinetics, but not strongly. The velocity used primarily in the EK-IA experiments, 100 $\mu$m s$^{-1}$, provides good reaction kinetics that is not significantly weaker than the solely reaction-limited case. Therefore, under the above reaction conditions, this velocity may still be used, to lower the amount of Joule heating, as long as a sufficiently low LOD can be reached.

Another advantage of using lower velocity is that the consumption of sample is reduced, which is especially important when the available sample is limited, such as for pediatric patients, and for the case of embryo culture media described in Chapter 7. In situations where a rapid assay is not required, using a lower flow velocity and longer incubation time allows a deeper reaction, at the same analyte consumption. For example, in the case illustrated in Fig. 8.2 (d), compared to 600-s reaction at the velocity of 100 $\mu$m s$^{-1}$, 1800-s reaction at 30 $\mu$m s$^{-1}$ increases the surface concentration of bound antibody by 71%.

The above close look at the role of velocity on reaction kinetics thus enables optimization of experimental conditions for different purposes, such as low LOD, short assay time, and low analyte consumption. However, the above simulation is based on specific values of the parameters as listed at the beginning of this section, such as the reaction rate constants and the surface concentration of antigen. Variations in these
parameters can affect the velocity-dependence of binding kinetics. To understand how the other parameters change the weight of velocity on reaction kinetics and how these parameters act together, a modified Damköhler number was introduced as an indicator of the type of reaction kinetics.

### 8.3.2 Damköhler number

The Damköhler number \((Da)\) indicates the relative rates of reaction and mass transport. Existing studies on the reaction kinetics of biosensors mostly use the conventional form of \(Da\) for biomolecular surface reaction (Gervais & Jensen, 2006; Sigurdson et al., 2005; Vijayendran et al., 1999),

\[
Da = \frac{k_{\text{on}}R_s}{D/h},
\]

in which mass transport is only from diffusion. In those studies, convective mass transport, if present, is investigated separately via the Peclet number,

\[
Pe = \frac{u h}{D},
\]

the ratio of convective and diffusive transport (Gervais & Jensen, 2006; Sigurdson et al., 2005). This approach, however, does not directly relate the convective transport to the reaction kinetics. Therefore, it is proposed that in the case of flow-based surface binding reaction, a modified Damköhler number can be used to indicate the ratio of reaction rate and the mass transport rate inclusive of both the convection and diffusion effect, in the formula of,

\[
Da = \frac{k_{\text{on}}R_s}{D/h + u}
\]  

\[(8.7)\]

The above expression represents the ratio of the rate of the two serial processes in the Ab-Ag binding reaction in a microchannel: \((D/h + u)\) is the rate that antibody molecules are delivered to the reaction surface via vertical diffusion and lateral
convection in the 2-D plane; $k_{on}R_t$ is the rate that antibody binds to the immobilized antigen on the surface.

The Damköhler number thus provides a quick estimate of the kinetics of the reaction: if $Da$ is $\sim 1$, transport and reaction are equally important to the reaction kinetics; Otherwise, the overall rate of the binding reaction is more limited by the slower process of the two: if $Da > 1$, the kinetics is more transport-limited, and the kinetics can be effectively enhanced by improving the transport of antibody via increasing flow rate; if $Da < 1$, the kinetics is more reaction-limited, and not as sensitive to the increase of flow rate as the former case.

For the case simulated and shown in Fig. 8.2 in the previous subsection, $Da$ at $u = 100 \mu m s^{-1}$ can be calculated as,

$$Da = \frac{k_{on}R_t}{D/h + u} = \frac{1 \times 10^6 M^{-1}s^{-1} \cdot 1 \times 10^{-8} mol \cdot m^{-2} \cdot 10^{-3} m^3 L^{-1}}{4 \times 10^{-11} m^2 \cdot s^{-1} / 2 \times 10^{-3} m + 1 \times 10^{-4} m \cdot s^{-1}} = \frac{1 \times 10^{-5}}{2 \times 10^{-6} + 1 \times 10^{-4}} = 0.098$$

This $Da$ number of $\sim 0.1$ indicated that the rate of mass transport is one order of magnitude greater than that of the reaction and the kinetics is clearly reaction-limited. This explains why further increase in flow velocity does not enhance the reaction kinetics greatly. Also, the Peclet number, $Pe$, is 50 in this case, indicating that the contribution from convection is significantly stronger than from diffusion to the overall mass transport.

$Da$ is equal to 1 at a flow rate of 8 $\mu m s^{-1}$. When antibody concentration is 1 nM, the binding curve at this condition should be between the two top dashed lines (10 $\mu m s^{-1}$ and 3 $\mu m s^{-1}$, respectively) in Fig. 8.2 (b). As the velocity is further reduced to 1 $\mu m s^{-1}$, $Da$ is 3.33 and transport-limitation comes to play a major role in the reaction kinetics. $Pe$ is 0.5 in this case and convection is at a lower level than diffusion in mass transport. As
illustrated in Fig. 8.2 (b), the binding curve at this condition, the bottom dashed line, is very close to that of the diffusion-only case.

If the values of the other parameters change, e.g., at elevated levels of the association-rate constant, $k_{on}$, and/or the surface concentration of antigen, $R$, then according to Eq. (8.7), the flow rate need to be increased accordingly to maintain a comparable reaction kinetics. This case will be discussed in detail below. Also, if the analytes are smaller molecules, e.g., antigens or drugs molecules, the diffusion coefficient, $D$, becomes greater, according to the Stokes-Einstein relation, $D = \frac{K_B T}{6\pi\eta r}$, where $K_B$ is the Boltzmann’s constant, $T$ is the absolute temperature, $\eta$ is the dynamic viscosity and $r$ the radius of the sphere. In this situation, the relative contribution of convection to the overall mass transport will decrease unless the flow rate is increased.

The modified Damköhler number thus relates transport and reaction parameters to the reactions kinetics. It can serve as a rough estimation, while numerical simulation is useful in disclosing details. This strategy is used in the following discussions.

### 8.3.3 Optimization of reaction conditions towards a lower LOD

LOD is a crucial criterion in evaluating the performance of IA. An IA must have a sufficiently low LOD to be able to detect analytes from a sample solution. Therefore, with a lower LOD as the target of optimization, a comprehensive investigation will be conducted on the effect of the various parameters on the reaction kinetics.

As already seen in previous chapters, the LOD in practice is usually determined as the lowest concentration yielding a signal above the “mean ± 3SD” of the signal intensity from negative controls. Therefore, the LOD of an IA can be lowered from two aspects: first, minimizing the signals from negative controls, approaches for which include
suppressing non-specific binding of interfering molecules to the surface, and reducing background fluorescence of the substrate. It should be noted that there should be a large room for optimization in this aspect, as fluorescent detection can reach a limit of \( \sim 0.5 \) analyte per \( \mu \text{m}^2 \), i.e. \( \sim 10^{-12} \text{ mol m}^2 \) (Zimmermann et al., 2005), which is significantly lower than the surface concentration shown here. The second approach to achieve a lower LOD is to improve the signal from binding reaction at low concentration of analyte, as will be the focus of this section.

From the reaction side, according to Eq. (8.6), the surface concentration of bound antibody, \( B \), is dependent upon the reaction time, the kinetic parameters and surface concentration of antigen. These parameters will be examined respectively below. Also, as demonstrated in Section 8.3.1, bound signal also increases with improvements on the transport side, i.e., higher flow velocity of antibody solution. Here, the effect of velocity will again be investigated, not separately but combined with that of the kinetic parameters and the surface concentration of antigen.

1) Reaction time

Figure 8.3 (a) shows the binding curves for different concentrations of antibody, at a flow velocity of 100 \( \mu \text{m s}^{-1} \). While for high concentrations of antibody, e.g. 10–100 nM, reaction reaches equilibrium rapidly, at low antibody concentrations, reaction proceeds slowly and does not approach equilibrium in the 1500-s computed time range. In this case, the concentration of antibody molecules bound to the surface depends on the duration of reaction. Longer reaction produces more bound antibody, and, hence, a lower LOD.
Two examples are shown in Table 8.1, using the data from the two dashed lines in Fig. 8.2 (a). In the first case, it is assumed the reaction is stopped at 300 s and the detection limit is 0.3 nM. The surface concentration of bound antibody, $3.4 \times 10^{10}$ mol m$^{-2}$, can also be reached by the 0.1 nM antibody, at an extended reaction time of 1005 s. The LOD is thus lowered by 3 times when reaction time is 3.3 times longer. Accordingly, the LOD becomes 1 nM and 3 nM, when reaction time is shortened to 88 s and 29 s, respectively. Therefore, the relative reaction time change is roughly inversely proportional to relative change of LOD, as shown in the plot in Fig. 8.3 (b). This relationship also applies to another case shown in the lower half of Table 8.1, in which the LOD is assumed to be 0.1 nM at a reaction time of 300 s.

The above results also demonstrate the flexible switching between rapidity and sensitivity for an existing IA by controlling the reaction time, a useful feature for IA in practice. The relationship between $\Delta t/t$ and $\Delta$LOD/LOD, as shown here, should be established first, to allow estimation of the reaction time for a targeted LOD.

2) Kinetic parameters

Different from reaction time, which can be flexibly adjusted, kinetic parameters, i.e., the association and dissociation rate constants, are intrinsic to a certain Ab-Ag binding reaction and indicate the affinity of binding. Improving kinetic parameters, i.e. increasing $k_{on}$, or decreasing $k_{off}$, or both, thus means to increase the affinity. This improvement can be achieved by using purified and more specific reagents, which, in the case of this study, can be the use of purified antigen rather than whole lysate proteins. In addition, using non-adsorptive approach for the immobilization of capture molecules, for
In this study, antigen, can also increase the affinity of binding by preventing denaturation and preserving the activity of antigen.

In spite of the efforts involved, it is worthwhile to improve the kinetic parameters, as the binding reaction can be greatly enhanced. As shown in Eq. (8.6), kinetic parameters exist in the coefficients of both the time-evolving term and the overall bound concentration. The effect of kinetic parameters on the coefficients is exemplified in Table 8.2, assuming an increase of the equilibrium constant, $K$, from $10^9$ to $10^{10}$, via a 10-fold increase of $k_{on}$ or 10-fold decrease of $k_{off}$, respectively. Four low antibody concentrations, from 0.1 to 3 nM, are investigated.

The increase of $k_{on}$ leads to increases of both the coefficient of time and of bound concentration. Namely, the increase of $k_{on}$ not only accelerates the process to reach equilibrium but also elevates the surface concentration at equilibrium. Also, the higher the antibody concentration, the greater the increase of the coefficient of time. This relationship is depicted in Fig. 8.4. The factor of growth of $(k_{on}C + k_{off})$ increases from 1.82 to 7.75 when antibody concentration is increased from 0.1 nM to 3 nM. On the other hand, the coefficient of bound concentration grows more slowly at higher antibody concentration, as also illustrated in Fig. 8.4. The factor of growth of $k_{on}C/(k_{on}C + k_{off})$ reduces from 5.5 to 1.29 when antibody concentration increases from 0.1 to 3 nM.

The 10-fold decrease of $k_{off}$ improves the coefficient of bound concentration the same way as a 10-fold increase of $k_{on}$, as this coefficient is a function of $K$ only. However, the decrease of $k_{off}$ has a negative effect on the coefficient of time, which means the time-evolving process to reach equilibrium is even slower when the kinetics is improved by reducing $k_{off}$ only. The higher the antibody concentration, the less the negative effect. The
overall trend of the factor of growth of \((k_{on}C + k_{off})\) against antibody concentration is the same as the one for the increase of \(k_{on}\) shown in Fig. 8.4, but the factors are all ten times smaller.

Taking antibody concentrations of 0.1 and 1 as examples, the binding kinetics, with the parameters used above, was simulated and the results are shown in Fig. 8.5. The kinetic data are from the starting point of the reaction region, to exclude depletion effect. Decreasing \(k_{off}\), due to the counteracting effects shown above, does not produce significant improvement on the bound concentration, especially for low antibody concentration (0.1 nM). On the other hand, the binding kinetics improves much more pronounced at a higher \(k_{on}\). Especially, bound concentration increases significantly at antibody concentration of 0.1 nM and the time to reach equilibrium is greatly shortened at antibody concentration of 1 nM, as discussed above.

As \(k_{on}\) increases 10 times from \(10^6\) to \(10^7\), the \(Da\) number increases from 0.1 to 1, which means the kinetics changes from being reaction-limited to a situation where transport plays an equally important role. As discussed earlier, increasing velocity can greatly enhance the reaction kinetics at such conditions. This effect is also shown in the binding curves in Fig. 8.5. The increase of velocity by 3 or 10 folds produces further significant improvement in the binding kinetics. A 10-fold increase of velocity brings \(Da\) back to 0.1. As expected, increasing velocity does not make a big difference in the binding kinetics of the 10-fold lower \(k_{off}\), as \(Da\) does not change and the kinetics keeps being reaction-limited.

To evaluate the effect of the kinetics improvement on LOD, the binding curves of threefold or fivefold higher antibody concentration at original kinetic parameters are also
included in Fig. 8.5. Clearly, for both antibody concentrations, even without velocity increase, the 10-fold higher $k_{\text{on}}$ produces a comparable or better binding kinetics than that of the threefold higher antibody concentration. A three-fold velocity increase to 300 µm s$^{-1}$ is sufficient to gain comparable or better kinetics than that of the fivefold higher antibody concentration. Therefore, for the range of antibody concentration examined here, a 10-fold greater $k_{\text{on}}$ can effectively reduce the LOD by over 60%. When combined with appropriate velocity increase, the LOD can be lowered by ~80% or even more. Also, from the trend shown here, the lower the LOD, the more difficult it is to refine it further by improving the kinetic parameters.

3) Surface concentration of capture molecules

Surface concentration of capture molecules is of primary importance in an IA. As shown in Eq. (8.6), at idealized conditions, the amount of bound molecules, $B$, is directly proportional to the surface concentration of capture molecules, $R_s$. That is why the optimization of antigen-coating condition is the usually first stage in IA development, in the experimental studies shown in Chapters 4 and 5. Further improvement of the surface concentration of antigen can be achieved by changing the antigen immobilization approach to non-adsorptive ones, such as covalent bonding. The total number of high-affinity probing antigen increases when immobilized antigen is not denatured. Surface concentration can also be increased by enlarging surface area via creating surface roughness (Rossier et al., 2000) or using porous substrates (Yakovleva et al., 2002).

The directly proportional relationship between the concentrations of probing antigen and of bound antibody, as described in Eq. (8.6), applies only when the reaction is highly reaction-limited. Figure 8.6 shows the binding curves of 100 nM antibody at
four different surface concentrations of antigen. At $R_t$ of $10^{-9}$ and $10^{-8} \text{mol} \cdot \text{m}^{-2}$, the Da number is 0.01 and 0.1, respectively. The shapes of binding curves at these two conditions are almost identical. However, at increased $R_t$ of $10^{-7}$ and $10^{-6} \text{mol} \cdot \text{m}^{-2}$, Da grows to 1 and 10, respectively. Transport-limitation becomes increasingly strong, as apparent from the longer time to reach equilibrium in the curves. Therefore, the increase of $R_t$ would also be most effective when combined with velocity change, for the case of increasing $k_{on}$ shown in the previous section.

Still, two low antibody concentrations of 1 nM and 0.1 nM are chosen as examples to simulate the binding kinetics with a 10-fold increase of $R_t$ from $10^{-8}$ to $10^{-7} \text{mol} \cdot \text{m}^{-2}$ and the results are shown in Fig. 8.7. As expected, the higher $R_t$ does not accelerate the binding process to reach equilibrium but greatly increases the amount of bound antibody. When velocity keeps at 100 $\mu\text{m} \cdot \text{s}^{-1}$, surface concentration of bound antibody increases by 3.2 and 4.2 times, for antibody concentrations of 0.1 and 1.0 nM, respectively, at reaction time of 1500 s. At increased velocity of 1000 $\mu\text{m} \cdot \text{s}^{-1}$, the increases of bound antibody further grow to 6.8 and 8.0 times, respectively. Even a moderate velocity increase to 300 $\mu\text{m} \cdot \text{s}^{-1}$ brings about an additional ~ twofold increase, compared to the original antibody concentrations. The corresponding increase of velocity thus plays an important role, in making the most out of the improvement of $R_t$. It should also be noted that in case that $R_t$ is improved by modifying the antigen immobilization approach, e.g., to covalent bonding, both the surface density of antigen and the affinity constant increases. It is then more important to use higher velocity to match the much-increased reaction kinetics, as it is the product of and $k_{on}$ and $R_t$ that indicates the rate of binding reaction, in the expression of $Da$ (Eq. 8.7).
The binding curves of higher antibody concentrations of 3, 5 and 10 folds, at original surface concentration of antigen, are also included in Fig. 8.7, to evaluate the effect of the improvement of $R_t$ on LOD. At antibody concentration of 1 nM, the amount of bound antibody already exceeds the surface concentration at equilibrium at the original $R_t$, after ~600 s of reaction, as shown in Fig. 8.7 (a). At antibody concentration of 0.1 nM, the binding kinetics at 100 $\mu$m s$^{-1}$ and 300 $\mu$m s$^{-1}$ are close to those at antibody concentrations of 0.3 nM and 0.5 nM, respectively, at original $R_t$. Therefore, for the range of antibody concentration examined here, a 10-times greater $R_t$ can effectively reduce the LOD by over 60%, similar to the case of increasing $k_{on}$ discussed earlier. When combined with the increase of velocity, the LOD can be lowered by ~80% or even more. Also, similar to that of increasing $k_{on}$, the trend here also shows that the lower the LOD, the more difficult to refine it further.

8.3.4 Depletion effects

The depletion of analytes (antibody) has been mentioned several times in earlier chapters and was seen from experimental results in Section 5.3.3. It is thus helpful to explore the depletion effects briefly here, to provide a big picture of the development of this phenomenon and its effect on the IA.

Here, “depletion” is not used to describe the cases where the solution is completely depleted of analytes, but rather, the situation that the concentration of analyte in solution keeps decreasing along the flow in a microchannel, because of the consumption of analytes in the binding reaction on the channel surface, which is also known as the moving front phenomenon. This effect is shown clearly in Fig. 8.8, a 2-D concentration profile of antibody solution during the binding reaction ($C_{Ab} = 1$ nM, $t =$
600s). Because of this depletion effect, the binding kinetics of the downstream reaction sites is weaker and equilibrium is reached later, than for the upstream reaction sites.

This phenomenon is illustrated in detail with the profiles of the surface concentration of bound antibody along the 100-µm long reaction region at different times of reaction, as shown in Fig. 8.9 (a) and (b). The curves in Fig. 8.9 (a) are from the initial stages of reaction, 100–600 s. Bound concentration decreases along the flow direction and the negative gradient becomes steeper as reaction proceeds. That is because the rate of binding reaction is higher upstream than downstream, as shown in the profile of the reaction rate in Fig. 8.9 (c). Though the negative gradient of reaction rate diminishes quickly with time due to the decrease of free binding sites upstream, a decreasing trend still exists at 600 s. However, as the reaction upstream proceeds further toward equilibrium, the reaction slows down dramatically and the reaction on the downstream sites occurs at a more rapid rate, as shown in the reaction rate profile at 2000–2500 s, in Fig. 8.9 (d). The bound concentration profile at this time range, shown in Fig. 8.8 (b), indicates that the negative gradient along the reaction region gradually flattens out in the late stages of reaction.

Therefore, if the reaction is stopped before reaching equilibrium, the bound concentration will vary along the reaction region. This heterogeneity is especially strong if the reaction is still at an early stage when stopped. In IA practice, depletion tends to occur when the antibody concentration is low, because equilibrium is established rapidly at high antibody concentration. For example, at antibody concentration of 100 nM, no gradient of bound concentration was present at 80 s. This phenomenon is observed from the experimental results as given in Section 5.3.3: after 600-s incubation, an overall
intensity decline was present along the flow direction, only for antibody concentrations less than 0.2 $\mu$g mL$^{-1}$ (1.3 nM), while at concentrations higher than 2 $\mu$g mL$^{-1}$ there is no such a trend.

It is easy to understand that since the depletion effects occur as a result of analyte consumption along the flow direction, the effects will be weaker when the reaction is less transport-limited, i.e., at higher velocity, or lower $k_{on}$ or $R_t$.

Due to the depletion effects discussed above, using extra reaction sites along the flow direction and averaging the bound signals increases the effects of analyte transport on the result and reduces the accuracy of results. It is better to use only one reaction site to collect bound signal.

Another situation where the depletion effects could be of concern is where the solution-phase antibody is present not as analyte, but as a detection antibody to bind multiple surface-bound samples. This case includes the second binding reaction in the EK-IA using detection antibody, or the assay for surface-coated antigen as conducted in Chapter 4. The bound signal would not be indicative of the amount of surface-bound analyte if the antibody solution were depleted along the flow. However, there was little chance that depletion would have occurred in those cases, because of the high concentration of antibody solutions. The antibody solutions used for detection purposes in the experimental studies, including both the bacterial antibodies and the anti-species secondary antibodies, have concentrations in the range of 10–60 $\mu$g mL$^{-1}$, i.e., 85–400 nM. With the parameters used in this chapter, such high concentrations would have brought the reactions on all of the multiple binding sites to equilibrium, during the 300-s incubation. This topic will not be further investigated here, but optimization of the
reaction for detection purposes (rather than for capture purposes as discussed above) can also be conducted using numerical simulation, either to economize reagent consumption or to reduce the incubation time.

8.4 Summary

This chapter investigates the reaction kinetics of microfluidic EK-IA, using numerical simulation as the primary tool. A modified Damköhler number ($Da$), which accounts for the reaction rate and mass transport rate inclusive of both convection and diffusion, is proposed and used throughout the chapter to provide an estimation of the transport-limitation on reaction kinetics.

Under transport-limited kinetic conditions, i.e., $Da > 1$, increasing flow velocity can greatly improve the reaction kinetics; whereas the binding rate is not as sensitive to the increase of velocity if the kinetics is strongly reaction-limited. With the reaction conditions used in the simulation, a velocity of 100 $\mu$m s$^{-1}$, typically used in the EK-IA experiments, provides good reaction kinetics ($Da = 0.1$) not significantly weaker than the ideally reaction-limited case.

The reaction kinetics depends strongly on the association rate constant and the surface concentration of binding sites. A 10-fold increase of either of the two parameters can lower the LOD by over 60%. When the change of these parameters shifts the kinetics to a transport-limited one, corresponding increase in the flow velocity demonstrates significant further enhancement of the binding kinetics.
The LOD can also be lowered by extending the reaction time. The relative change of the reaction time is inversely proportional to the relative change of LOD. The reaction time can thus be flexibly adjusted in practice, according to the targeted LOD.

The depletion effects caused by the decrease of antibody concentration along the flow are also depicted. Due to the stronger reaction kinetics upstream than downstream, putting extra reaction sites along the flow and averaging the bound signal can introduce transport-induced errors to the results. However, when the solution-phase antibody is not the analyte but a detecting reagent, depletion is not a concern, since the antibody concentrations are usually high enough to have the reactions on the multiple reaction sites all reach equilibrium.
Figure 8.1 Schematic of a heterogeneous immunoassay for detecting antibody in a microfluidic channel (not in scale). Sample solution containing antibody enters the channel from the inlet at the left and flows through the channel with a plug-like velocity profile. Antigen is immobilized at the bottom surface, within a region with width \( w \). The dimensionless parameters take the values of: \( w = 100 \ \mu m \), \( h = 20 \ \mu m \).
Figure 8.2 The effect of velocity on Ab-Ag binding kinetics. The kinetic data are from the mid-point of the reaction region (50 µm from the start point). (a), (b) Kinetic curves of the binding process at antibody concentration of 100 nM (a) and 1 nM (b), respectively. (c) Time to reach equilibrium at different flow velocities, from the data in (a). (d) Surface concentration of bound antibody as a function of velocity, from the data in (b), at two different durations of binding.
Figure 8.3 (a) Binding curves for different concentrations of antibody. The flow velocity is 100 µm s\(^{-1}\) for all the cases. (b) The reaction time to reach certain bound concentrations, as indicated by the two dashed lines in (a), as a function of antibody concentration.
Figure 8.4 The growth of the coefficient of time, $k_{\text{on}}C + k_{\text{off}}$, and overall coefficient of bound concentration, $k_{\text{on}}C / (k_{\text{on}}C + k_{\text{off}})$ against antibody concentration, at the increase of $k_{\text{on}}$ from $10^6$ to $10^7$. 
Figure 8.5 Comparison of the binding kinetics at a 10-fold increase of equilibrium constant. (a) Antibody concentration is 1 nM. (b) Antibody concentration is 0.1 nM. The bottom dotted lines represent the original binding curve before kinetics improvement. The solid lines represent the binding curves at a 10-fold greater \(k_{\text{on}}\). The dashed lines represent the binding curves at a 10-fold lower \(k_{\text{off}}\). The lines in “△” and “○” represent the binding curves at the original kinetic parameters, but increased concentration to 5-fold and 3-fold, respectively.
Figure 8.6 The binding kinetics of 100 nM antibody at four different surface concentrations of probing antigen.
Figure 8.7 Comparison of the binding kinetics at a 10-fold increase of the surface concentration of antigen from $10^{-8}$ to $10^{-7}$ mol m$^{-2}$. (a) Antibody concentration is 1 nM. (b) Antibody concentration is 0.1 nM. In both graphs, the bottom dashed line denotes the original binding curve before the surface concentration of antigen increases. The three solid lines represent the binding curves at a 10-fold greater $R_t$. The three lines in “○”, “□” and “△” represent the binding curves at the original surface concentration of antigen, but increased antibody concentration to 3, 5, or 10-fold, respectively.
Figure 8.8 The concentration field of antibody in the microchannel, after 600-s of reaction. The concentration of antibody is 1 nM.
Figure 8.9 Reaction kinetics along the reaction region during the binding reaction of 1 nM antibody. (a), (b) Profiles of surface concentration of bound antibody, at the moments of 100, 200, 300, 400, 500 and 600 s (a), and 2000, 2100, 2200, 2300, 2400 and 2500 s (b), respectively, from bottom to top. (c),(d) Profiles of the rate of binding reaction, at the moments of 100, 200, 300, 400, 500 and 600 s (c), and 2000, 2100, 2200, 2300, 2400 and 2500 s (d), respectively, from top to bottom.
Table 8.1 Relationship between antibody concentration and reaction time, for targeted bound concentrations.

<table>
<thead>
<tr>
<th>Surface concentration of bound antibody, mol m(^{-2})</th>
<th>Antibody concentration, nM</th>
<th>Reaction time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.40 (\times 10^{10})</td>
<td>3</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1005</td>
</tr>
<tr>
<td>1.14 (\times 10^{10})</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>1140</td>
</tr>
</tbody>
</table>
Table 8.2 The effect of a 10-fold increase of $K$ by varying $k_{on}$ or $k_{off}$ on the coefficients of reaction in Eq. (8.6).

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Antibody concentration, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>$k_{on}C + k_{off}$ s(^{-1})</td>
<td>Original</td>
</tr>
<tr>
<td></td>
<td>$k_{on}*10$</td>
</tr>
<tr>
<td></td>
<td>$k_{off}/10$</td>
</tr>
<tr>
<td></td>
<td>Factor of growth at $k_{on}*10$</td>
</tr>
<tr>
<td></td>
<td>Factor of growth at $k_{off}/10$</td>
</tr>
<tr>
<td>$k_{on}C / (k_{on}C + k_{off})$</td>
<td>Original</td>
</tr>
<tr>
<td></td>
<td>$k_{on}*10$</td>
</tr>
<tr>
<td></td>
<td>$k_{off}/10$</td>
</tr>
<tr>
<td></td>
<td>Factor of growth at $k_{on}*10$</td>
</tr>
<tr>
<td></td>
<td>Factor of growth at $k_{off}/10$</td>
</tr>
</tbody>
</table>
Chapter 9 Conclusions and future work

9.1 Conclusions and contributions of this thesis

This thesis has been devoted to the development of a high-throughput electrokinetically-controlled heterogeneous immunoassay (EK-IA) microfluidic chip for clinical application, and is best broken into three main parts. The first part (Chapters 2-5) is experimental work focusing on the development and improvement of the EK-IA, through a series of stages, to a parallel, multiplexed and automated electrokinetically-controlled IA prototype. The second part (Chapters 6-7) is experimental work on the application of the EK-IA to the testing of clinical fluids, including human serum and embryo culture media. The third part (Chapter 8) is theoretical work on the reaction kinetics study the EK-IA. Specifically the major conclusions and contributions of this thesis are:

1. The EK-IA microchip developed (Chapter 5) is the first high-throughput (multi-sample and multi-analyte) heterogeneous IA based on the EK platform. It is superior to other heterogeneous IA microchip from the following aspects:
   a) Taking advantage of the EK-driven flow, the entire assay is automatically conducted by applying fluidic control over the $\mu$FN. In designing the $\mu$FN and obtaining the fluidic control parameters, the tool of CFD has been effectively used.
   b) Owing to the use of CFD for precise fluidic control, a highly integrated $\mu$FN is developed for the high-throughput EK-IA, with minimum number of channels and wells. This integrated $\mu$FN not only possesses a small footprint, but also
significantly reduces reagent consumption and the work of manual solution operations.

c) Compared with other EK-based heterogeneous IA, the EK-IA developed in this thesis not only has the desirable feature of high-throughput, but also has achieved a much lower LOD, which is well comparable with other heterogeneous IA microchips. This result demonstrated that the EK-driven flow, though having some limitations for conducting certain assays, is a suitable platform for heterogeneous IA.

2. The clinical applicability of the EK-IA has been successfully proved by testing human sera for the infection status of both \textit{H. pylori} and \textit{E. coli} O157:H7. This low-cost, rapid (22 min), multiplexed, automatic and low-volume (8 \textmu L) EK-IA thus shows great potential for the point-of-care, with further integration of the power supply and detection system, onto a portable device.

3. Aimed to provide a practical guideline for optimizing the performance of the EK-IA (and flow-based surface reaction in general), theoretical study has investigated the effect of both operational parameters (including velocity and incubation time) and inherent parameters (including kinetic parameters and probe density) on the reaction kinetics (Chapter 8). A modified Damköhler number ($Da$) has been proposed and effectively used to directly relate convective mass transport to reaction kinetics.

9.2 \textbf{Proposed extensions of the thesis work}

Several aspects for extending the current work are briefly addressed below.
Continuing research on the application of EK-IA for detecting specific biomarkers in embryo culture media. As mentioned in the introduction of Chapter 7, this is a very suitable application area where the introduction of EK-IA might bring a breakthrough. The advantages of the EK-IA could be fully exploited for the targeted application, including small sample consumption, multiplexed detection, short assay time and parallel assay. Therefore, this project should be further developed. This project should be able to move forward greatly, if the most critical technical problem of capture antibody (cAb) immobilization could be solved. As mentioned in Chapter 8, using covalent bonding rather than adsorption should increase both the association rate constant and the surface density of probing molecule, thereby significantly improve the reaction kinetics. Considering the proper orientation of antibody, the immobilization can be done by covalent bonding of protein A or protein G first as an anchoring layer to the wall, which then binds the cAb.

Adopting integrated detection scheme. The current EK-IA system depends on expensive fluorescence microscopy, which restricts its application to well-equipped laboratories. Though a portable EK-IA with laser-induced fluorescent detection has been developed (Xiang et al., 2006), modification with that system to detect the matrix of signals from the high-throughput EK-IA is yet to be proved. Another option is to use the highly integrated waveguide-based fluorescent detection.

Expanding functions of the EK-IA. The powerfulness of using numerical simulation as a tool for the design and optimization of an EK-IA and for obtaining controlling
parameters has been demonstrated in Chapter 5. This tool can be used to design more functional EK-IA chip with complicated microfluidic networks. For example, the function of serial dilution of the sample can be incorporated as well, to further eliminate manual work. Further improvement can also be extended to the assay of whole blood samples, with modules of blood cell filtration (e.g. using membrane), serum dilution, and assay of serum components.
REFERENCES


immobilized antibodies and chemiluminescence detection. *Analytical Chemistry* 74(13), 2994-3004.


