

Immunoassays in microfluidic systems

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Abstract Immunoassays have greatly benefited from miniaturization in microfluidic systems. This review, which summarizes developments in microfluidics-based immunoassays since 2000, includes four sections, focusing on the configurations of immunoassays that have been implemented in microfluidics, the main fluid handling modalities that have been used for microfluidic immunoassays, multiplexed immunoassays in microfluidic platforms, and the emergence of label-free detection techniques. The field of microfluidic immunoassays is continuously improving and has great promise for the future.

Keywords Microfluidics/microfabrication · Immunoassays/ELISA · Biosensors · Bioanalytical methods · Fluid handling/automation multiplex analysis

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Introduction

The immunoassay is a technique that exploits the sensitivity and specificity of antibody–antigen interactions for the detection of relevant analytes. Immunoassays are used for the quantification of proteins and small molecules in a number of different fields such as medical diagnostics, proteomics, pharmaceutical research and biological research. Immunoassays can be classified into main two types: heterogeneous and homogeneous. In heterogeneous immunoassays, antibodies are immobilized on a solid support and interact with the antigen at the boundary layer. In this format, unbound antibodies and other reagents can be easily removed. In homogeneous immunoassays, antibodies interact with antigens in solution. In this case, the bound and unbound antibodies are discriminated based on physical [1, 2] or chemical [3] changes arising from the binding event. Heterogeneous and homogeneous immunoassays can be further divided into competitive and non-competitive modes. In competitive mode, target antigens (from the sample) compete with exogenous labelled antigens for a limited number of antibody binding sites. Thus, the generated signal is inversely proportional to the antigen concentration. This mode is particularly important for small antigens with limited numbers of epitopes (binding sites). In non-competitive mode, antigens are captured by an excess of antibodies and are detected after subsequent binding of a second set of labelled antibodies that bind to the antigen at a different epitope. This forms a “sandwich” immunoassay, in which the signal is proportional to the antigen concentration. This mode is only compatible with large analytes (>1,000 Da) that have more than one epitope [4].

Heterogeneous immunoassays are conventionally performed in microtiter plates with 96 or more sample wells. This is labour-intensive as it requires at least six sequential

steps: (1) sample preparation, (2) antibody and analyte delivery, (3) mixing and incubation (4) washing unbound antibodies, (5) substrate delivery and (6) detection. Incubation time can require several hours because analyte molecules must diffuse across long distances before they encounter antibodies on the surface. To improve throughput of these processes, robotic systems can be used for fluid handling, but this solution is only available to wealthy laboratories, and requires significant maintenance efforts and a large laboratory footprint.

One way of circumventing problems associated with conventional immunoassays is miniaturization in microfluidic systems. The most common microfluidic paradigm relies on networks of enclosed micron-dimension channels. At these small scales, fluids exhibit laminar flow, i.e. fluidic streams that flow parallel to each other, and mixing occurs only by diffusion [5]. Microfluidic immunoassays offer at least three advantages over conventional methods [4]: (1) increased surface area to volume ratios speeds up antibody–antigen reactions; (2) smaller dimensions reduce the consumption of expensive reagents and precious samples; and (3) automated fluid handling can improve reproducibility and throughput. These advantages can potentially improve the performance and reduce the operating cost of conventional immunoassays.

This review focuses on some of the major developments in microfluidic immunoassays between the years 2000 to 2010. Reviewing the field in its entirety would be challenging; here, we highlight unique contributions in four areas: microfluidic immunoassay configurations, fluid handling modalities, multiplexed platforms, and label-free detection strategies.

Microfluidic immunoassay configurations

Microfluidic immunoassays have been implemented in both heterogeneous and homogeneous configurations. In heterogeneous configurations, antibodies are either immobilized on the surface of the microfluidic device or on micron-dimension beads (“microbeads”) embedded in the device. In homogeneous configurations, bound and unbound antibodies are mostly discriminated by their electrophoretic ratio mobility in microchannels. This section will illustrate several emerging strategies that determine the performance of surface-, microbead- and electrophoresis-based immunoassays.

Heterogeneous surface-based immunoassays

Antibody immobilization

The method of antibody immobilization on the microfluidic substrate can dramatically influence the performance of a surface-based immunoassay. The substrate can be made from

materials such as glass, polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), silicon, silicon nitride, polystyrene and cyclic polyolefin; the pros and cons of the various substrates have been reviewed elsewhere [4–6]. The simplest form of immobilization is physical adsorption of antibodies/antigen onto the surface [7–9]. Physical adsorption can reduce antibody binding activity because of unfavourable orientation, steric hindrance and denaturation [6]. In response to these challenges, many covalent and bioaffinity immobilization strategies have been developed for immunoassays in microfluidic platforms. Yakovleva et al. [10, 11] compared different strategies for covalent attachment of antibodies and bioaffinity proteins (protein A or G) on silicon substrates and found that covalent attachment with long flexible linkers such as polyethyleneimine (PEI) [12] and dextran (DEX) [13] were more favourable than shorter linkers such as 3-aminopropyltriethoxysilane (APTES). These long flexible linkers circumvent problems associated with antibody accessibility and steric limitations. Other strategies of immobilization include the use of lipids [14–16], DNA [17, 18], poly(ethylene glycol) (PEG), polyacrylate chains [19], protein G hydrogels [20] and nanofibrous membranes [21, 22]. To preserve the functionality of immobilized antibodies, sugars [23, 24], sol-gels [25] and desiccants [26] have been used to protect antibodies against denaturation. Recently, Wen et al. [27] developed a novel antibody immobilization method on PMMA surface that enhanced antibody binding efficiency and capture activity. In this work, biotin-poly(L-lysine)-*graft*-poly(ethylene glycol) (biotin-PLL-*g*-PEG) was used as a surface linker to minimize the repulsive force between antibody and surface. The PMMA surface was activated by oxygen plasma and grafted with poly(acrylic acid) (PAA) to add functional carboxyl groups. The carboxylic groups formed electrostatic interactions with biotin-PLL-*g*-PEG and PLL-*g*-PEG molecules, and biotinylated protein A was linked to the surface via NeutrAvidin bridges (Fig. 1a). Protein A is a bioaffinity protein that selectively immobilizes the Fc regions of the antibody, enabling favourable orientation. The non-adhesive PEG chains on the surface prevented denaturing of the NeutrAvidin and reduced non-specific adsorption of other proteins. This strategy resulted in higher antigen capture efficiency than PEI covalently linked to protein A. In addition, the long-branched chain of biotin-PLL-*g*-PEG combined with PLL-*g*-PEG helped distribute protein A on the surface to minimize steric hindrance and enabled efficient binding of antibodies.

Analyte delivery and washing

Innovative strategies for analyte delivery and washing can improve the sensitivity, duration and limit of detection of surface-based immunoassays. Although diffusion distances

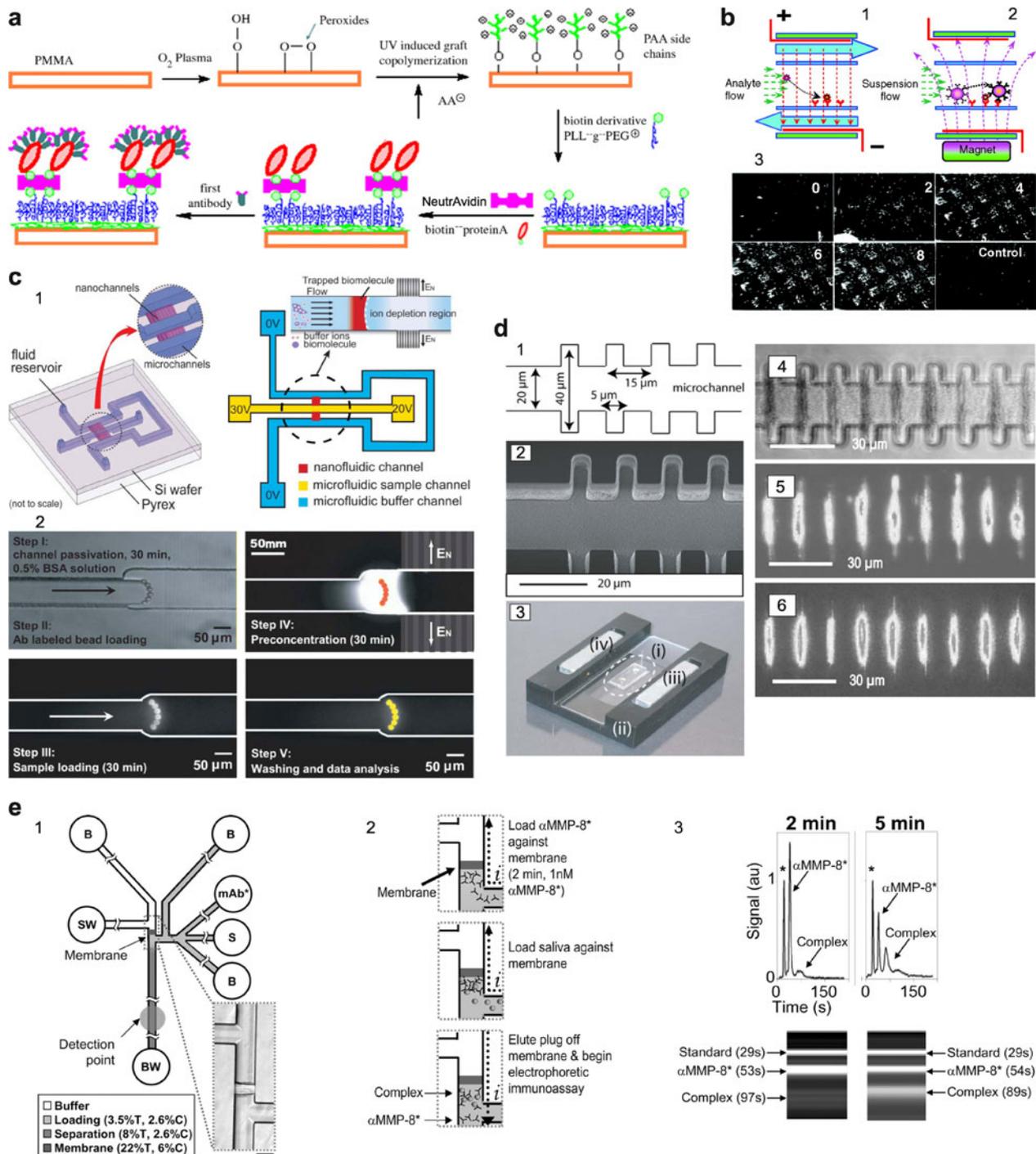


Fig. 1 Microfluidic immunoassay configurations. **a** Schematic of antibody immobilization strategy using biotin-PLL-g-PEG and biotin-protein A linked by NeutrAvidin. (Reprinted from [27], with permission from Elsevier.) **b** 1 Electrophoretic attraction of charged analytes to antibody array from bulk phase. 2 Magnetic attraction of magnetic bead labels to antibody array and removal of non-specifically bound labels by shear force. 3 Dark-field images show magnetic bead label on antibody array at various capture times (mins). (Reprinted with permission from [34]. © 2007 American Chemical Society.) **c** 1 Schematics of the nanofluidic preconcentration device. The middle sample channel is connected to the U-shaped buffer channel by a nanochannel array. 2 Bead loading, immunosensing and

preconcentration procedure. ([77], Reproduced by permission of The Royal Society of Chemistry.) **d** 1–3 Magnetic retention microfluidic device with two permanent magnets (iii, iv). 4 Optical image of the self-assembled magnetic chains. 5 Fluorescent image of bead from off-chip incubation protocol. 6 Fluorescent image of bead from full on-chip protocol (higher intensity). (With kind permission from Springer Science+Business Media [89].) **e** 1 Schematic of gel-electrophoresis device. (%T total acrylamide, %C bis-acrylamide cross-linker). 2 On-chip loading and enrichment of antibody and analyte. 3 Electropherograms and gel-like plots show that enrichment increased signal of complex. ([112], © 2007 National Academy of Sciences, USA)

in microchannels are significantly reduced in comparison to conventional microtiter well plate formats, analytes can still be transport-limited in microchannels at low sample concentrations [28]. One can conceivably lower the dimensions of the devices even further—but this increases fluidic resistance to impractical levels. To circumvent this problem, Hofmann et al. [29] developed a flow confinement method for rapid delivery of small sample volumes to capture antibodies. In flow confinement, a sample flow is joined with a perpendicular makeup flow of water or sample medium. Under laminar flow conditions, the makeup flow confines the sample into a thin layer above the sensing area and increases its velocity. Another strategy to improve analyte capture is to integrate mixing elements in the microfluidic device [30]. Golden et al. [31] demonstrated that embossing patterned grooves on the microchannel can increase immunoassay sensitivity by at least 26%. The grooves induced fluid mixing in the channel which enhanced delivery of analyte to the capture zone and prevented the depletion of analytes at the boundary layer.

Another strategy to improve the performance of surface-based immunoassays is the use of active forces. Mulvaney et al. [32, 33] developed a washing technique termed fluid force discrimination (FFD) to significantly reduce non-specific adsorption and achieved limits of detection of attomolar concentrations. In FFD assays, analytes captured on the surface are labelled with antibody-coated magnetic beads. Subsequently, non-specifically bound beads are removed by applying shear forces. The density of beads that remains bound is proportional to the analyte concentration and can be determined with either optical counting or magnetoelectronic detection of the magnetic labels. In another example, Morozov et al. [34] exploited active forces (electric, magnetic and mechanical) to achieve a zeptomole detection limit within 3 min (Fig. 1b). First, electric fields were generated to electrophoretically draw the analytes to the surface and promote capture. Second, antibody-coated magnetic beads were flowed into the channel while being attracted to the surface with a magnet, causing them to slide over the surface. Finally, the non-specifically bound beads were removed by shear forces, similar to FFD.

Heterogeneous microbead-based immunoassays

Microbeads are frequently used in microfluidic immunoassays as they offer a dramatic increase in surface area to volume ratio and serve as a simple mechanism to reproducibly deliver antibodies to desirable locations [4, 35]. On the other hand, microbeads have the risk of adsorbing to device surfaces, clogging channels, increasing flow resistance, and scattering light [4]. Microbeads can be either magnetic or non-magnetic—this often determines the method of implementation in the microfluidic device.

Non-magnetic microbeads

In non-magnetic microbead-based immunoassays, a physical retention microstructure is necessary to facilitate the removal of unbound analyte or antibodies. Kitamori and co-workers [36–41] pioneered the use of antibody-coated polystyrene beads trapped by a dam structure for heterogeneous immunoassays. Subsequent work by other groups has included variations in detection strategy, fluidic modality and microbead material [42–54]. In a related configuration, discrete microbeads can be immobilized in arrayed microstructures which enable simultaneous detection of multiple analytes [55–59]. As an alternative to physical retention, microbeads can be immobilized by dielectrophoresis [60, 61] or electrostatic forces [62, 63]. The need for microbead immobilization can be avoided with special detection mechanisms involving immunoagglutination [64–69], resistive-pulse sensors [70], deflection velocity sensors [71, 72] or microflow cytometry [73].

Several unique bead-based immunoassay implementations have resulted in superior assay performance. In one example, Yang et al. [74] used superporous agarose beads as a solid support for enhanced detection of goat IgG. Here, the porous beads were covalently conjugated to protein A which immobilized the capture antibody in a favourable orientation. The porosity of the beads lowered the fluidic resistance and increased the effective surface area, thereby enhancing the sensitivity of the assay. In another example, Shin et al. [75] implemented a solid-phase extraction strategy to increase the sensitivity of a competitive immunoassay for C-reactive protein (CRP). In this work, CRP was captured by antibody-coated microbeads packed against a frit and subsequently was eluted in acid buffer. This technique improved the sensitivity by 20-fold which facilitated the use of an inexpensive on-chip photodiode for detection. A similar strategy was successfully employed by Peoples et al. [76] to detect CRP in human serum and cerebrospinal fluid. In a very unique design, Wang et al. [77] demonstrated a nanofluidic-based electrokinetic preconcentrator in a bead-based immunoassay format (1, Fig. 1c). In this work, antibody-coated polystyrene beads were trapped by a dam structure just before the nanofluidic preconcentrator (2, Fig. 1c). When a field is applied across the nanofluidic channels, an ion depletion region is created which can trap biomolecules. If the ion depletion force is balanced by an external flow, biomolecules can be accumulated in the vicinity of the microbeads to enhance antibody–analyte interactions. Within 30 min of preconcentration, the immunoassay sensitivity was improved by 500-fold. The duration of preconcentration can be used to modulate the dynamic range which enables the analysis of protein concentrations that vary over many orders of magnitude.

Magnetic microbeads

The use of magnetic microbeads in microfluidic platforms for immunoassays is an emerging trend, as it eliminates the need for physical retention microstructures. In typical magnetic bead-based immunoassays, antibody-coated magnetic beads are immobilized on the device surface [78–80] or directly on an integrated electrochemical sensor [81–85] for the duration of the assay. In this case, the full utility of the beads is not realized because the beads are localized during antibody–antigen interaction. Ideally, microbeads should be dispersed or resuspended to reduce the diffusion distances between analyte and antibody. On the other hand, immobilizing/resuspending magnetic beads at different stages of the assay requires more sophisticated fluidic handling [86–88]. To circumvent this problem, Lacharme et al. [89] developed a unique magnetic bead retention technique. In this scheme, microchannels with varying cross-sections were used to retain magnetic beads (1–2, Fig. 1d). In the presence of a homogenous magnetic field applied perpendicularly to the channel axis, magnetic beads self-assembled in chains along the channel (3–4, Fig. 1d). This magnetic bead retention strategy facilitated highly efficient mixing and enhanced antibody–antigen interaction. Using this system, two immunoassay protocols for the detection of mouse monoclonal antibodies were compared. In the first protocol, capture antibody and analyte were incubated off-chip, while exposure to the detection antibody was performed on-chip. In the second protocol, the complete immunoassay was executed on-chip. The full on-chip protocol was faster, consumed fewer reagents and was more sensitive compared with the off-chip incubation protocol (5–6, Fig. 1d).

Homogeneous capillary electrophoresis (CE)-based immunoassays

In homogenous configurations, bound and unbound antibodies have been discriminated by differences in diffusion characteristics [2, 90], isoelectric point [91], fluorescence polarization [3, 92], fluorescence resonance energy transfer [93] and enzyme activity [94]. But the most popular form of homogeneous immunoassay is based on capillary electrophoresis (CE), in which immune complex and free antibodies are discriminated based on their electrophoretic mobilities. CE-based microfluidic chips have become very popular because of their compatibility with electrokinetic fluid manipulation, rapid electrophoretic separation and enormous potential for multiplexing [4]. Harrison and co-workers [1, 95–98] pioneered the development of microfluidic CE for immunoassays. In their most elaborate design, they integrated six functional microfluidic CE manifolds on a single chip

and achieved simultaneous quantification of anti-estradiol and ovalbumin in less than 1 min [98]. Subsequent development from other groups focused on implementing electrochemical detection [99–101], increasing throughput [102, 103] and miniaturizing device footprint [104]. In a unique application, Kennedy and co-workers [105–108] developed high-throughput CE-based devices for long-term on-chip monitoring of insulin secretion from islet of Langerhans. Notably, they developed integrated strategies to continuously perfuse fresh reagents and electrophoresis buffers to extend the operation time of their CE devices for up to 24 h [105].

Recently, gel-electrophoresis-based microfluidic immunoassays have gained attention because of their enhanced ability to discriminate between bound and unbound analytes [109–113]. In contrast to non-sieving CE microfluidic chips, gel electrophoresis can separate molecules based on electrophoretic mobility and molecular weight. This is particularly important for large analytes since their charge to mass ratios are very similar to those of the corresponding immune complexes. To ensure that the immune complexes remain intact during gel electrophoresis, the sieving gels must be non-denaturing; examples of such gels include methylcellulose [111] and polyacrylamide [113]. Using different compositions of polyacrylamide gels, Herr et al. [112] developed a gel-electrophoresis-based immunoassay microchip that integrated sample pretreatment (mixing, incubation and enrichment) and electrophoresis to rapidly quantify matrix metalloproteinase-8 (MMP-8) in saliva. The microchip operates on three photopatterned polyacrylamide elements: (1) a large-pore-size gel for sample loading and preparation, (2) a size-exclusion membrane for sample enrichment and mixing, and (3) a small-pore-size gel for electrophoretic separation of bound and unbound MMP-8 (1, Fig. 1e). First, the antibodies and analytes were electrophoretically loaded against the size-exclusion membrane for a fixed duration. Then, the sample plug was eluted off the membrane for electrophoretic separation. The enrichment process enhanced the antibody–antigen interaction by minimizing diffusion distances (2, Fig. 1e). As a result, a longer enrichment time resulted in a higher immune complex signal (3, Fig. 1e).

Fluid handling modalities for immunoassays in microfluidic platforms

Conventional immunoassays are labour-intensive as they require sophisticated fluid handling steps at various stages of the assay [114]. To miniaturize immunoassays in microfluidic systems, analogous fluidic operations have been developed which use fluid handling forces that fall under three major categories: electric, pressure and passive. Each

category has several unique benefits that can be exploited to improve the performance or versatility of immunoassays.

Electric fluid handling

Electrokinetic flow

In microchannels, electric forces for flow are generated by electrophoretic and electroosmotic interactions of applied fields with ionic species in the fluid [6]. In electrophoresis, charged molecules are manipulated in the presence of electric field by electrostatic forces. In electroosmosis, a layer of fluid enriched in solvated ions is attracted to the oppositely charged walls; in the presence of electric field, the solvated ions and their waters of hydration are driven toward the oppositely charged electrode while dragging the bulk fluid via viscous forces to form a uniform plug-like flow [5]. The direction of this flow can be controlled by applying appropriate voltage polarity to the channel reservoirs and/or changing the net charges on the channel surface. Consequently, electrokinetic flow does not require valves or pumps and is amenable for automation. The use of this fluid modality for surface-based heterogeneous immunoassays was pioneered by de Rooij and co-workers [115, 116] and further popularized by Li and co-workers [7, 117]. Recent simulations by Hu et al. [118] suggest that electrokinetically driven immunoassays have better reaction kinetics than pressure-driven assays because of the uniform plug-like velocity profile afforded by electroosmosis. Although electrokinetic flow has many salient features, there are strict requirements for buffers and reagents used in the channels and materials used for device construction. For example, fluids used in such systems must be conductive; however, if the ionic strength is too high, Joule heating can undermine the performance of the assay. This often precludes the use of biological liquids such as blood and urine [119]. Furthermore, the device substrate material should be non-conductive to prevent electrical breakdown [6].

In addition to fluid transport, electrokinetic forces can be used for electrophoretic separations and sample stacking. Electrophoresis is a useful phenomenon that forms the foundation of CE-based homogeneous immunoassays, in which antigen and immune complexes are discriminated by their electrophoretic mobilities. The sensitivities of CE-based immunoassays can be enhanced by a sample stacking technique termed isotachopheresis (ITP). Using this technique, Mohamadi et al. [111] developed a highly sensitive immunoassay for quantifying human serum albumin (HSA) in a gel-electrophoresis-based microfluidic device (Fig. 2a, b). In this work, a mixture of fluorescently labelled HSA and its immune complex with a monoclonal antibody was preconcentrated

by ITP and resolved by electrophoresis in a methylcellulose solution. ITP preconcentration was facilitated by the inclusion of high-mobility ions in the leading electrolyte (LE) and low-mobility ions in the trailing electrolyte (TE), which were respectively loaded in the buffer waste reservoir (BW) and buffer reservoir (BR) (1, Fig. 2a). The sample mixture was electrokinetically injected into the channel from the sample reservoir (SR) by applying high electric potential at sample waste reservoir (SW) (2, Fig. 2a). Subsequently, a sample plug was injected in the orthogonal separation channel by changing the potential at the reservoirs (4, Fig. 2a). During ITP stacking, the disparate electric field intensities in the TE, sample plug and LE caused the sample plug to focus in a narrow band (3–5, Fig. 2a). Concomitantly, the HSA and immune complex formed distinct bands by electrophoretic separation (6, Fig. 2a). An 800-fold signal enhancement was achieved with respect to control experiments without ITP preconcentration. Figure 2b shows fluorescent images of electrokinetic sample injection and sample preconcentration by ITP. The full process of injection, preconcentration and separation were controlled by an automated sequential voltage switching program. Subsequent work on ITP-CE-based immunoassays by Satomura and co-workers [120, 121] integrated of all assay steps on-chip by using ITP to efficiently mix antibodies and analyte in addition to preconcentration and separation.

Digital microfluidics

In an emerging paradigm of electric fluid manipulation called digital microfluidics (DMF), fluids are controlled as discrete droplets in contrast to continuous flow in channels. In DMF, droplets of reagents and samples are manipulated on an array of electrodes which are insulated by a hydrophobic dielectric layer. By applying sequences of AC or DC electric potentials between ground and actuation electrodes, droplets can be driven to move, merge, split and dispense from reservoirs by a combination of electrostatic and dielectrophoresis forces [122–124]. Sista et al. [86, 125] demonstrated the use of DMF for magnetic bead-based heterogeneous immunoassays to quantify human insulin and interleukin-6 (IL-6) (Fig. 2c, d). The full immunoassay protocol was implemented in six steps: (1) a droplet containing magnetic beads with antibodies, reporter antibodies and blocking proteins (prepared off-chip) was merged and mixed with a droplet of analyte on-chip; (2) the pooled droplet was shuttled on six sets of electrodes for 2 min to allow for antibody–antigen binding (1, Fig. 2c); (3) the reaction mixture was delivered over the magnet to immobilize the magnetic beads (2, Fig. 2c); (4) the unbound supernatant was removed by splitting the excess liquid from the beads (3, Fig. 2c); (5) unbound molecules were further washed by passing five

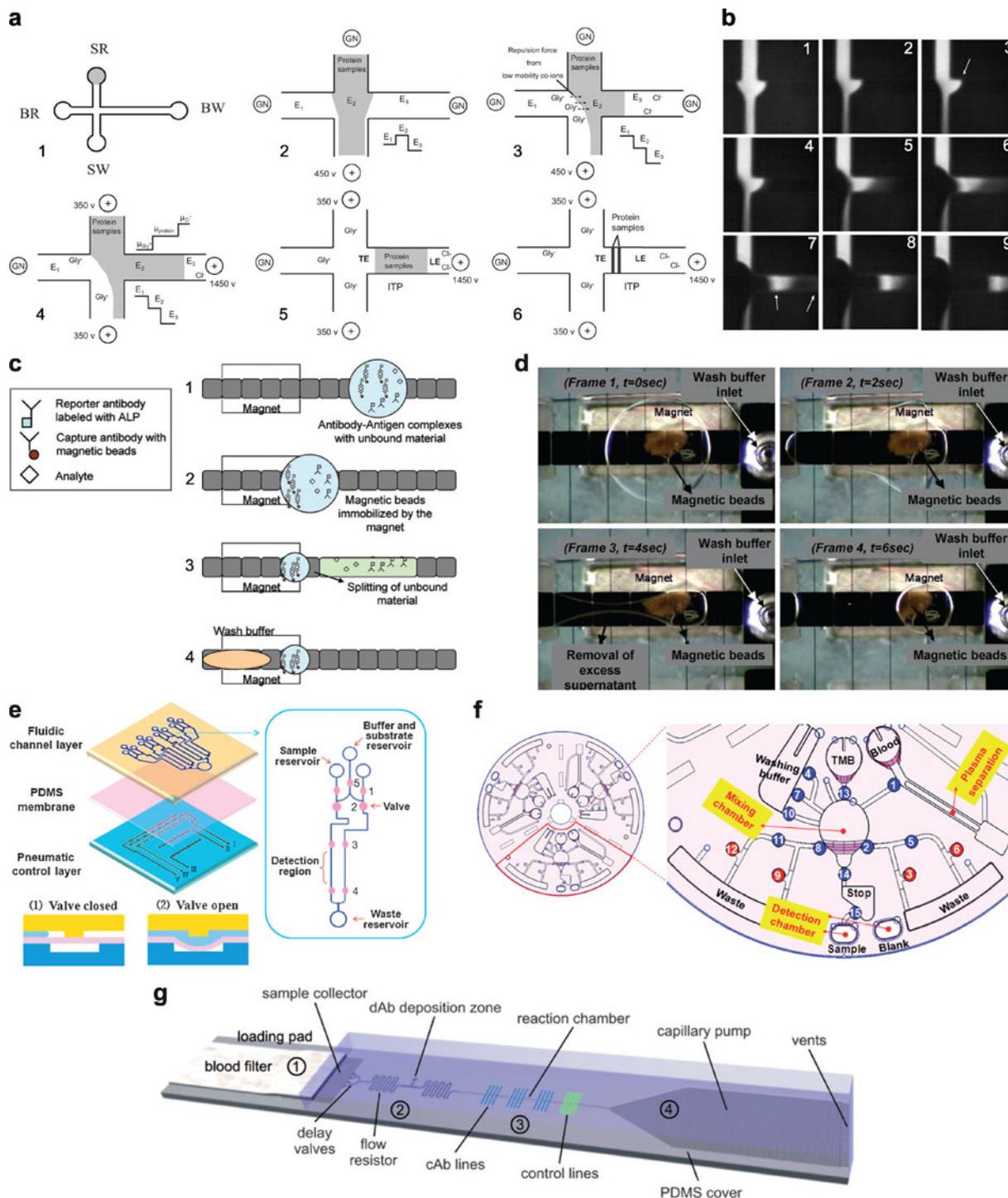


Fig. 2 Fluid handling modalities for immunoassays in microfluidic platforms. **a** Schematic of electrokinetically driven preconcentration of protein sample in a gel-electrophoresis channel. **b** Fluorescent images of electrokinetic sample injection and sample stacking. (Reprinted with permission from [111]. © 2007 American Chemical Society.) **c** Protocol for heterogeneous magnetic bead-based immunoassay on a digital microfluidics platform. **d** Video sequence of magnetic bead washing by removing the excess supernatant on-chip. ([86], Reproduced by permission of The Royal Society of Chemistry.) **e** Layout of the pneumatically driven integrated microfluidic device for surface-

based immunoassay. ([136], Reproduced by permission of The Royal Society of Chemistry). **f** Centrifugal-driven disc design detailing layout and function. *Blue numbers* represent normally closed valves and *red numbers* denote normally open valves. ([54], Reproduced by permission of The Royal Society of Chemistry.) **g** Concept of capillary-force-driven one-step immunoassay. 1 Sample is separated from blood, 2 sample redissolves deposited detection antibodies, 3 sandwich immunoassay formed, 4 unbound antibody and analyte removed. ([26], Reproduced by permission of The Royal Society of Chemistry)

droplets of wash buffer over the magnetic beads (4, Fig. 2c); and (6) using the interfacial tension of the receding edge of the droplet, the magnetic beads were moved away from the magnet and resuspended for detection. Figure 2d shows frames from a movie demonstrating bead washing by removal of excess supernatant.

Pressure-driven fluid handling

Pressure-driven flow is the most popular fluidic modality for immunoassays in microfluidic platforms. In its simplest form, pressure-driven flow can be created with either (i) a vacuum pump by opening an inlet to atmospheric pressure and applying vacuum at the outlet or (ii) by applying positive pressure at the inlet and opening the outlet to atmospheric pressure [5]. Pressure-driven flow can also be controlled by thumb-actuation [42, 126, 127] and chemical reactions [128] in devices targeted for low-cost point-of-care analysis. In contrast to electrokinetic flow, this modality is compatible with a wide range of substrate materials and solvent compositions—even non-conductive solvents and conductive substrates [6]. However, pressure-driven flow has a parabolic velocity flow profile which causes sample plug dispersion and peak broadening, rendering it less attractive for separations [129]. Moreover, channel dimensions cannot be too small because high pressures are required to counter the fluidic resistance in such channels [5].

Pneumatic valves

Recently, pressure-driven flow based on integrated pneumatic valves has become popular for immunoassays because such systems are well-suited for integration and automation [130–136]. This paradigm relies on mechanical elastomeric valves that are formed by multilayer soft-lithography with PDMS [137]. These structures can be used to isolate reagents and samples from each other for storage or reactions. A mixing component can be formed by placing several pneumatic valves in a circular loop [114]. Also, by arranging at least three pneumatic valves in a row, peristaltic pumps are formed for fluid propulsion [138]. These micro-pumps are well-suited for sequential reagent delivery, making them a good fit for immunoassays. Using pneumatic valves, Kong et al. [136] constructed an integrated microfluidic chip for high-throughput analysis of clenbuterol. This microchip was integrated with 36 normally closed pneumatic valves which facilitated the delivery of reagents and isolation of reaction mixture. The valves were fabricated by sandwiching a PDMS membrane layer between a fluidic channel layer and a pneumatic control layer (Fig. 2e). The valves were controlled by adjusting the pressure in the pneumatic layer using an external diaphragm vacuum and

compressor which were controlled by computer-regulated solenoids. A competitive immunoassay was performed on the surface of the detection region and detected by using laser-induced fluorescence. Although pneumatic valve devices have an impressive potential for throughput and automation, fabrication of multilayer devices is not simple and the operation of these devices requires external vacuum pumps and compressors [129].

Centrifugal fluid handling

Centrifugal-based microfluidic platforms are typically formed from round substrates (often matching the footprint of compact discs, CDs) containing channels and microchambers that rely on spin frequency to drive fluid movement. Fluid movements between microchambers are typically gated by capillary or hydrophobic valves [119, 139]. By spinning the disc with a motor, the centrifugal force overcomes the capillary or surface forces of these valves, enabling fluid to be pumped sequentially from the centre of the CD to the edge with increasing spin frequency [6]. Like all pressure-driven flow techniques, centrifugal flow devices are insensitive to physicochemical properties of fluids such as pH, ionic strength or chemical compositions. Because of their geometries, these devices are easily adapted to existing optical detectors and well-suited to do multiple assays in parallel. Various fluidic functions such as valving, decanting, calibration, mixing, metering, sample splitting, and separation, can be implemented on such platforms [119]; the ability to implement blood separation on-chip is particularly attractive. There are several examples of CD-based immunoassays implemented in either bead- or surface-based heterogeneous formats [45, 49, 54, 139–141]. In the most innovative approach, Lee et al. [54] developed a centrifugal bead-based immunoassay for the detection of antigen or antibody of hepatitis B virus (Fig. 2f). The device was capable of plasma separation; to control the flow between chambers, the authors used a valving strategy based on the melting of ferrowax. Ferrowax comprises paraffin wax with embedded iron oxide nanoparticles which enable faster melting of the wax in the presence of low intensity laser light. These valves can be either normally closed or normally open, depending on the requirement of the procedure. The immunoassay is fully automated—after injecting a blood sample, the disc was inserted into a programmable blood analyzer that has an integrated detector, servo motor, and laser diode for valve control.

Passive capillary force fluid handling

Passive fluid handling is becoming popular because of portability, low dead volume, ease of operation and low

power consumption [129, 142]. Although Hosokawa et al. [143–145] implemented passive microfluidic immunoassays by using degassed PDMS, the most prevalent and versatile passive driving force is capillary force [8, 26, 52, 146–149]. In a particularly interesting example, Gervais et al. [26] developed a one-step, simple to use microfluidic immunoassay platform that is passively driven by capillary forces. The microfluidic device comprises a sample collector, delay valves, flow resistors, detection antibody (dAb) deposition zone, a reaction chamber with immobilized capture antibodies (cAb), a capillary pump, and vents (Fig. 2g). The flow rate of the device depends on the total flow resistance and capillary pressure which are determined by the intricate microstructure of the capillary pumps, sample collector, delay valves and flow resistors [150]. The capillary valve and sample collector were designed to minimize flow resistance and maximize capillary pressure. The delay valves were designed to minimize the risk of entrapping air by consolidating the flow stream from the sample collector before progressing to the flow resistors. The flow resistor is a convenient component which can be easily added or removed in the design to modulate flow rate. Toward a one-step immunoassay, all necessary reagents were integrated in the device. In the deposition zone, dAb were deposited by inkjet; these dAb were then redissolved as fluid flowed through the device during the assay. In the reaction chamber, the PDMS surface is patterned with cAbs to facilitate the capture of incoming analyte. The addition of a blood sample to a loading pad triggers a cascade of precise fluidic events, resulting in a completely autonomous heterogeneous immunoassay. Capillary force is also used in lateral flow devices which produce qualitative or semi-quantitative results for primary screening at point of care; the pros and cons of these devices are reviewed elsewhere [151].

Platforms for multiplexed immunoassays

Multiplexed immunoassays are those that facilitate detection of multiple analytes from a single sample, and this form of analysis is gaining importance in fields such as medical diagnosis and proteomics [152]. Surface microarrays and microbeads are the two most common platforms used for multiplexed immunoassays. These platforms rely on their reduced size to increase the amount of analytes that can be analyzed from a single sample, and microfluidic techniques facilitate sample handling for these platforms.

Surface microarray-based multiplexing

Antibody microarrays enable the spatial encoding of antibodies on a surface, which can be analyzed by spatially

resolved imaging methods. Microfluidics has been used in surface microarrays both as a sample handling method and also for creating the microarrays.

Microfluidics has been used as a fluid handling method to deliver reagents to surface microarrays. Multiple microfluidic channel designs have been developed such as linear channels that can cover a single row of a microarray [23], flow chambers that can cover an array of immobilized protein ($9 \times 3 \text{ mm}^2$) [153], or combinations of multiple flow chambers to cover larger arrays (4 arrays, each array $2 \times 2 \text{ mm}^2$) [154]. By using microfluidics for sample delivery, faster surface reaction rates are achieved because of reduced diffusion distances within microchannels. Although microfluidic chambers allow simultaneous access to multiple spots of an array, there are some fabrication challenges that limit its use. The aspect ratios of the chambers need to be taken into consideration, especially for plastic microfluidic devices. Large aspect ratios, i.e. wide and shallow chambers, can cause elastomeric materials like PDMS to sag during the bonding stages, blocking the flow of solution in subsequent steps. Another factor that is usually considered in these experiments is flow rates. Because flow in microchannels is laminar, mass transfer is diffusion limited and it is important to have a continuous flow of reagents in order to ensure the presence of analyte at the boundary layer. As noted in the previous section, for pressure-driven systems, it is often advantageous to use large channels to avoid back-pressure; however, such systems are less attractive because of increased reagent and sample consumption. A balance must be reached between the size of the microfluidic device and the flow rates to obtain optimal assay performance.

Microfluidic networks for surface patterning

Microfluidic systems can be used to pattern proteins on surfaces. Delamarche et al. [155] reported a particularly well-known method, using the moniker microfluidic networks (μ FNs). This procedure is demonstrated in Fig. 3a; a first μ FN is used to deliver the proteins that are immobilized on the surface of the substrate in the form of strips. A second μ FN, oriented perpendicular to the initial set of channels, is used to deliver the solutions required for the assays. The immunoassay signals are optically imaged, usually using fluorescence, to obtain the results of the assay. The samples are driven through the channels by capillary action, simplifying the instrumentation required for patterning the surface. A number of immunoassays have been implemented by using this patterning method [8, 156–159]. In these assays, the individual assay spots usually cover an area of $20 \times 20 \mu\text{m}^2$ and a 64-component array can occupy an area of $300 \times 300 \mu\text{m}^2$. Electrokinetic flow control has also been used on a surface that was patterned

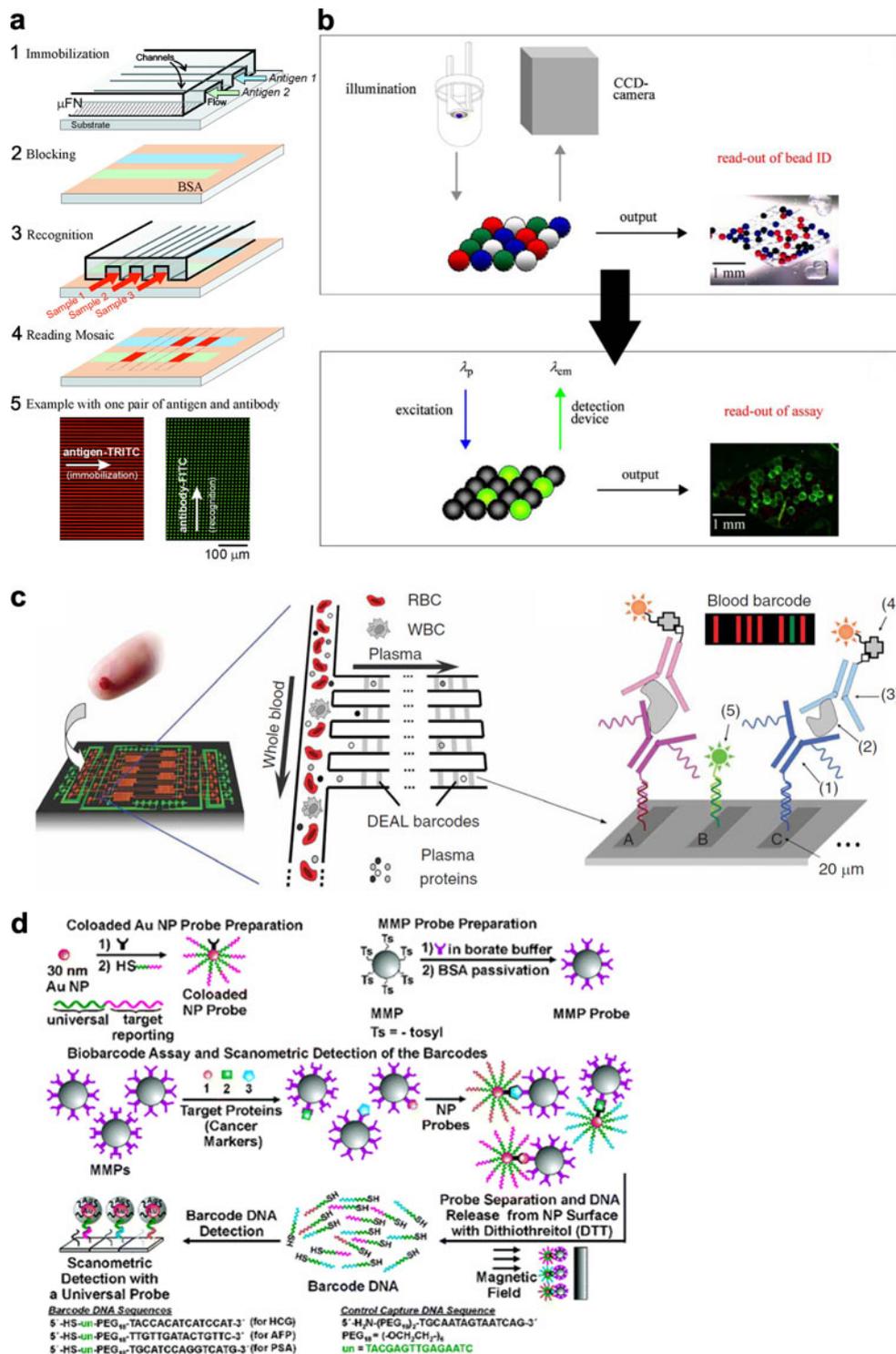


Fig. 3 Multiplexed immunoassays on microfluidic platforms. **a** Patterning of surfaces using microfluidic networks. (Reprinted with permission from [157]. © 2001 American Chemical Society.) **b** Fluorescently labelled beads for multiplexed bead based immunoassays in a centrifugal microfluidic platform. (Reprinted from [165], with permission from Elsevier.) **c** DEAL (DNA encoded antibody library) for generating barcodes that can be used for surface-based

multiplexed immunoassays. Microfluidics used for sample purification and delivery of proteins to detection spots. (Reprinted with permission from Macmillan Publishers Ltd: Nature Biotechnology, [160], © 2008.) **d** Biobarcode assay (BCA) for multiplexed immunoassays and signal amplification by silver staining of gold nanoparticles. (Reprinted with permission from [170]. © 2006 American Chemical Society)

by using μ FNs [117]. In this work, a microfluidic device capable of handling 10 samples simultaneously was positioned perpendicular to the patterned stripes and electrokinetic pumping was used to deliver samples to the immobilized antibody surface. Kartalov et al. [130] also used μ FNs for protein patterning and immunoassays; but in this work, pneumatic valves were used to control the solutions that were introduced and also the sequence in which all the reagents were dispensed. Using this device, the authors detected 5 different proteins from 10 samples simultaneously.

μ FNs have a number of advantages. First, such systems are relatively easy to implement and customize due to the availability of established microfabrication protocols. Second, the solution composition requirements are less stringent because evaporation in a closed channel is easier to control, whereas in spotting techniques, the solution is exposed to the environment. Third, the mass transfer of proteins is much faster in the microscale, allowing immobilization to occur in minutes rather than hours.

μ FNs also have certain disadvantages. First, the increased surface area to volume ratios of microfluidic channels increases the interaction of proteins with the channels, leading to non-specific adsorption of proteins. This is usually overcome by passivating the microchannels and/or by ensuring a continuous flow of reagents through the microchannel [5]. Second, protein spotters can produce spots of proteins with higher densities as compared to the strips patterned by the microchannels. Third, protein spotters can process multiple slides in a single run, while the μ FN-based patterning method is limited by the number of μ FNs available.

DNA-directed immobilization for antibody microarrays

Another method used to pattern antibodies for microfluidic immunoassays is a DNA-directed approach known as DNA encoded antibody library (DEAL). In this approach, antibodies are attached (or encoded) with single-stranded DNA (ssDNA) with complementary sequence to a second strand that is immobilized on a DNA microarray. When the DNA-labelled antibody is introduced to the array, the complementary DNA strands bind to each other, leading to the immobilization of antibodies at a specific location on the array. Bailey et al. [18] used this method to detect multiple proteins in a microfluidic channel. In this approach, PDMS channels were positioned over a DNA array and the microfluidic channels were used to introduce solutions required for the immunoassay. DNA-directed immobilization is particularly advantageous for microfluidic-based systems because DNA is a more robust molecule than antibodies, allowing for the use of harsh conditions required for certain fabrication steps such as the assembly

and proper sealing of a channel on a solid substrate. In addition, there are no problems associated with allowing the DNA spots to dry, while antibodies can lose their structure and function if dehydrated. One cause for concern is the potential effect of the ssDNA on antibody function. The authors report that up to 3 DNA strands per antibody has minimal effect on antibody function.

Another unique experiment demonstrated by Bailey et al. was the ability to perform a homogeneous immunoassay in solution, and then immobilize the entire immune complex on the surface using the specific DNA strands. This allows for multiplexing because the immobilization is driven by the selectivity of the DNA strands. But the authors obtained a lower sensitivity by this method, which was explained by competitive binding of unbound antibodies to the surface, which reduces the amount of detectable complexes. The same group used the DEAL method to develop a multiplexed immunoassay [160] as shown in Fig. 3c. The DNA array in this case was patterned using the μ FN method, allowing for a denser array. In addition, their microfluidic device was capable of handling blood samples and it could separate large particles, such as red blood cells, from the smaller proteins of interest using the Zweifach–Fung effect [161]. The device was used to analyze 12 different proteins present in blood with adequate sensitivity.

Microbead-based multiplexing

Another platform commonly used for multiplexed immunoassays is microbeads. For a general review on the integration of microbeads with microfluidic technologies, we suggest Derveaux et al. [162]. This section focuses on the use of beads for multiplexed immunoassays in microfluidic platforms.

The principle for bead-based multiplexing is to encode the beads such that the identity of the antibody immobilized can be determined by decoding the signal from the beads. The simplest approach is to isolate different sets of beads in different compartments [59, 163]. In these experiments, beads with antibodies are isolated in different compartments, and a single sample solution is allowed to contact the beads in all compartments. This method is limited by the number of different compartments that fit on a single device, and one must ensure that there is enough sample to distribute across the different compartments. Other methods for encoding beads include optical, electronic, physical and graphical; these schemes are reviewed by Braeckmans et al. [164] and are not discussed in detail here. The most common method employed for multiplexed immunoassays in microfluidic platforms is fluorescence encoding (optical). In the macroscale, microbeads are typically analyzed in a sequential manner using flow cytometers. But microfluidics

helps to miniaturize the device footprint allowing the use of imaging methods to analyze the beads, in addition to the flow-cytometer-type approach. Beads are usually physically confined to a monolayer for simultaneous imaging; this is convenient in microfluidic devices because of the similar size scale of the microfluidic structures and the beads. A few immunoassays have been performed by using this readout method [134, 165]. Riegger et al. [165] implemented a multiplexed immunoassay to detect two analytes using beads that were labelled with fluorophores. A centrifugal microfluidic platform was used for fluid handling and also to physically confine the beads to a monolayer for imaging (Fig. 3b). The maximum multiplexing capability is limited by the number of dyes that can be spectrally distinguished. An alternative method is to use a two-dimensional encoding strategy such as the xMAP technology of the Luminex (<http://www.luminexcorp.com>) system. In this method, beads are labelled with two colours of dyes and they are combined at 10 different intensity levels, yielding 100 unique codes. The dyes are chosen such that they can be excited with the same light source, simplifying the instrumentation. These beads are most often used for multiplexed immunoassays in flow cytometer systems [166], but have been applied in a microfluidic platform by Diercks et al. [134]. In this work, the beads were trapped in a microchannel and imaged using a confocal microscope. The authors detected four different analytes from a 2.7-nL sample. The microfluidic device had a unique structure in which the beads were trapped in a chamber within a channel loop, allowing the continuous flow of sample using only the initial plug introduced in the loop, which helped improve reaction kinetics while using a small volume.

Quantum dots (fluorescent nanoparticles) have also been used for labelling microbeads in both the one-dimensional [165] and two-dimensional (2D) labelling schemes [167]. Quantum dots are especially advantageous for the 2D labelling scheme because of their narrow emission peaks and their broad, size-independent absorption spectrum, allowing all colours to be excited by using a single light source. Han et al. [167] demonstrated the 2D labelling scheme using quantum dots and claim that this method has potential for an encoding scheme with 10,000–40,000 unique codes (using 5–6 different colours at six intensity levels). The use of these beads in a microfluidic platform was demonstrated by Klostranec et al. [168], but in this experiment, only three codes were created using two different colours of quantum dots in order to detect three analytes. In contrast to previous approaches in which an imaging method was used for the bead readout, Klostranec et al. used an approach similar to a flow cytometer, using the effect of hydrodynamic focusing in microchannels to focus the beads into a single stream such that they could be

analyzed in series. The concept of using microfluidics to analyze beads in a manner similar to a flow cytometer was also demonstrated by Holmes et al. [61], who demonstrated a dielectrophoresis (DEP)-based bead focusing method to focus beads into the centre of the flow channel.

The biobarcode assay (Fig. 3d) [169] is another interesting application of microbeads for multiplexed immunoassays. In this method, magnetic microbeads are used to implement a heterogeneous immunoassay with the detection antibody labelled with gold nanoparticles, which are also conjugated with double-stranded DNA (dsDNA). After the immunoassay, the dsDNA is denatured, and the resulting ssDNA can either be detected by using a sandwich DNA assay or PCR methods for high sensitivity. This biobarcode assay can also be used for multiplexed assays as demonstrated by Stoeva et al. [170], in which microbeads are used to perform the immunoassay, and ssDNA is analyzed in a DNA microarray. The proteins are identified by the spots on the DNA array that are hybridized. Goluch et al. [78] showed that this technique can be integrated into a microfluidics device to detect prostate specific antigen in samples. This form of immunoassay is unique since it combines the best of both multiplexing methods. The immunoassay is implemented on magnetic beads, allowing for easier sample control, and in addition, it benefits from the high multiplexing capability of surface microarrays.

Label-free detection strategies for multiplexed immunoassays

Although fluorescence is the most common detection scheme in microfluidic platforms, there is a growing trend for the use of label-free sensing technologies. The advantage of these methods is that some reagents and assays steps (e.g. addition of fluorescently labelled secondary antibody) can be avoided, reducing the cost and assay duration. Some of the label-free sensing technologies also facilitate real-time measurements allowing for kinetics quantitation. In this section we briefly discuss some of the label-free methods used for microfluidic-based multiplexed immunoassays. For a more detailed discussion on this topic, see Qavi et al. [171].

Surface plasmon resonance (SPR)-based detection

Surface plasmon resonance is one of the most widely used label-free detection schemes in microfluidic platforms. In this method, light is used to excite surface plasmons in a thin film of metal, which occurs as a function of wavelength and angle of incidence. The surface plasmons have an evanescent character, making the technique highly sensitive to refractive index changes at the metal–liquid

interface, such as when an antigen binds to an immobilized antibody. There are a number of techniques that make use of SPR for multiplexed measurements. One of these is surface plasmon resonance imaging (SPRi), in which light at a fixed wavelength and angle of incidence is shined on a thin metal film, and the reflected light is recorded by a CCD camera. Binding events on the surface cause a local change in the refractive index, changing the surface plasmons at a specific spot on the surface. For a well-optimized system, a lateral resolution of $2\ \mu\text{m}$ is possible [172]. Kanda et al. [173] developed microfluidic networks for patterning a gold surface and detected the binding of anti-bovine IgG to bovine IgG patterned on the surface. In another example, Luo et al. [133] demonstrated the use of a pneumatic valve microfluidic device to pattern an array of gold patches with antibodies and also to perform surface binding experiments over a 96-component array on the same chip. Krishnamoorthy et al. [174] also used SPRi to detect surface binding events in a microchip that used electrokinetic flow focusing to selectively deliver samples over a single row of an array. The high lateral resolution and surface sensitivity of this method make it a good fit for surface microarrays in microfluidic chips.

Extraordinary optical transmission (EOT) is another detection scheme that uses the phenomenon of plasmon resonance. To perform an EOT experiment, light is shined on an array of subwavelength holes (nanohole array) in a thin metallic film and the transmitted light is monitored (Fig. 4a). The intensity of light transmitting through the apertures is low for such structures, but the coupling of light with the surface plasmons allows certain wavelengths to pass through the nanohole array at intensities much greater than predicted. The dependence of this phenomenon on plasmon resonance makes the wavelength of light transmitted sensitive to binding events on the surface. The use of these sensors for immunoassays has been demonstrated by Sharpe et al. [175] and the integration of this sensing technique on microfluidic chips has been demonstrated by De Leebeek et al. [176]. In these cases, white light was incident on the nanohole arrays and the transmission spectrum was collected to monitor the binding event, but this configuration would not be useful for multiplexed measurements. Ji et al. [177] demonstrated the use of nanohole arrays in imaging mode with a fixed wavelength, with the transmitted intensity being monitored by using a CCD camera. Using this method the authors were able to monitor 25 nanohole arrays simultaneously. The use of EOT is particularly advantageous for microfluidic systems because of their small footprint and also because of their transmission mode of operation. The transmission mode of operation allows the adaptation of microscopes to be used for performing SPR measurements, as opposed to the requirement of a dedicated SPR sensing system.

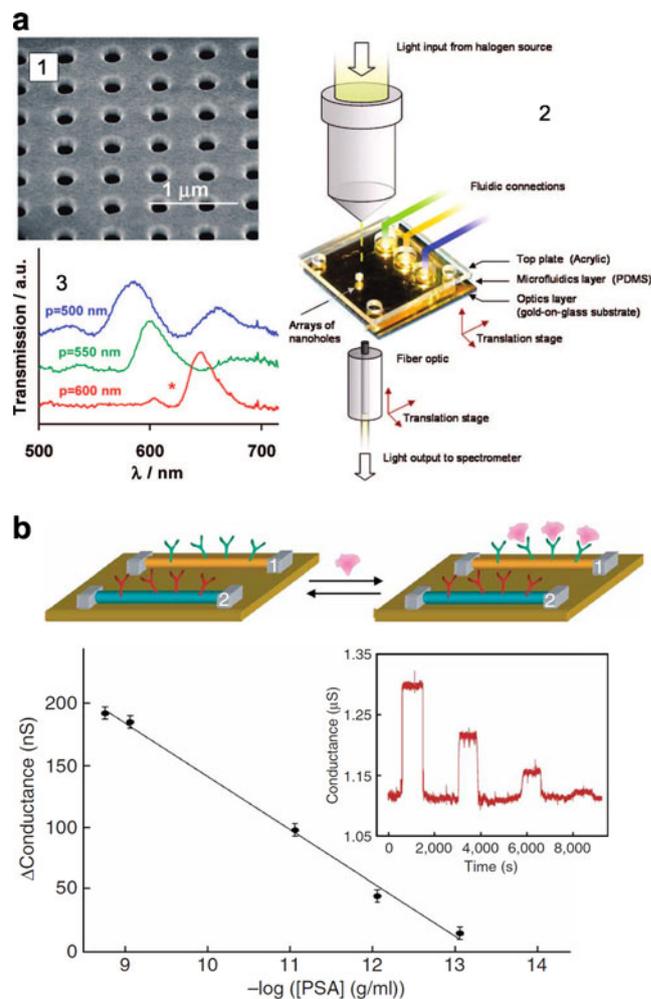


Fig. 4 Label-free detection schemes for multiplexed immunoassays in microfluidic platforms. **a** Extraordinary optical transmission of light through nanohole arrays on metal films as a detection scheme for surface-based assays. (Reprinted with permission from [185]. © 2008 American Chemical Society.) **b** Silicon nanowire field effect transistors for label-free sensing of immunoassays. (Reprinted with permission from Macmillan Publishers Ltd: Nature Biotechnology [178], © 2005)

Silicon nanowire field effect transistors

Another label-free technique that has been combined with microfluidics is a silicon nanowire (SiNW) field effect transistor. Field effect transistors comprise a semiconductor connected to source, drain and gate electrodes; the last of these controls the conductance of the detector. SiNWs behave as semiconductors and have been used as field effect transistor immunosensors in which the gate electrode is replaced by a layer of capture antibodies. The dielectric environment of the silicon nanowire changes as a function of antibody binding, resulting in a change in the conductance of the detector (Fig. 4b). Zheng et al. [178] demonstrated this sensing mechanism for a multiplexed immunoassay in a microfluidic format. The microfluidic

device was used to deliver sample and reagents to the nanowire arrays, facilitating detection of four cancer markers in serum. As is the case for nanohole arrays, the small footprint of such systems allows for the integration of several independent sensor elements in microfluidic devices for multiplexed detection.

Imaging ellipsometry

Imaging ellipsometry has also been used as a detection scheme for multiplexed immunoassays. In imaging ellipsometry, elliptically polarized light is shined on a reflective surface (which may contain layers of adsorbed molecules), and the reflected ray (now linearly polarized because of the reflection of light by the surface features) is analyzed to determine the thickness of the adsorbed layers. By using a spatially sensitive detector such as a CCD camera, an image of the surface can be obtained and can be used to monitor surface binding events. Wang et al. [179, 180] used imaging ellipsometry coupled to a microfluidic device to detect five different markers of hepatitis B. This is a very sensitive technique, capable of monitoring small surface thicknesses, and is also known to have a spatial sensitivity in the micrometer regime.

The above-mentioned methods are only a small subset of available label-free detection technologies. There are many other label-free detection methods that have not yet been implemented in an array format for microfluidics, including interferometry [181], wavelength interrogated optical sensing [182], liquid crystals [183] and microcantilevers [184]. We propose that these techniques and others may have promise for multiplexed immunoassays in microfluidics in the future.

Conclusions and outlook

In this review, we described the state-of-the-field of microfluidic immunoassays in four areas: microfluidic immunoassay configurations, fluid handling modalities, multiplexed platforms, and label-free detection strategies. In reviewing the various microfluidic immunoassay configurations, we observed that microfluidic platforms are particularly attractive because of the ease of integrating active forces (in place of simple diffusion), which can enhance antibody–analyte interaction, leading to faster analysis times and improved sensitivity. We also observed that the various fluid manipulation techniques that have been used in microfluidic systems have pros and cons; for example, electrokinetic flow has limits in compatibility with fluid makeup, which makes alternatives such as pressure-driven flow or digital microfluidics more attractive for complex biological samples. We proposed that multi-

plexing represents the latest and most exciting development in microfluidic immunoassays, and observed that while surface microarrays and microbeads can both be used for this purpose, microbeads are more practical because of the flexibility this system offers. On the other hand, we noted that microarrays are suited for high-density multiplexed analysis and that this format is compatible with label-free detection schemes.

In the final analysis, we see great potential for growth in microfluidic immunoassays, which leads us to make several predictions. The current techniques for enhancing antigen–antibody interaction will continue to improve and will eventually be adapted for multiplexed immunoassays. Fluid handling techniques will become more versatile as multiple modes will be integrated onto a given platform. Multiplexed immunoassays coupled with label-free detection strategies will eventually lead to devices capable of real-time multi-analyte quantification. Surface immobilization strategies will continue to improve, with the aim of achieving an ideal surface that offers high antibody activity and low non-specific binding. These developments will increase the functionality, sensitivity and throughput of microfluidic immunoassays to accelerate progress in fields such as proteomics and drug screening. As devices become smaller, they will become more compatible with point of care (POC) applications, which has the potential to revolutionize the way medical care is delivered, particularly in resource-poor areas. The main challenge that remains for microfluidic immunoassays is to reduce device complexity, making systems more robust, cost-effective and user-friendly. The integration of microfluidics with immunoassays has demonstrated advantages and the future of this field is promising.

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