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Development of microfluidic-based heterogeneous immunoassays

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1. ABSTRACT

Microfluidic-based heterogeneous immunoassays are reviewed in this article by first introducing the principle of immunoassay, followed by a discussion of microfluidic-based technology. Microfabrication, surface modification, solution dispensing and detection technology are discussed and their applications to biomolecular detection reviewed. In the future, improved manufacturing processes and integrated assay systems in an automatic fashion with a reduced assay time and reagent consumption will allow for the effective detection of biomolecules that are of interest in medical diagnostics, drug discovery and bioterrorism.

2. INTRODUCTION

Microfluidics technology has seen tremendous evolution during the last 10 years, because it forms the basis for miniaturization of many conventional laboratory analytical processes, including on-chip DNA analysis, cytometry, immunoassay and integrated detection technologies (1-4) The need for miniaturization is driven by technological advances in microfabrication and the recent explosion in biotechnology with high throughput, small volume, combinatorial screening representing the new paradigm for proteomics and drug discovery (5).

Table 1. Comparisons between lab-on-a-chip and conventional immunoassay

Characteristics	**Lab-on-a-chip**	**Conventional ELISA**
Assay time	≤ 1 hour	Hours to days
Reagent consumption	25 nl-2 μl (100 nl – 10 μl)	> 50 μl
Solution delivery	Flow-based	Pipetting
Throughput	Multiplexed (OR multi-analyte)	Single-analyte
Sensitivity	Comparable to conventional assay	pg to μg/ml

Recent advances in microfabrication have led to the development of integrated microfluidic devices, the so-called lab-on-a-chip (6) Microfabrication techniques, such as photolithography and micro-machining, have been employed successfully for making a biochip on many different substrates, including glass, silicon and poly (dimethysiloxane) (PDMS) (7) Lab-on-a-chip devices are microscale laboratories on a chip of several centimeters in dimension, with a network of microchannels etched into the surface. The width or height of a typical microchannel ranges from 10 to 200 μm. These lab-on-chips can perform the specialized functions comparable to their room-sized counterparts. The potential advantages of these lab-on-chips include markedly reduced reagent consumption, improved reaction kinetics, high throughput and automation (7) These features are particularly suitable for miniaturization of immunoassay. Table 1 illustrates the advantages of miniaturization (8)

To date, there have been major advances in the areas of microfluidic capillary electrophoretic separation (9), electrochromatography (10), polymerase chain reaction (11), on-chip mixing (12) and enzymatic reactions (13), detection of macromolecules (14), DNA hybridization (15, 16) and combinations of these strategies (17) (Figure 1) These approaches have potential applications in many fields of medical and biomedical research, particularly related to proteomics (18) and drug development (19)

Immunoassays are currently the predominant analytical technique for the quantitative and qualitative determination of a broad variety of antigens of clinical, medical, biotechnological and environmental relevance (20, 21) The solid-phase heterogeneous immunoassay generally confers high sensitivity and excellent specificity. Major clinical diagnostic strategies utilizing immunoassays include: measurement of hormone and vitamin levels, detection of markers of acute cardiac damage (e.g., troponin T and I) and cancer markers (e.g., carcino-embryonic antigen, α-fetoprotein, P53), therapeutic drug monitoring and detection of drugs of self abuse, autoimmune antibody detection and the diagnosis of microbial infections (e.g., *Helicobacter pylori*, human immunodeficiency virus, hepatitis viruses A to E) (22) Thus, the diagnostic capability of immunoassay is important in both adult and pediatric medicine. Immunoassays remain an important tool for clinical diagnostic purposes.

The scope of this review article is restricted to the development of microfluidic heterogeneous immunoassay technology over the last five years and the relevant design considerations and applications. For more general reviews of protein- or DNA-based microfluidic devices, the reader is referred to references 1-5.

3. CONSIDERATIONS FOR IMMUNOASSAY DEVELOPMENT

Immunoassays can be classified as homogeneous or heterogeneous. In homogeneous immunoassays, the antibody and antigen are free to associate in solution, with direct determination of antibody-antigen complex formation in the solution phase without the need for a solid phase. Such assays, although simple to perform, tend not to be sufficiently sensitive and accurate to meet many needs (23) On the other hand, heterogeneous immunoassays, with one reactant immobilized onto a solid support (membrane or plastic) are highly versatile and allow for multiplexing and parallel analysis of large sample sets, which makes this approach attractive for automation.

All heterogeneous immunoassay configurations are composed of three components: the capture system, the analyte (i.e., the substance that the assay is designed to measure; either antibody (Figure 2a) or antigen (Figure 2b)), and the detection system. The antibody-antigen reaction takes place on a solid surface. Solid phases can be grouped into at least three categories, consisting of plastic material (polystyrene-based), membranes (e.g., nitrocellulose) and beads. Solid-phase reactants can be immobilized by three general procedures: (1) passive adsorption, resulting from strong hydrophobic interactions between proteins and the synthetic surface; (2) covalent attachment to functionalized solid-phases; and (3) immunochemical and other nonadsorbent, noncovalent attachment (e.g., by using streptavidin-biotin linkages) (24) Passive adsorption is most widely used for enzyme-linked immunosorbent assay (ELISA) on microtiter plates and nitrocellulose membranes.

Before probing for the antigens of interest, it is essential to block all other protein-binding sites on the solid surface to prevent non-specific binding (NSB) to the probe. In addition, other sources of NSB include impurities in reagent preparations (e.g., cross-reactive species or antigens), proteins or lipids in the sample (or from hands) that bind label or exposed surface areas (unblocked area) to which assay reagents can directly adsorb (23) A variety of blocking agents are used for polystyrene microtiter plates and membranes, including dilution solutions of bovine serum albumin, nonfat dry bovine milk, casein, gelatin and serum (25) Nonionic detergents, including Tween-20, Nonidet P-40 and Triton X-100 have also been used, which are inexpensive and readily available. While blocking agents may mask nonspecific sites, they may also contribute to new NSB sites. Therefore, testing of compatibility of blocking solutions should be performed with the reagents used.

Primary antibody, in the form of antiserum, affinity-purified polyclonal antibody or monoclonal antibody, is used to probe for the antigen of interest.

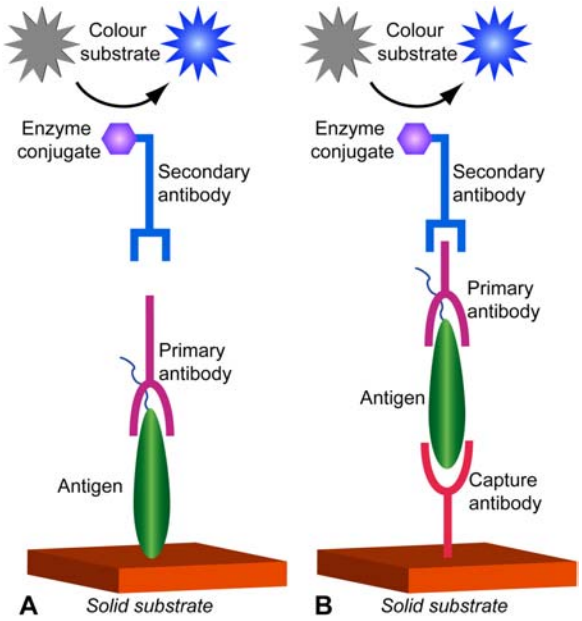

Figure 1. Schematics for generalized ELISA detection of (a) antibody and (b) antigen. Reproduced from Lin (2005) (8).

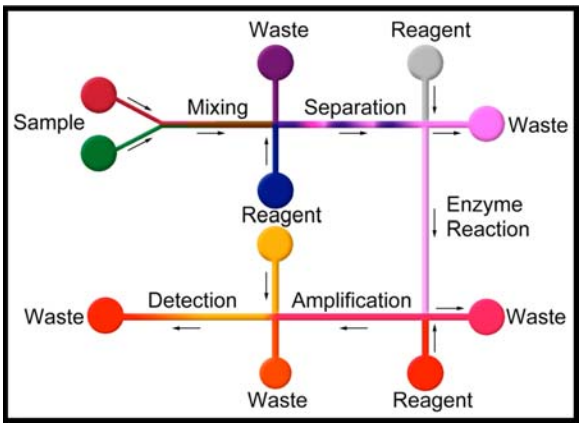

Figure 2.. Schematics of a lab-on-a-chip. Reproduced from (8).

Polyclonal antiserum contains a diverse population of antibody molecules capable of binding a variety of epitopes on the immunizing antigen. In contrast, a monoclonal antibody is an antibody preparation in which all the molecules are identical and have precisely the same variable and constant amino acid sequences in both heavy and light chains. Monoclonal antibody recognizes only a single, highly specific epitope for which it was selected. By contrast, polyclonal antibodies can cross-react to structurally related antigens (26) Although monoclonal antibody offers excellent specificity, it is more expensive to produce compared to polyclonal antibody. Polyclonal antisera can be initially partially purified to yield immunoglobulin (Ig) G, which can then be further purified against particular cross-reactant by using chromatographic methods (27) In addition, pre-adsorption against cross-reacting antigens can be used to reduce cross-reactions (28)

A secondary antibody, usually polyclonal anti-IgG antibody, can be used to amplify the signal as more than one secondary (25); hence, increasing the sensitivity of the assay. As secondary antibodies are typically labeled for detection, this same antibody can be used for many primary antibodies without the necessity of labeling each one. In addition, by labeling the secondary antibody, it avoids the need for labeling the primary antibody by radiolabeling or conjugation, which may lead to nonspecific binding or loss of specificity (25)

There are many labels for antibodies, including, for example, radioisotope labels (e.g., ^{125}I, ^{3}H), enzyme-conjugation (e.g., horseradish peroxidase, alkaline phosphatase), chemiluminescence, fluorescence and nanoparticles (29) Radioisotopes are highly sensitive, but require a scintillation counter for detection of radioactivity (25) Another disadvantage is that precautions must be taken during the handling and disposal of radioisotopes. Enzyme labels may give rise to several different signal sources, depending on the substrate. The most common signal is color. The enzyme catalyses substrate to its colored end-product without changing itself chemically. Therefore, it can keep converting substrate until the reaction is terminated; for example, by changing the local pH. The rate of generation of the color is relatively fast, typically in 30 to 60 minutes. The optical readout is recorded by a spectrophotometer if the immunoassay is performed in microtiter plates. For membrane-based reactions, a precipitating substrate is needed. The readout is quantified by using densitometry. Fluorescent signals are generated by fluorophores, which absorb light at one wavelength and emit light at a longer wavelength, when the appropriate wavelength of light is applied. If the difference between the two wavelengths is large, it is easier to measure the emitted light without interference from incident light (30) Fluorescence is directly affected by temperature, polarity and pH. If washing is not effective before the signal generation stage, interferences may arise from light scattering, background fluorescence and quenching from the sample. At extreme light intensities, photobleaching can occur. Nanoparticles, such as colloidal gold, also have been used for immunoblotting and dot-blotting (31) Particle sizes of 15 to 40 nm are suitable for staining blots. Pink bands are produced, which can be enhanced by developing with a silver stain. More recently, the light scattering properties of submicroscopic metal particles, such as gold nanoparticles, in the size range from 40 to 120 nm, have been investigated (32) These particles scatter incident white light to generate monochromatic light which can be seen either by the naked eye or using dark-field microscopy (33) The intensity generated by a nanoparticle is 100,000 times that of a fluorescein-labeled molecule (33) This approach has been used with success in DNA hybridization arrays, immunohistochemistry and immunoassays (33)

4. MICROFLUIDIC-BASED IMMUNOASSAY DEVELOPMENT

4.1. Materials for microfluidic applications

Silicon, glass and polymer materials are the most common materials used in microfluidic-based technologies. Silicon is structurally very strong. Much of the early

microfluidics work was done using silicon as a substrate, as the technology for photolithography, etching and bonding silicon wafers had already been developed in the microelectronics industry for use in microchips and other micro-electro-mechanical systems (MEMS) Precise patterning can be created in the nanometer scale (34-36) Silicon has high thermal conductivity, which is suitable for applications involving heat transfer, such as the polymerase chain reaction (PCR) It is heat-stable and, therefore, suitable for applications with high-temperature sterilization. Silicon is hydrophobic, capable for adsorption of biomolecules, such as protein, oligonucleotides and DNA. However, there are several disadvantages of using silicon. It is not optically transparent in the wavelength range typically used for optical detection (1) The semiconductivity of silicon may cause problems in electroosmosis, which is a common method for fluid pumping (3) Also, the fabrication process is lengthy and expensive (1)

Glass overcomes many of the above problems encountered by silicon. It is transparent and thus allows for optical detection. Glass possesses a negative surface charge, which, in combination with its good heat conduction ability, making glass surfaces a favorable material for electroosmotic flow (37)

However, the fabrication steps are similar to silicon, which are time consuming and expensive. The wet etching process typically produces a curve bottom, which can complicate optical detection (3)

Polymers are increasingly used in microfluidic research. Advantages include reduced cost and simplified manufacturing procedures. In addition, there is a wide range of available materials, which allows the manufacturer to choose for specific applications. Such polymers include, for example, polystyrene, poly-ethylene (PE), poly-ethylene tere-phthalate glycol (TETG), poly (methylmethacrylate) (PMMA) and poly (dimethylsiloxane) (PDMS) (38) Much of the exploratory research in microfluidic systems has been carried out using PDMS.

PDMS is a silicone elastomer, which has recently been demonstrated as an excellent material for use in microfluidic devices because of inertness, ease of casting, rapidity in prototyping by lithography, good sealing properties, optical transparency, hydrophobicity leading to its ability for protein adsorption and low-cost (39) It is one of the most developed polymers for microfluidics (40) Solid PDMS has been used as implant material for the treatment of urinary incontinence (41) and maxillofacial prosthetic reconstruction (42), and it has good biocompatibility characteristics (43) PDMS comes in the form of liquid (i.e., a pre-polymer), which requires a curing agent to form cross-linking after curing at 75^{o}C for more than one hour. From its liquid form, PDMS can produce a replica of a master mold with a sub-0.1-μm fidelity (40) It has been shown that PDMS can be used to form complex microchannel networks (44) The procedure for the fabrication of masters by soft lithography is standardized and reviewed in detail by McDonald and Whitesides (40)

One advantage of PDMS is that it can seal either to itself or to other surfaces (e.g., glass), reversibly or irreversibly without distortion of the channels (45) PDMS can conformally contact other smooth surfaces, forming a reversible seal. To form an irreversible seal, PDMS and the second surface need to be exposed to an air or oxygen plasma, which cause the surface of the PDMS to introduce silanol groups (Si-CH_3), which form Si-O-Si bonds with plasma-treated glass (45) The silanol groups allow plasma-treated PDMS surfaces to become hydrophilic. However, this oxidized surface is unstable in air and reverts to being hydrophobic in approximately 30 minutes (37) PDMS is more hydrophobic than polystyrene and, hence has a high avidity for proteins (46) Plasma-treated microchannels can be used for passive protein adsorption after exposure to air for more than 30 minutes to take advantage of this reversible hydrophobicity property. Under certain circumstances, where long-term stability of the hydrophilic layer is desired, the oxidized PDMS microchannel can be stored in contact with a polar liquid (45) The EOF mobility of an oxidized PDMS channel can be preserved in this way for 14 days without apparent loss (47)

4.2. Surface modification and protein immobilization

Protein molecules need to be immobilized onto the surface of the microchannel, similar to conventional immunoassays. In the microenvironment, high surface area/volume ratios magnify the effects of NSB. Due to the high surface area/volume ratio, a higher concentration of BSA (5% compared to 1% used in conventional immunoassays), which has been used to pre-coat the surface to block NSB (48) needs to be used in order to achieve effective blocking (49)

The orientation of the protein/antibody on the surface of microchannel is also important. Direct adsorption usually produces random orientation and the active site of protein might be concealed. Direct adsorption of antibody to this protein surface could block the active sites on the surface, resulting in NSB of antigen and the secondary antibody on the surface. As a result, the sensitivity and specificity of the assay can be reduced (3) Microchannels have been modified with a three-layer biotin-neutravidin sandwich coating, made of biotinylated IgG, neutravidin and biotinylated dextran (50) By replacing biotinylated dextran with any biotinylated reagent, the modified surface can be readily functionalized with biochemical probes, such as antibodies. The resulting hydrophilic surface resists non-specific protein adsorption and can support electroosmotic-driven flow. Similarly, covalent binding of primary antibody to surface adsorbed protein A has been reported in a heterogeneous immunoassay (51)

Direct covalent attachment of protein/antibody developed by dramatization of PDMS surfaces with (3-aminopropyl)-triethoxysilane (APTES) The amino groups generated by APTES are either derived into carboxyl group by succinic acid anhydride (SAA) and the protein/antibody then captured either through a cross-linker, 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) or directly reacted with cross-linker, glutaraldehyde (GA)

(52) Although both GA- and EDC- based methods have good covalent immobilization capability, the EDC method has a higher efficiency, possibly due to intermolecular cross-linkage of proteins (52) A solution-based silanization reaction has been developed instead of using oxygen plasma or UV radiation to induce hydrophilicity on the PDMS surface (53) An acidic H_2O_2 solution and a sequential silanization reaction using neat silane reagents is used to modify the microchannel surface embedded in PDMS matrices. Subsequently, functional groups can be introduced, including polyethylene glycol (PEG), amino group, isothiocyanate, peptide, DNA and protein.

Microbeads trapped in a microchannel are used for antibody-antigen reactions, because it can further increase the surface area for immune-mediated reactions (54-56) For instance, Sato *et al.* (54, 55) have integrated an heterogeneous immunoassay system for human secretory immunoglobulin A (s-IgA) and carcinoembryonic antigen (CEA) in cancer diagnosis. Liu *et al.* (56) developed a bead-based microfluidic device and demonstrated a sensitive enzyme-linked immunosorbent assay (ELISA) with quantum dots as the labeling fluorophore for rapid detection of a marine iridovirus.

4.3, Solution and reagent delivery

Solution dispensing strategies have been developed, which can be categorized into pressure-driven flow (57), capillary driven flow (58) and electroosmotic-driven flow (59) Pressure-driven flow is a traditional way of solution delivery. Pressure-driven flow is mediated by a pressure drop across the length of the microchannel using external syringe pumps or pneumatic pressure, and is very similar to classic Poiseuille flow (POF) The flow rate depends on both the pressure drop across the channel and channel resistance. For microchannels, the presence of an electrical double layer near the solid-liquid interface results in an electro-viscous effect on the pressure-drive liquid flow, which can cause further flow resistance (57) The main advantage of pressure-driven flow is that such circuits have little compliance, which makes controlling the exact amount of pumped fluid, and knowing the exact location of a fluid meniscus, much easier (19) The pump used can be as simple as a roller in the blister pouch design (60) or as complicated as a miniaturized syringe or acoustic pump (61) The former is simple, low cost and readily available; however, it offers little opportunity for further miniaturization or for high-throughput testing. The latter is more costly and, for acoustic pumping the choice of materials is limited to piezoelectrics. Ultra-precise syringe pumps and diaphragm pumps with piezoelectric activators can offer suitable low flow rates (62-64) Integrated micropumps have been designed, such as a diaphragm pump with ball valves to produce a flow rate of 2 μl/min (65) Pumps with lower flow rates have also been designed. For example, a microfabricated peristaltic pump was constructed using multilayer soft lithography that can reach flow rates as low as 3 nl/min (66) A gravity-driven microfluidic system has been developed by using height difference between inlet and outlet to achieve a flow rate of 10-1000 nl/min (67)

Capillary flow is naturally induced by surface tension between capillary surfaces and the liquid. Flow can be controlled passively by chemical modifications of surface hydrophobicity and geometrical changes in capillaries (68) Such a design can prove suitable for hand-held diagnostics.

Electrokinetic techniques, such as electroosmosis and electrophoresis, have the advantages that they scale favorably for miniaturization. Electrokinetic pumping is the method of choice for transporting and separating liquid samples in microchannels (3) Electroosmotic flow (EOF) occurs in devices with channel walls made of materials that are charged under a polar medium, such as a buffer solution. The fluid comprising the double-layer proximal to the charged surface will contain a higher-than-bulk fluid concentration of counter-ions, resulting in a charged fluid. If an electric potential is applied parallel to the channel walls using a pair of electrodes, this charged fluid is attracted to the electrode of opposite charge, resulting in fluid flow (69) EOF is dependent of the interaction between channel walls and the enclosed fluid. The flow is efficient in channels of less than 0.1 mm in diameter (19) EOF has been used successfully with a range of materials, including glass (70) and PDMS (59) By changing the polarity of the electrodes in a microfluidic network, accurate amount of solutions can be dispensed, allowing for the development of complex microchannel networks (39) An advantage of EOF over POF is that the former has a plug profile, which causes less dispersion in the cross-stream direction than the parabolic profile arising in a traditional pressure-driven system (71) Electroosmotic flow can range between 1 mm/min to 5 cm/min. EOF rates can be estimated by using current monitoring methods based on differences in conductivity of the sample solution and running buffer which is used to displace the sample in the microchannel (69)

Disadvantages of EOF include the sensitivity of flow velocity to the surface properties of channel wall, the discrimination of different molecules due to electrophoresis, as well as Joule heating (72) The Joule heating effect, together with electrolysis at high current load, particularly render EOF ineffective for high-salt or high-concentration buffers (73), such as those for used for DNA hybridization and cell lysis. Although these drawbacks limit the application of EOF to some bioassays, most are not problematic in heterogeneous immunoassays, provided that only one reagent is transported at a time and the buffer concentration used is below 100 mM (20) The applied electric field strength is on the order of 100-1,000 V/cm. An expensive high voltage power supply is often used. Recently, a portable high voltage power source has been developed (74) employing a 12-V battery and potentiometers to produce a maximum voltage of 620 V. Dodge *et al.* (75) first utilized electrokinetic-driven flow for reagent delivery in an immunoassay. Protein A was immobilized on the channel surface and its affinity for immunoglobulin (IgG) tested by using competitive immunoassay. Linder *et al* (76) also employed electrokinectic-driven flow to conduct a competitive

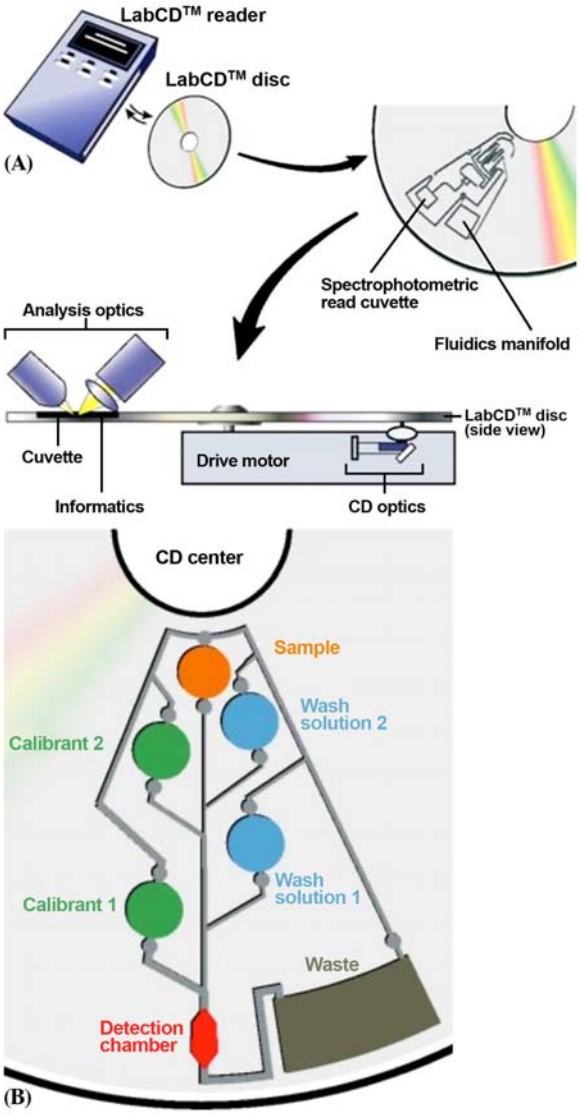

Figure 3. (a) LabCD™ instrument and disposable disc. Here, the analytical result is obtained through reflection spectrophotometry. (b) Schematic illustration of the microfluidic structure employed for the ion selective optode CD platform. The fluidic structure contains five solution reservoirs, a detection chamber, and awaste reservoir. The reservoirs contain the first and second calibrant, wash solutions, and thesample. Upon increasing rotation rates, calibrant 1, wash 1, calibrant 2, rinse 2, and thensample were serially gated into the optical detection chamber. Absorption of the calibrants and sample was measured. Reprinted, with permission, from (77)

immunoassay for human IgG in a biopassivated cross microchannel.

Centrifugal force has also been utilized to move fluids in microfluidic systems. Microchannels are designed radially on a compact disk (CD)-like platform and the fluid is driven by centrifugal force to flow through the microchannel under the rotation of the CD (Figure 3) Many different assays have been tested with such a platform, including immunoassays (77)

Fluid flow within microchannels is laminar (69); hence, diffusion is the rate-limiting step of interactions between molecules (6) As the dimensions of the reaction chamber decrease from a few millimeters in microtiter wells to a few micrometers in microchannels, the time taken to diffuse across the reaction chamber decreases from a few hours to a few seconds (6) Hence, miniaturization increases reaction kinetics.

4.4. Detection methods

Different assay techniques use varying signaling pathways to recognize an antibody/antigen binding event. Most applications use a label to increase the sensitivity of detection. Current labels include both optical and electrochemical approaches (4)

Enzymes, such as alkaline phosphatase and horseradish peroxidase, are important because they can be used to efficiently 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, because the number of detectable molecules can be exponentially higher than the number of antigens (22) Optical labels include color substrate, fluorescence and metals. Colorimetric detection is widely used in conventional ELISA because it is easily quantified by either simple instrument or using the naked eye. A colorimetric precipitating substrate is used in a microchannel-based immunoassay (78) for the detection of *Helicobacter pylori* infection.

Chemiluminescence, which is light emission from the oxidation of luminal to the production of aminophthalate ion catalyzed by an enzyme, is another popular detection method for use in conventional immunoassays and western blotting. It can also be used in microfluidic-based immunoassays (79) The most commonly observed form of detection in microfluidics is fluorescence, primarily due to high sensitivity and the ease of integrating a label conjugated to a protein/antibody molecule. Common fluorescent labels include fluorescein isothiocyanate, Cy5 and tetramethylrhodamine isothiocyanate (TRITC) (4) Fluorescence detection usually involves the use of fluorescent microscope, with a charge-coupled device (CCD) camera or photomultiplier tube (4) Recently, the fluorescence detection strategy has been miniaturized by using laser-induced fiber optical fluorescence detection (Figure 4) (80) This study demonstrated the portability of laser detection module for the detection of enterohemorrhagic *Escherichia coli* O157:H7 antigens. Colloidal gold particles can be used as a label conjugated to antibody immobilized on polystyrene beads within microchannels and detected by using a thermal lens microscope (81) The light scattering properties of nanoparticles can also be detected by dark-field microscope (82) Gold nanoparticle-antibiotin conjugate has been used to detect both *H. pylori* and *E. coli* O157:H7 antigens in microchannels (83)

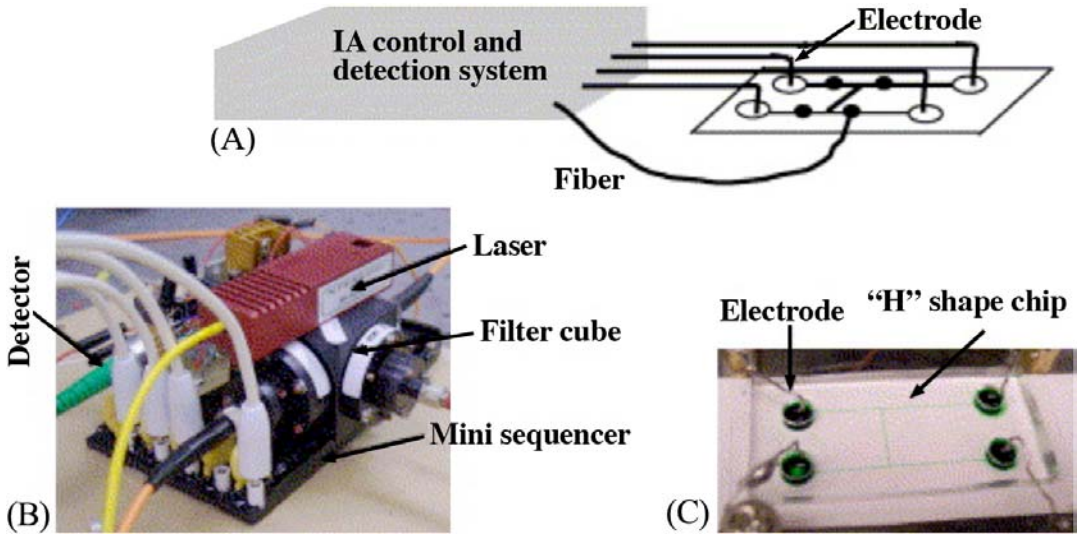

Figure 4. (a) Configuration of an immunoassay test system. Four outputs of a mini-sequencer are connected with electrodes in the wells. Fluorescence excitation and emission are conducted through the fiber under the chip. Fluorescence intensity of two spots on each arm is measured. (b) Picture of the assembled miniature immunoassay control and detection systems. (c) Picture of the "H"-shaped immunoassay chip. Reproduced with permission from (80).

Electrochemistry is another commonly used detection method. Oxidation-reduction reactions of the redox label take place in solution and the resultant electroactive species occur at the surface of the electrode. The potential of the electrode is held at a specific value and the current is measured as a function of time (amperometry) (4) As the electrode diameter is decreased in microfluidic applications, there is an increase in the collection efficiency of the electroactive species at the surface (83) Electrochemical detection has been applied in microfluidic-based immunoassay for detection of D-dimer (84) and ferritin (68)

Surface plasmon resonance (SPR) is widely used as an indirect means of immunodetection. It is a surface sensitive optical technique that is used to study a thin layer on a metal surface. SPR imaging systems apply a collimating monochromatic light beam oriented such that it incidents on a gold film through a prism or a grating-coupling. The angle of incidence is close to the SPR resonance angle and the reflectivity pattern is captured by a charge-couple device (CCD) device (85) The SPR device is capable of performing real-time, high-throughput biomolecular interaction analyses. The major advantages of this approach include the fact that prior labeling of the analytes is not required and that it can rapidly monitor dynamic interactions in real-time (86) The immunosensor is based from the angle shift in SPR derived from the formation of an immune complex on an antibody- or antigen-modified metal surface. For instance, Kurita *et al.* (87) demonstrated an on-chip enzyme immunoassay for the cardiac marker, B-type natriuretic peptide (BNP), using a microfluidic device combined with a portable SPR system (Figure 5) In addition, a microarray immunoassay with a two-dimensional SPR system was developed capable of detecting 1x 10^{-4} mg/ml of IgG by using IgG and anti-IgG interactions (88)

5. APPLICATIONS

Detection of specific antigens and macromolecules based on microfluidic heterogeneous immunoassays has been developed. The detection of viruses such as human immunodeficiency virus (89) and marine iridovirus (56) and bio-macromolecules such as interferons (64, 81), human immunodeficiency virus (89), parathyroid hormone, interleukin-5 (90), C-reactive protein, cardiac markers (91), D-dimer (84), ferritin (68), *H. pylori* antigens (49, 78, 92), and *E. coli* O157:H7 antigens (62, 80, 82, 92) have been realized. Multi-analyte array detection has proven to be feasible for miniaturization of heterogeneous immunoassays (92, 93) A micromosiac immunoassay strategy was developed by Bernard *et al.* (94) and Wolf *et al.* (91) based on patterning different molecules (antibody or antigen) on parallel regions on a surface by means of a microchannel network (95) This approach enables localized immunoreaction between the antigen and antibody in a multi-analyte immunosassay format. Recently, an electroosmotic-driven multi-analyte assay has been developed (92) A microchannel network bearing a pattern of parallel straight channels is used to carry out multiple antigen immobilization. Primary and secondary antibodies, washing buffer solution and waste collection are located in different wells of the H-shaped microchannel network and EOF utilized for solution delivery (Figure 6a-c) Different concentrations of *E. coli* O157:H7 antigens can be detected by using fluorescence microscopy (Figure 6d-e) The detection limit is 3 μg/ml, which is comparable to conventional immunoassays. Different microbial antigens can be immobilized onto a parallel straight microchannel network. Specific primary antibodies are then used for antigen detection (Figure 6f-i) The solution dispensing process and incubation are automated, taking just 26 minutes to complete the entire operation, from dispensing of primary antibody to washing off of the

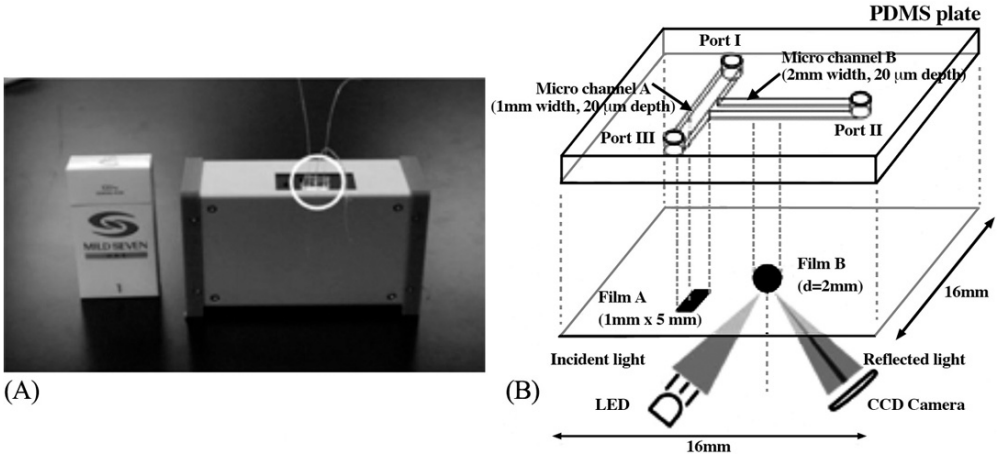

Figure 5. (a) Photograph of a portable immunosensor system with surface plasmon resonance equipment. (b) Schematic representation of a PDMS-based microfluidic immunosensor. Reproduced with permission from (87)

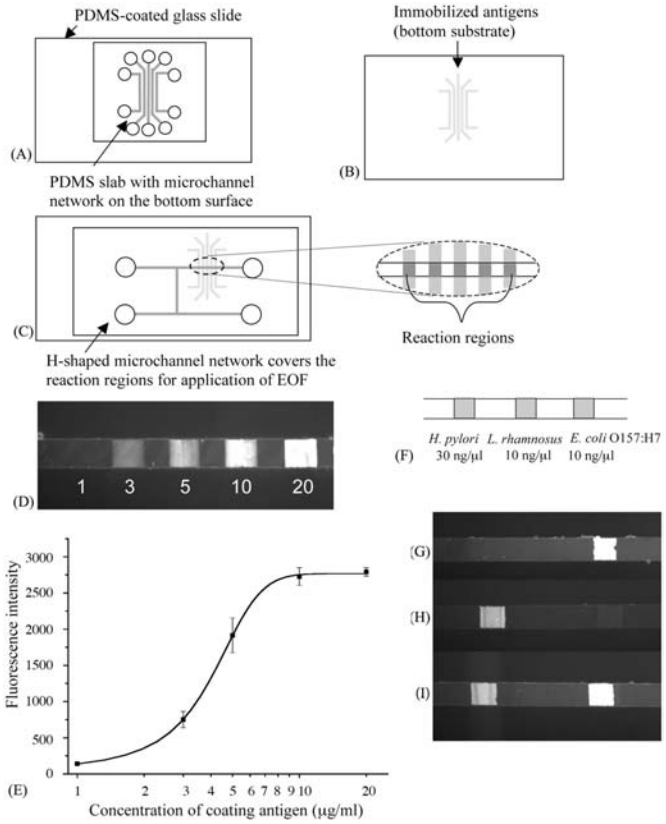

Figure 6. Preparation processes for immunoassays. (a) antigen immobilization using the parallel straight microchannel network; (b) antigen molecules pattern of the surface after a 15-minute immobilization; (c) H-shaped microchannel network put across the antigen pattern. Intersections of the parallel regions of antigen coating and the H-shaped microchannel network become the reaction regions in immunoassay. Fluorescence signal depends on the concentration of coating antigen. (d) Fluorescent image of the immunoassay. Concentrations of coating *E. coli* O157:H7 lysate antigens were (μg/ml, from left to right): 1, 3, 5, 10, 20. (e) Quantified intensity-concentration dependence. Specificity of immunoassays. (f) Sites were coated with different species of antigen (from left to right): *H. pylori* (30 μg/ml), *L. rhamnosus* (10 μg/ml), *E. coli* O157:H7 (10 μg/ml) (g) The primary antibody solution contained only anti-*E. coli* O157:H7 antibody; (h) the primary antibody solution contained only anti-*H. pylori* antibody; (i) the primary antibody solution contained a mixture of anti-*H. pylori* and anti-*E. coli* O157:H7 antibodies. (g)- (i) are the fluorescence microscopy images. Reproduced with permission from (92)

secondary antibody. Furthermore, multiple parallel immunoreactions can be carried out in a single experiment, providing capacity for high throughput screening of antibody-antigen reactions.

6. CONCLUSIONS

Microfluidic devices have been developed for performing various immunoassays that can be employed for the detection of biological agents. Advances in microfabrication, surface modification and detection have greatly aided the development of these devices and facilitate portability. In addition, integrated devices now exist for carrying out multiple reaction steps within the lab-on-a-chip. Although a great deal of research has been conducted, there are still few commercial applications of such devices in the biotechnology industry. One reason for this lag is that all of the necessary technologies-such as fabricating structures, modifying surfaces, integrating detection and the chemistry of the immunoassay-need to be combined and optimized in order to compete with existing methods that have been developed over decades. More robust manufacturing process and integrated assay systems, including sample agent loading, microfluidics and signal detection need to be developed and refined. Decreased reliance on external equipment is necessary in order to make the microfluidic devices portable. Point-of-care devices are important for medical diagnostics. More recently, there is greater demand for such portable devices for the detection of agents of bioterrorism. This would require further development of on-chip raw sample pre-treatment capability, miniaturized optical sensors and detectors and low consumption power source. Reliability and sensitivity also need to be further verified in a variety of broader applications. The need for high throughput characterization of drug candidates and rapid methods of analysis during drug testing are important driving forces for the future development of microfluidic devices.

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