REVIEW

Microfluidic-based biosensors toward point-of-care detection of nucleic acids and proteins

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Abstract This article reviews state-of-the-art microfluidic biosensors of nucleic acids and proteins for point-of-care (POC) diagnostics. Microfluidics is capable of analyzing small sample volumes (10^{-9}–10^{-18} l) and minimizing costly reagent consumption as well as automating sample preparation and reducing processing time. The merger of microfluidics and advanced biosensor technologies offers new promises for POC diagnostics, including high-throughput analysis, portability and disposability. However, this merger also imposes technological challenges on biosensors, such as high sensitivity and selectivity requirements with sample volumes orders of magnitude smaller than those of conventional practices, false response errors due to non-specific adsorption, and integrability with other necessary modules. There have been many prior review articles on microfluidic-based biosensors, and this review focuses on the recent progress in last 5 years. Herein, we review general technologies of DNA and protein biosensors. Then, recent advances on the coupling of the biosensors to microfluidics are highlighted. Finally, we discuss the key challenges and potential solutions for transforming microfluidic biosensors into POC diagnostic applications.

Keywords Microfluidics · Biosensor · Point-of-care detection · Protein · DNA

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1 Introduction

Biosensors combine a molecular recognition element with a signal conversion unit (Mohanty and Kougianos 2006). Some biosensors have been successfully commercialized for clinical applications such as electrochemical blood glucose sensors (Kissinger 2005). Molecular biosensors are more preferred as a clinical diagnostic tool than other methods partially because of real-time measurement, rapid diagnosis, multi-target analyses, automation, and reduced costs (Luong et al. 2008). As a recent advance in molecular biology has led to our better understanding of potential disease-related protein biomarkers and DNA mutations, biosensors became a promising technology for early diagnosis (Teles and Fonseca 2008; Schasfoort 2004; Wang 2006). The spatio-temporally regulated gene is a critical process for proper functions of all living organisms. Genetic and epi-genetic modifications of the regulatory processes are the underlying causes in many diseases (Situma et al. 2006). Therefore, monitoring the mutation and modification of DNA and the expression level of protein biomarkers is critical for the early diagnosis of diseases (Wang 2006). Recently, there has been an increasing interest to integrate advanced biosensors into lab-on-a-chip systems by introducing microfluidics (Haeberle and Zengerle 2007). The lab-on-a-chip systems take advantage of several intrinsic characteristics of microfluidics including laminar flow, low consumption of costly reagents, minimal handling of hazardous materials, short reaction time required for analysis, multiple sample detection in parallel, portability, and versatility in design (Choi and Chae 2009a). With microscale fluid regulators (e.g. valves, mixers, and pumps) integrated on the lab-on-a-chip platform, the analytical performance of biosensors toward point-of-care

(POC) diagnostics can be greatly enhanced (Henares et al. 2008).

POC testing is one of the most promising areas for biosensor applications and readily provides the clinician essential information of proper treatments. In developing areas and disaster scenes where only very limited resources are readily accessible, POC system is an attractive tool to diagnose patients for proper clinical management (Yager et al. 2006; Rasooly 2006). Nevertheless, the stringent requirement of POC diagnostics presents new challenges for biosensor technologies. For instance, detecting target analytes with high sensitivity and selectivity is a key challenge in microfluidic-based POC because of the ultra-small sample volumes. Another challenge is to merge the detection component with other fluid regulatory elements on a single platform. There are many prior art on microfluidic-based biosensors and this article does not review all. For those who are interested in microfluidic applications and POC systems, developed before 2005, the authors suggest them to read the following review articles (Ahn et al. 2004; Bashir 2004; Yager et al. 2006; Lee and Lee 2004; Sanders and Manz 2000; Jakeway et al. 2000; Chovan and Guttman 2002; Colyer et al. 1997; Vilkner et al. 2004; Feng et al. 2009; Crevillen et al. 2007; Henares et al. 2008; Abgrall and Gue 2007; Situma et al. 2006). In this article, we focus reviewing recently reported biosensors within past 5 years that are integrated with microfluidics for detecting DNAs and proteins toward POC applications. We will also address challenges in the development of the microfluidic-based biosensors and their system level integration.

2 Promises

2.1 DNA biosensor

2.1.1 Why DNA for diagnostics?

DNA detection of POC systems facilitates "personalized" healthcare. If the sequence of a complete genome of an individual is available, for instance, the patient can adapt the prescriptions pharmacogenomically and set up a personalized treatment plan that maximizes the efficiency of the treatment. Besides the whole genome sequencing, DNA-based diagnostics allow tumor mutation profiling (Milos 2009) as well as identifying highly specific disease markers. In this section, we will provide a brief overview of the current methods used for DNA-based diagnostics and elaborate on their potential for microfluidic integration. In addition, we will point out what current limitations on integrating DNA biosensors are and which of the new

approaches in DNA analysis has the potential to transform POC microfluidic diagnosis.

2.1.2 General DNA sensing technique for diagnostics

There is a significant demand in high-throughput DNA analysis tools to assist diagnostics in research and clinical applications. The "standard" approach involves the multiplication of the sample DNA via polymerase chain reaction (PCR) and the subsequent fluorescence detection of DNA fragments based on their electrophoretic mobility on a gel substrate (Ugaz et al. 2004). This protocol requires the use of individual facilities for each of the steps involved, which needs well-equipped laboratory and highly trained operators. A different approach, which is valuable for DNA sequencing, is the use of fluorescence hybridization assays. Such assays are performed by selectively immobilizing single-stranded DNA (ssDNA) or RNA strands on a solid substrate, typically glass. The immobilized ssDNA fragments are complementary to the ssDNA to be probed. The probe DNA is tagged with fluorescent markers and added to the chip carrying the immobilized test DNA. Since the probe DNA carries fluorescent tags, hybridization events can be detected via optical interrogation and probing. Fluorescence hybridization assays are very well automated, requiring little manual operation. A detailed overview covering the commercially available screening tools with their respective detection limits, lateral resolution, and detection technology used is provided by Bally et al. (2006).

2.1.3 Microfluidic DNA sensor for POC diagnostics

The main reason why DNA-based diagnostics has not found more widespread use is the requirement of a complete tool-chain (PCR, electrophoresis, and fluorescence scanner) and the high cost of integrated tools for hybridization assays. Gascoyne et al. (2004) compared different methods including DNA hybridization for Malaria detection. It was noted that genetic detection methods would offer significant advantages over microscopy assays if they would be implemented in a micro total analysis format.

Learning from the success of the "standard" approach in DNA-based analysis, the development of microfluidic lab-on-a-chip for DNA-based diagnostics has focused mainly on replicating the standard approach, involving PCR and gel electrophoresis, by integrating the different steps into a microfluidic platform for POC applications. Miniaturization in the small-size platform is useful since the classic macroscopic liquid handling leads to sample dilution and the use of multiple instruments causes sample loss and bears the risk of contamination (Liu and Mathies 2009). Several extensive reviews on these microfluidic devices

exist, involving PCR and eventually with an integrated capillary electrophoresis step (Zhang et al. 2006, Pereira et al. 2008; Liu and Mathies 2009). Microfluidic devices based on PCR and fluorescence detection have led to a significant improvement in total processing time, while featuring low limits of detection. For example, Easley et al. (2006) reported a fivefold reduction in assay time, reducing the total essay time to 30 min. Another approach using centrifugal flow reports an assay time of only 15 min (Peytavi et al. 2005). Microfluidic on-chip PCR has recently demonstrated an enhanced sensitivity over conventional methods for the detection of fetal chromosomal aneuplodies, a clinically relevant diagnostic (Lun et al. 2008). However, a complete micro total analysis platform for DNA diagnostics has not been successfully commercialized for a POC diagnostic device. A probable reason for the limited commercial success of the PCR-based microfluidic total analysis platforms is that they rely on a fluorescence assay, which requires optical excitation, involving gas lasers or high-power broadband sources. The detection of the fluorescence is performed using confocal microscopes, all involving classic Abbe optics which is limited in resolution by the numerical aperture. In addition, detectors such as photomultipliers or charge-coupled devices (CCDs) have to be used to provide a sufficient limit of detection. Thus, integrating the optical readout into the microfluidic platform is a very difficult challenge. Recent approaches using optical excitation by light emitting diodes (LEDs) and detection using photodiodes (PDs) are very promising (Kaigala et al. 2008; Ramalingam et al. 2009; Pjescic et al. 2010). Kaigala et al. reported on a fully integrated analysis system involving PCR and capillary electrophoresis, enabling a detection limit of 465 nM RNA in solution with a signal-to-noise ratio (SNR) of 23. They specified that the cost of the components used in their setup is approximately US$1,000, reasonable for a POC device. A device that relies on the capillary drive rather than electrophoresis was demonstrated by Ramalingam et al. showing pM detection limits on DNA fragments originating from the severe acute respiratory syndrome (SARS) virus genome. Pjescic et al. demonstrated that SNR of 200 can be accomplished using an LED/CCD combination for fluorescence detection, which is comparable to that from a confocal microscope setup, with a limit of detection of 166 pM and an assay time of only 15 min.

Despite the advanced development of microfluidic DNA assays involving on-chip PCR and capillary electrophoresis, recent reviews are very skeptical about the market potential of such devices for POC diagnostics (Milos 2009). They are perceived to be very complicated integrated devices (Andresen et al. 2009), requiring unique and expensive reagents for the PCR steps (Sabounchi et al. 2008). Thus, recent efforts have focused on technologies that allow a lower limit of detection, reduce the number of PCR cycles or even relax the need of a PCR step completely. Such devices should be capable of detecting a single molecule or performing direct sequencing, using "circulating" DNA, i.e. DNA at concentration levels naturally occurring in body fluids (Milos 2009). On the line of such effort, recent studies focused on label-free technologies by replacing the readout of fluorescence intensity with electrical readout (Meller et al. 2001; Howorka and Bayley 2002).

The label-free DNA hybridization detection techniques can be divided into two main approaches. The first is based on the detection of the change in surface charge upon ssDNA hybridization. DNA strands are negatively charged due to the phosphate ions in their backbone (Nair and Alam 2007). Since the initial ssDNA strand is immobilized on a surface, hybridization of a second ssDNA strand leads to a charge accumulation at the site of the immobilized initial strand. This change in the spatial charge distribution can be detected electrically either using a semiconducting nanowire-based transistor (Fig. 1) or a planar field-effect transistor (Fig. 2a). The second hybridization detection method utilizes the change in diameter of the hybridized DNA strand when compared to the original ssDNA (Fig. 2b). Natural or artificial nanometer-sized pores or channels can be used as constrictions, which will limit the ionic current between two electrolytic baths. If the DNA strand passes through such an aperture, it will further reduce the ionic current, based on its diameter, allowing determining which part of the original single strand is hybridized.

Direct detection of DNA hybridization is an elegant method with the potential of being able to quantify the amount of mismatched base pairs. When the assay is performed on a Metal-Oxide-Semiconductor Field Effect Transistor (MOSFET) gate, it is possible to estimate the amount of the drain current change upon a hybridization event (Barbaro et al. 2006; Ingebrandt and Offenhausser 2006; Landheer et al. 2007; Sakata et al. 2004). Experimental results, however, show that the drift of the operating point of the MOSFET and the expected signature of the DNA hybridization are on the same order of magnitude, complicating a straightforward evaluation of the electrical signal. Nanowire-based charge detection appears to be more robust (Curreli et al. 2008; Gao et al. 2007; He et al. 2008; Li et al. 2004; Nair and Alam 2007; Stern et al. 2007a; Tolani et al. 2009; Wang 2005; Zhang et al. 2008), since nanowires offer a higher sensitivity toward changes in surface charge than classical Ion-Sensitive Field Effect Transistor (ISFET) structures. ISFET-based sensors can sense as small as 1 μM analyte concentration (Ingebrandt and Offenhausser 2006), whereas nanowire-based sensors can detect as low as 10 fM (Patolsky et al. 2006). Electrochemical impedance spectroscopy offers robust
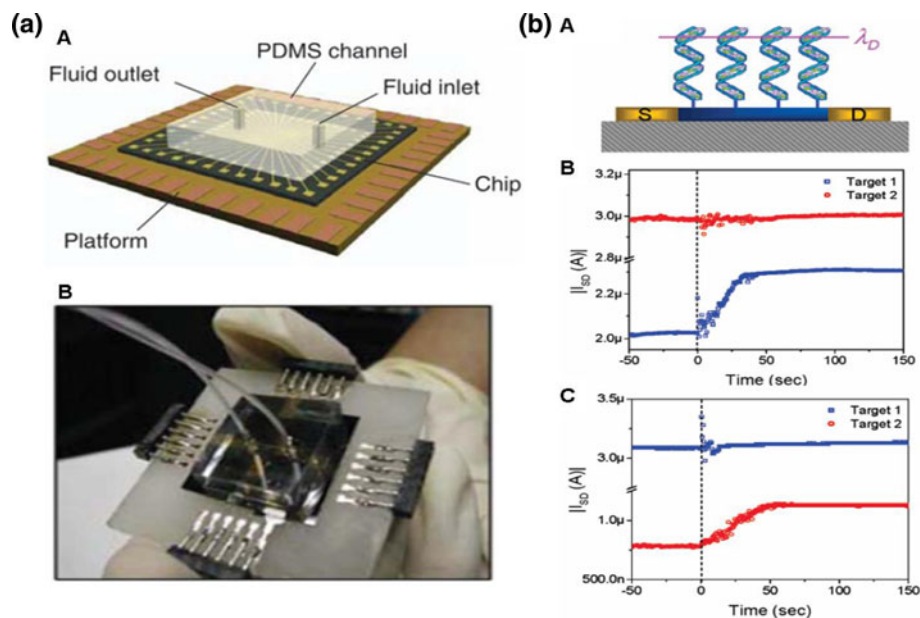
Fig. 1 Examples of microfluidic biosensors using semiconductor nanowires. **a** Prototype nanowire sensor biochip with integrated microfluidic sample delivery (Patolsky et al. 2006). **b** The experiment by Stern et al. demonstrated that nanowires can be used to detect DNA hybridization. In panel *A*, a cross section schematic of the device shows the immobilized DNA on top of the nanowire with its source and drain contacts. Panels *B* and *C* show the source drain current change for two devices that have been functionalized with specific probe DNA strands upon addition of target DNA. The device in Panel *B* has been functionalized with probe 1, the one in *C* with probe 2, respectively. If 10 pM of the respective complementary target is added to the solution, the drain current increases (*bottom trace*), while the current stays constant if a non-complementary strand is introduced (*top trace*). The DNA sequences used were DNA-T(1): 5′-CCT GCA GTG ACG CAG TGGCG-3′; DNA-T(2): 5′-AAG GTG GAA AAT GTA ATC TA-3′;DNA-P(1): 5′-CGC CAC TGC GTC ACT GCA GG-3′; DNAP(2): 5′-TAG ATT ACA TTT TCC ACC TT-3′. In order to observe the drain current change, the solution has to be of a low ionic concentration, 5 mM, that the Debye length is larger than the length of the DNA fragment (Stern et al. 2007b)

detection, less sensitivity to baseline drift, and can be applied to planar and nanowire devices (Gebala et al. 2009; Ghoshmoulick et al. 2009; Ingebrandt et al. 2007; Ito et al. 2007; Kafka et al. 2008; Peng et al. 2007; Vamvakaki and Chaniotakis 2008). Electrochemical detection of the hybridization reaction monitors the direct oxidation of the DNA bases and records surface properties such as capacitance and resistance by adding a redox-active compound, such as ferri/ferrocyanide to the DNA-modified electrode (Peng et al. 2007; Kafka et al. 2008; Gebala et al. 2009). Detection limits in the range between 1 µM down to 100 pM were reported using electrochemical impedance spectroscopy. All the surface-sensitive label-free DNA hybridization methods, however, suffer from the fact that in order to detect the difference in charging state upon hybridization, the charges must not be screened by the solution, i.e. the Debye length must exceed that of the DNA strand, in order to detect the difference in charging state upon hybridization.

This limits the salt concentration in solution to values of $0.005\times$ phosphate buffered saline (PBS) which is significantly below the physiological concentration, making it impossible to work with clinically relevant fluids. Even in low ionic strength solutions, the maximum DNA strand length is limited by the Debye length, irrespective of the transduction technology used (Stern et al. 2007b, 2008). The benefit of all the charge-based measurement technologies described is that they are easily integrated with microfluidic systems. In most studies, this has been successfully demonstrated or is feasible without degrading the sensor performance, which is what makes the technology easy to use and low-cost diagnostic tools enabled by microfluidics.

Rather than probing the charge associated with DNA hybridization, the combination of two strands causes the molecule to expand. This geometric change can be observed electrically by the Coulter effect, which relies on the reduction of an ionic current through an aperture upon translocation of a particle of a size that is comparable with that of the aperture (DeBlois and Bean 1977). Coulter counting usually employs micron-sized apertures for the counting and sizing of cells. In order to apply this principle to the sizing of single- and dual-stranded DNA, the aperture must be of comparable dimensions since the current reduction upon translocation scales proportionally to d^3/D^4; where d is the diameter of the cell or molecule and D is the diameter of the aperture. This in consequence requires nanometer-size apertures with a known dimension.
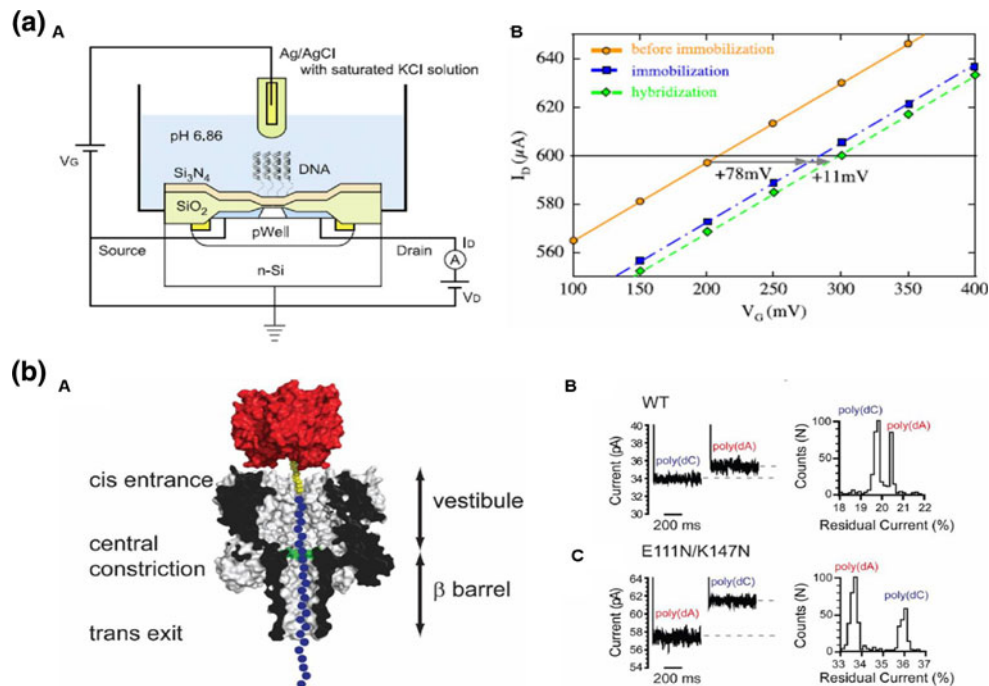
Fig. 2 a *A* Schematic diagram for measurements of electrical characteristics of a genetic field effect transistor (FET). The FET can be integrated into a microfluidic channel to allow in situ detection. *B* Electrical signal of immobilization of oligonucleotide probes, hybridization with target DNA on the FET (Sakata et al. 2004). **b** Direct DNA sequence readout using a natural nanopore (α-Haemolysin). The current versus time traces on the right show that a poly(dC) strand exhibits a different current signature when compared to a poly(dA) strand, allowing to draw a conclusions of which bases are present. By modifying the lumen of the a-HL pore the current signal can be improved (Stoddart et al. 2009; Copyright (2009) National Academy of Sciences, U.S.A)

Initially, natural nanopores were used to detect DNA translocation events (Howorka and Bayley 2002; Meller et al. 2001). Here, the natural nanopore α-Haemolysin showed that the diameter of its lumen is suitable for providing measurable differences in current upon DNA translocation (Fig. 2) (Maglia et al. 2008; Purnell et al. 2008; Butler et al. 2008; Purnell and Schmidt 2009). Nanopore-based sequencing methods are able to detect single DNA strands, and the signal can be distinguished from the background noise. The limit of detection in case of translocation-based sensors is thus not determined by the SNR, but rather by the probability of DNA reaching the pore and starting translocation. However, we can deduce the limit of detection from the concentration used in the experiments at which translocation events were observed and they lie in the range between 500 (Maglia et al. 2008) and 200 nM (Stoddart et al. 2009). The drawback of using a natural nanopore is that it is a biological entity with limited lifetime. In addition, the channel protein has to be incorporated in a lipid bilayer membrane, which itself is a challenging effort, maintaining a consistent and stable bilayer as a host environment. It is not necessary to use natural pores; artificial solid-state nanopores can be used to replace the channel proteins as apertures. Such apertures were successfully fabricated in a variety of materials, with plastic, glass, and silicon being prevalent (Joshi et al. 2010; Kim et al. 2006; Martin and Siwy 2007; Petrossian et al. 2007; Smeets et al. 2006; White et al. 2006). Translocation through plastic apertures has been studied in detail with very promising results (Harrell et al. 2006; Schiedt et al. 2005), with a reported detection limit of 10 nM. Glass pores are of high interest as well since they allow different surface functionalization chemistry, enabling temporary docking events. The biggest issue, however, is threading a long DNA strand through an aperture without the DNA molecule folding, risking an irreversible block of the aperture (Chen et al. 2004). Moreover, nanopores are very prone to blockage by particulates in solution. In comparison to nanopores, nanochannels exhibit a lower risk of the DNA strand agglomerating, allowing the measurement more probable (Mannion et al. 2006), which demonstrated sequencing at concentration as low as 58 pM. The direct DNA sequencing methods appear to be very promising; however, technology with the best potential for mass production is yet to emerge. Similar to the charge detection techniques, the size-based hybridization detection methods integrate well with microfluidics. Given the early stages of the research on nanopore-based particle sizing, the efforts have been mainly focused on nanopore fabrication and/or characterization. However, the use of microfluidics is

indispensable since fluid management (filtering, pressure, and exact flow control) is of extreme importance for the success of these approaches. Although no complete microfluidic device exists yet that provides fluid handling, pre-concentration, and electrical sequence detection all on one chip, the individual building blocks have been successfully demonstrated (Kovarik and Jacobson 2009). Such an integrated microfluidic device has a promising potential to change DNA-based POC diagnostics. The DNA detection method and specifications reviewed here are summarized in Table 1.

2.2 Protein biosensor

2.2.1 Why protein for diagnostics?

In contrast to the highly sophisticated DNA biosensors reviewed in the previous section, protein biosensors, e.g. immunosensors, are more straightforward to implement. Although both DNAs and proteins are used to correlate biomarkers to specific diseases, DNAs are often limited in providing a predictable and reliable diagnosis. This is because the correlation in the levels of expression between mRNAs and corresponding proteins is further regulated at the translation level (Huber 2002). Moreover, one gene may express multiple proteins with variable biological function, and the proteins expressed from the genes may undergo a number of post-translational modifications which may be important in various pathological processes. Proteins, on the other hand, are considered as effective diagnostic sources. This is because proteins are the final form of the gene product and hence directly associated with biological functions. Many protein biomarkers have been discovered for aid of early diagnosis (Sahab et al. 2007). Protein biosensors thus have a profound significance for POC diagnostic, and portable/disposable POC applications facilitate integration of the biosensors with microfluidics. In this section, we will introduce technologies for protein biosensing and discuss their strengths and challenges.

2.2.2 General protein sensing technique for diagnostics

The current standard in immunoassays is the Enzyme-Linked Immuno-Sorbent Assay (ELISA) (Lequin 2005). It has been in use for more than last three decades and proven very robust and reliable. However, ELISA suffers from several drawbacks such as a large sample volume of 50 μl per microwell, resulting in long diffusion and thus long incubation times on the order of days (Sato et al. 2000) as well as the need for specific enzyme-fluorophore combinations that do not interfere the antibody–antigen reaction (Bange et al. 2005). This has led to search for improved immunoassays, using either advanced labels or being

completely label-free. Classic ELISA assays do not involve microfluidics; the sample and reagent dispense is performed either via manual pipetting or via pipetting robots. While the pipetting robot technology is well accepted in the field, microfluidics offers quantifiable benefits over existing procedures. It turns out that microfluidics, in fact, can improve the sensitivity, speed, and reduce costs of an immunoassay over ELISA by overcoming the issues of the large sample volume. For example, Sato et al. (2000) quoted a time reduction for an immunoglobulin assay by a factor of 90 over the classical ELISA, resulting in an assay time of less than 1 h with a limit of detection of 1 μg/ml when employing a bead-based immunoassay in a microfluidic channel. Emerging label-free approaches have driven the research on improving immunoassays (Bange et al. 2005; Daniels and Pourmand 2007). These can be classified into direct assays using evanescent wave sensors (interferometry, surface plasmon resonance (SPR)) (Fan et al. 2008; Ince and Narayanaswamy 2006) and direct assays based on electrical impedance spectroscopy (Dong et al. 2007; Paenke et al. 2008; Yang et al. 2007).

2.2.3 Microfluidic protein sensor for POC diagnostics

Optical techniques are prevalent, which rely on the interaction between light and an adsorbed immunoselective adlayer. SPR is one of the most promising optical techniques, which relies on the measurement of the attenuated total reflection at the boundary between different refractive index materials. It is extremely sensitive to surface phenomena, making it a viable label-free sensor to probe small changes in monolayer properties. SPR sensors can be integrated into microfluidic devices; however, the requirements originating from the optical components mainly limit the miniaturization of the classical prism couplers. Fiber-, waveguide-based SPR, and SPR imaging are alternative approaches that bring bench-top prism-based SPR biosensor to miniaturized, integrated, and portable SPR devices. Fiber-based SPR sensors are an interesting approach and recent publications show that these sensors have a high potential for in situ measurements. Jang et al. detected prostate specific antigen (PSA) by using optical fiber SPR sensor (Jang et al. 2009) and the Fabry–Perot setup was embedded in a microfluidic channel (Lin et al. 2009). Waveguide-based SPR can also be built using various geometries, which can be integrated with microfluidics. A Mach–Zehnder waveguide SPR was fabricated in a planar process using established materials and MEMS (Micro-Electro-Mechanical-Systems) fabrication technology (Blanco et al. 2006; Sepulveda et al. 2006; Suzuki et al. 2005). SPR imaging (SPRi) is attractive because it allows simultaneous analysis of multiple bimolecular interactions. SPRi has been successful to detect

Table 1 Device specifications and detection parameters for microfabricated DNA sensors

Detection method	Device specifications	Linkers	Capture agent	Analyte	Solution delivery	Media	Ionic strength	Debye length	pH	Detection limit	References
Current	CMOS FET	MPTMS	ssDNA	ssDNA	Mixing cell	N/A	N/A	N/A	N/A	N/A	Barbaro et al. (2006)
Current	CMOS FET	APTES	ssDNA	ssDNA	Mixing cell	TE	6 mM	4 nm	8	1 μM	Ingebrandt and Offenhausser (2006)
EIS	CMOS FET	APTES	ssDNA	ssDNA	Mixing cell	NaCl	0.01–100 mM	100^{-1} nm	7	N/A	Ingebrandt et al. (2007)
EIS	CMOS FET	APTES	ssDNA	ssDNA	Mixing cell	NaCl	0.01 mM	100 nm	7	N/A	Ghoshmoulick (2009)
EIS	Au MEA	Thiol	ssDNA	ssDNA	Mixing cell	PBS	10 mM	3.2 nm	7.4	100 pM	Ito (2007)
EIS (FeCN)	Au MEA	Thiol	ssDNA	ssDNA	Mixing cell	PBS	100 mM	1 nm	7	350 nM	Kafka et al. (2008)
EIS (FeCN)	Au MEA	Thiol	ssDNA	ssDNA	Mixing cell	PBS	20 mM	2.3 nm	7.4	1 μM	Gebala et al. (2009)
EIS (FeCN)	Organic NW	N/A	ssDNA	ssDNA	Mixing cell	PBS	180 mM	0.75 nm	7.4	20 nM	Peng et al. (2007)
EIS	Porous Si	N/A	ssDNA	ssDNA	Mixing cell	PBS	25 mM	2 nm	7	1 mM	Vamvakaki and Chaniotakis (2008)
Current	α-HL NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8.5	N/A	Meller et al. (2001)
Current	α-HL NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8	500 nM	Maglia et al. (2008)
Current	α-HL NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8	200 nM	Stoddart et al. (2009)
Current	α-HL NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	7.5	17 μM	Purnell et al. (2008)
Current	α-HL NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	7.5	20 μM	Purnell and Schmidt (2009)
Current	MSPA NP	N/A	N/A	ssDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8	1 μM	Butler et al. (2008)
Current	A1203 NP	N/A	N/A	dsDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8	800 pM	Chen et al. (2004)
Current	Si NC	N/A	N/A	dsDNA	Microfl.	TE	445 mM	0.67 nm	8.3	58 pM	Mannion et al. (2006)
Current	PC NP	N/A	N/A	ssDNA dsDNA	Mixing cell	KCl/TE	1 M	0.32 nm	8	10 nm	Harrell et al. (2006)
Fluorescence (LED + CCD)	Glass/PDMS	N/A	N/A	RNA	Microfl.	MgSO$_4$	5 mM	4.5 nm	N/A	465 nM S/N = 23	Kaigalla et al. (2008)
Fluorescence (LED + CCD)	Glass/PDMS	N/A	N/A	ssDNA	Microfl.	KCl/TE	50 mM	1.4 nm	9	170 fM	Ramalingam et al. (2009)
Fluorescence (LED + CCD)	Glass/polyimide	N/A	N/A	ssDNA	Microfl.	TE	30 mM	1.9 nm	8.3	166 pM S/N = 200	Pjescic et al. (2010)

EIS electrochemical impedance spectroscopy, *LED* light emitting diode, *CCD* charge coupled device, *CMOS FET* complementary metal oxide semiconductor field effect transistor, *MEA* microelectrode array, *NW* nanowire, *α-HL* Alpha-Haemolysin, *MSPA* Mycobacterium smegmatis porin A, *NP* nanopore, *NC* nanochannel, *PC* polycarbonate, *PDMS* Polydimethylsiloxane, *MPTMS* 3-mercaptopropyltrimethoxysilane, *APTES* 3-aminopropyltriethoxysilane, *ssDNA* single-stranded DNA, *dsDNA* double-stranded DNA, *TE* Tris/ethylenediaminetetraacetic acid, *PBS* phosphate buffered saline (The Debye length was calculated based on the equation $\lambda D = 0.32/\sqrt{I}$, with I being the ionic strength)

adsorption and desorption of multiple proteins (Hook et al. 2009), monitor real-time reactions of antigen–antibody in arrayed format (Xinglong et al. 2005). Detecting breast cancer biomarkers have been demonstrated using SPRi (Ladd et al. 2009). Lee et al. developed an automatic, chip-based microfluidic device that has a multi-channel configuration to detect microarray immunoassay samples based on a SPRi detection system (Lee et al. 2007). However, the key challenge in all the SPR biosensor development lies not in the integration of the various components of the biosensors including sampling handling and electronics but maintaining sensitivity and robustness of the integrated SPR biosensors simultaneously (Hoa et al. 2007).

Besides optical detection of surface immunoreactions, impedance spectroscopy is a very interesting approach for a label-free immunoassay. Similar to the technology for DNA hybridization detection, a molecular linkage reaction in an immunoassay changes the capacitance of the adsorbed layer on the surface of an electrode or a Metal-Oxide-Semiconductor (MOS) transistor. Recently, the use of porous materials on top of an electrode has shown to significantly enhance the signal originating from an immunological linkage reaction, making it one of the future pathways toward improvement of an immunosensor. The electrical detection has benefits over a combined electro-optical technique: no optical transducers are necessary, which increase the cost and the complexity of the system. Recently, development on complete Complementary Metal-Oxide-Semiconductor (CMOS) circuits coupled with a immunoassay has also been reported, which are specifically designed to detect signals originating during an immunoassay. These circuits are based on well-established silicon device

technology (Ghafar-Zadeh et al. 2009). Despite all the progress on label-free immunoassays and the unprecedented benefit on reagent consumption, a recent comparative study on the performance of label-free immunoassays provides a very critical outlook; the selectivity of label-free assays is still inferior to that of the classical ELISA (Daniels and Pourmand 2007).

Besides the use of labels, one of the issues with ELISA is that it is performed in microtiter well plates, usually containing 96 or 384 wells per plate. These wells have a volume of 50 μl, which can lead to a substantial dilution of the initial sample, degrading the lower limit of detection. In addition, since the assay is driven by diffusion of the reactants to the antibodies immobilized on the microwell surface, the incubation times are comparably long, often up to a few days per assay. A strategy to reduce the assay time lies in reducing the well volume while increasing its surface area, which leads directly to miniaturization and microfluidic integration (Fig. 3) (Yang et al. 2007; Blagoi et al. 2008; Gao et al. 2005; Hoegger et al. 2007; Lin et al. 2004; Yu et al. 2009; Zhao and Shippy 2004).

One method to increase the surface area of the assay is to use a bead-based assay. Instead of functionalizing the well surface with antibodies, micron-sized beads can be functionalized to capture the antigen. A benefit here is that beads can be transported by a fluid flow, while the well plate surface remains static and the sensor has to be movable or being able to provide addressability (Fig. 4) (Holmes et al. 2007). By using magnetic beads, the beads can be separated from the initial testing medium, allowing the immunoassay to be performed in a different solution under more reproducible conditions (Fig. 5) (Do and Ahn

Fig. 3 Example of a micro-ELISA in microwells fabricated using SU-8 epoxy patterned on silicon. An IgG immunoassay has been performed inside of these microwells. a A schematic of the microwell chip, b a fluorescence image (top view) of the microwell plate, c a scanning electron micrograph of a single well and d a fluorescence microscopy image of the immunoassay on a part of the chip (C-reactive protein, Cy5 fluorescent dyes). The detection limit was reported to be 30 ng/ml and the assay time was 4 h. The microfluidic approach helps to reduce the solution volume, consequently reducing assay time (Blagoi et al. 2008)
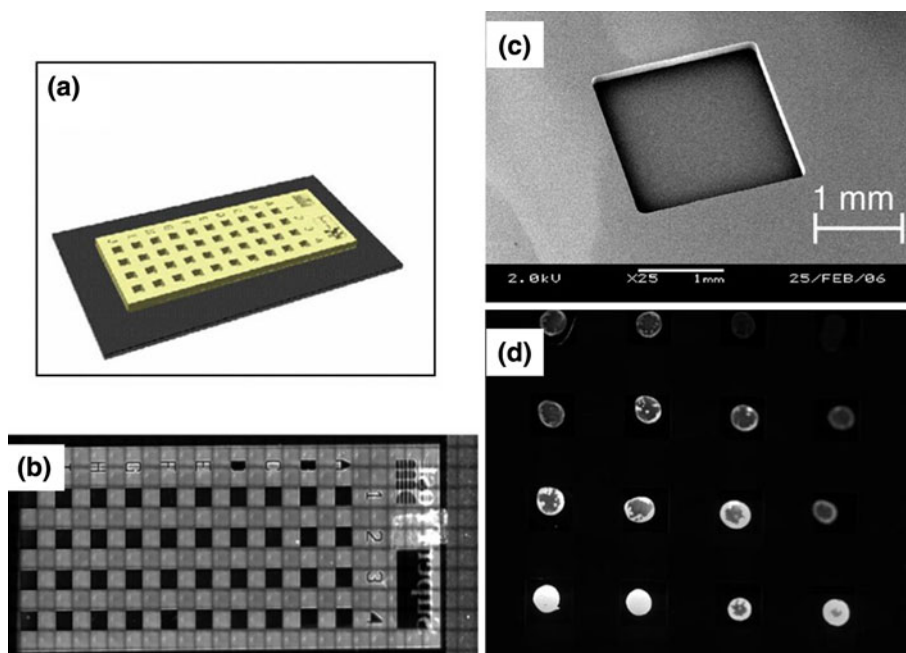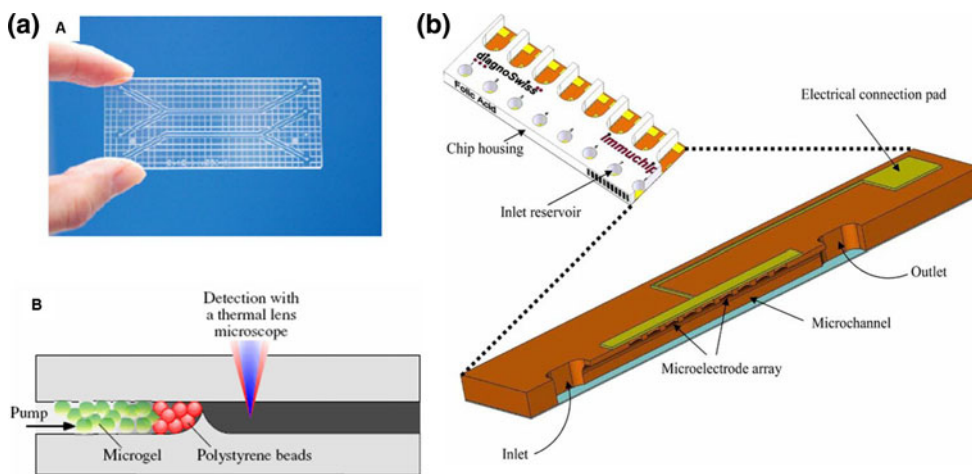
2008). Besides using beads to capture the antigen, an alternative to the enzyme-linked secondary antibody is the use of fluorescent beads that are linked to these secondary antibodies. By using labels with different fluorophores, a multiplex assay can be accomplished, reducing the number of wells needed on a microtiter plate since every secondary antibody carries its own "barcode" label (Derveaux et al. 2008; Earle et al. 2007; Rauf et al. 2009). This label can then be read out optically, allowing an independent quantification of concentration and type of antigen. Combining fluid flow of fluorescent beads with localized fluorescence quenching, the beads can be labeled "in situ" while flowing through a microfluidic channel (Birtwell and Morgan

2009). This allows a lab-on-a-chip without the need to provide beads with different fluorophores. Here, microfluidics is used to harvest the benefits of a bead-based immunoassay.
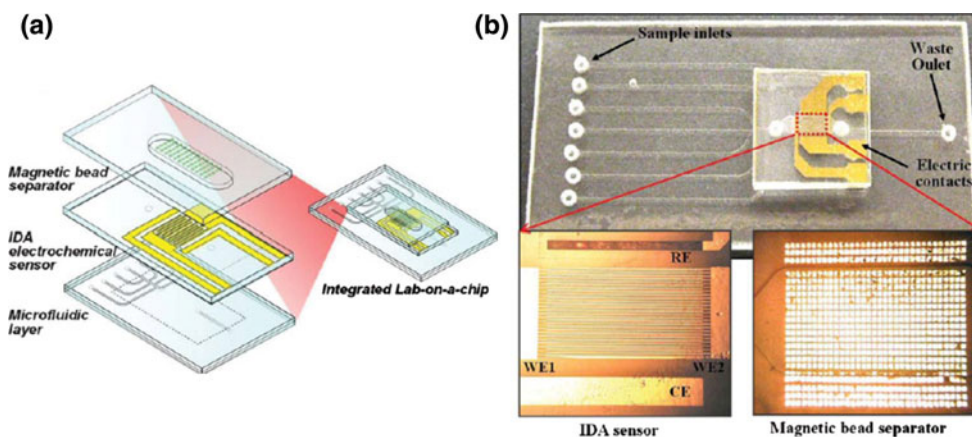
Bead-based immunoassays, however, do not necessarily have to rely on optical detection (Fig. 5) (de la Escosura-Muniz et al. 2008). Since the beads are micron sized, the resistive pulse detection technique can be used, similar to the classical Coulter counting setup for cell-based assays (Sexton et al. 2007; Uram et al. 2006a; Uram et al. 2006b; Uram and Mayer 2007). By using a pore size that matches size of the bead, minute changes in the bead diameter based on an immunological linkage reaction at the surface of the

Fig. 5 Microfluidic immunosensor based on the separation of magnetic microbeads, using impedance spectroscopy for detection. **a** The assembly of the microfluidic chip, consisting of a microfluidic bottom layer containing 7 parallel channels, an interdigitated array (IDA) middle layer providing electrical contact to the microchannels and a top layer containing a patterned permalloy to allow magnetic bead separation. All parts were fabricated using injection molding and bonded using an UV-curable adhesive. **b** The complete assembly with a magnified view of the electrode array and the permalloy magnetic bead separator (Do and Ahn 2008)

Fig. 6 Parallel microchannel device for the detection and determination of pollen. Due to the different surface charge of pollen and polystyrene test particles, the Coulter signature is different between the two types of particles, despite of similar sizes (Zhe et al. 2007)
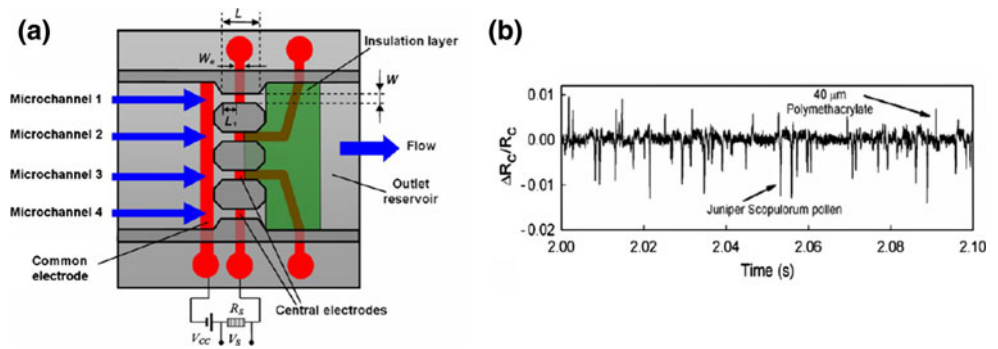
bead can be detected via the pulse amplitude. The resistive pulse sensing technique can be applied even for large particles such as pollen, which themselves can act as beads that capture antibodies and thereby increase their size (Fig. 6) (Zhe et al. 2007). Microfluidic integration of a pore array for this kind of pollen detection has been successfully demonstrated, enabling low-cost POC devices, including the supporting electronics (Jagtiani et al. 2006a, b). Looking at the recent accomplishments on the bead-based immunoassays, microfluidics has the potential to make a difference in the field of immunoassays. The challenge, however, that all immunoassay-based sensors face, independent of being classical ELISA or microfluidic-based assay, is the availability of a specific immunoreaction that allows selective detection of target analyte.

The indicator displacement assay has also been used to detect target proteins (Wilson 2009). While immunoassay techniques are based on specific recognition of antibody and antigen, the displacement assays is the pattern-based recognition of composite signal constructed from multiple differential binding interactions (Wright and Anslyn 2006). De et al. detected five proteins in undiluted human serum by using the competitive adsorption between green fluorescent proteins on gold nanoparticles and the target proteins (De et al. 2009). Choi et al. detected a cancer biomarker, Thyroglobulin, in a cocktailed protein mixture using the competitive protein adsorptions (Fig. 7) (Choi and Chae 2009b). Implemented in a microfluidic system, the target protein (thyroglobulin, Tg) displaces a preadsorbed weak-affinity protein, IgG, on one surface, while a pre-adsorbed strong-affinity protein, fibrinogen, is not displaced by the target protein on the other surface. Differential measurement using SPR allows the detection of thyroglobulin (Choi et al. 2008; Choi and Chae 2009b). While immunosensing technologies have to go through time-consuming and labor intensive immobilization processes, the sensor utilizing the competitive adsorption of proteins themselves can avoid the need to rely on bioreceptors as a capture probe and their attachment to transducers. This uniqueness can be a complementary solution to the conventional immunosensors.

Microfluidic paper-based analytical devices (μPADs) are a new class of POC diagnostic devices (Fu et al. 2010; Martinez et al. 2010). Paper is thin, flexible, light weight, flammable (disposable), compatible with biological samples, and can be easily modified by chemicals. Microfluidic channels are defined by hydrophobic barriers which are patterned by impregnating the paper with photoresist and exposing it to UV light (Martinez et al. 2007). Since fluids flow by capillary force, a bulky and complicated supporting pump is not necessary, which allows biosensors to be more readily transformed in lab-on-a-chip with capillary microfluidics (Fig. 8). μPADs has demonstrated clinically relevant concentrations of glucose and proteins in artificial urine quantitatively (Martinez et al. 2008, 2010). However, the application should be limited because the immobilization techniques on a paper are yet immature and dried bioreceptors could lose their activity with time (Mitchell 2002).

In spite of many studies in microfluidic-based protein biosensors, the robustness and reliability of them have not been fully explored yet, partially because of the complex three-dimensional protein conformation and the relatively poor knowledge of protein-to-surface interactions. Given that proteins have been more favorable as a diagnostic tool, clearly substantial amount of work needs to be performed in the development of microfluidic-based protein sensors.

3 Challenges

3.1 Non-specific adsorption (NSA)

Physiological samples consist of a complex mixture of compounds, including abundant proteins (on the order of mg/ml) that tend to adsorb nonspecifically to surfaces. It is extremely challenging to detect target biomarkers at concentrations on the order of, typically, ng/ml or less when other abundant nonspecific proteins are present. Reduction of non-specific adsorption (NSA) of biomolecules is crucial in biosensor developments especially for
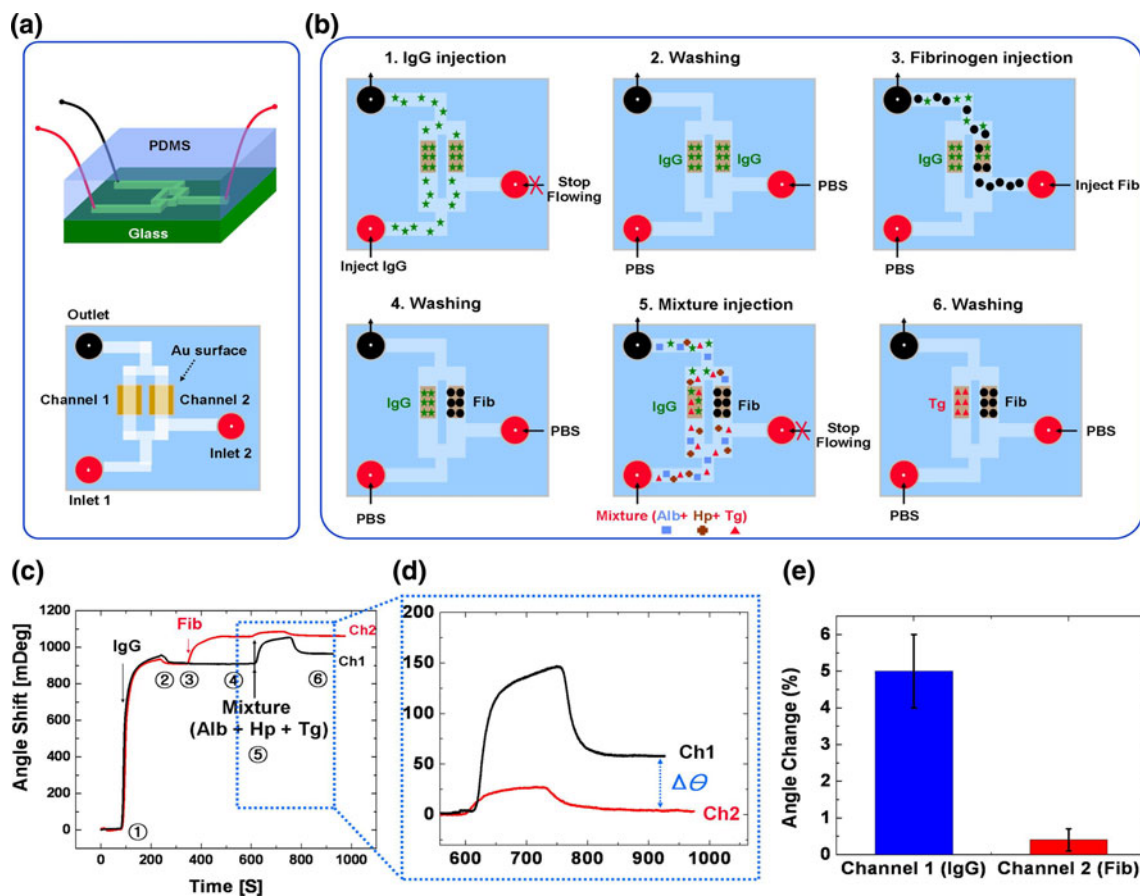
Fig. 7 a A custom-made microfluidic device to demonstrate the Vroman effect-based protein biosensor. **b** A schematic of operating principle. (1) IgG is injected from the inlet 1 to cover both surfaces, (2) washing process to remove unbound IgG, (3) fibrinogen flows from inlet 2 and displaces the pre-adsorbed IgG on one surface, (4) washing process to remove any residue on the surface, (5) a mixture of albumin, haptoglobin and Tg flows from inlet 1, (6) only Tg displaces IgG in channel 1 while any of proteins does not displace fibrinogen in channel 2. **c** SPR sensorgram of the displacement event; Tg detection of two engineered surfaces, pre-adsorbed by IgG and fibrinogen. **d** Normalized close-up SPR sensorgram after the Tg injection, **e** final angle changes (%) on both surfaces (angle change/ previous angle value × 100). Each has selectivity to a specific protein to be detected (Choi and Chae 2009b)

clinical diagnostics. Any biomolecular NSA provokes overestimation of the affinity value and consequently "false positive" errors in detection (Ogi et al. 2009). In addition, NSA masks the signal from analytes of interest, reducing sensitivity of the sensor (Masson et al. 2006). Various approaches have been exploited to reduce NSA on biosensing surfaces (Lahiri et al. 1999; Chapman et al. 2000; Israelachvili 2005). Polyethylene glycol (PEG) or OH-terminated SAM (Self-assembled monolayer) has been used as a one of the most promising blocking materials but it has been reported that detectable levels of NSA was observed after the modification (Munson et al. 2004). An alternative to minimize NSA includes blocking the vacant sites using bovine serum albumin (BSA). BSA, however, may limit interactions between the biosensor and biological samples, thereby causing false negative responses (Bolduc and Masson 2008) or can be displaced by other proteins, or form a multilayer with oppositely

charged proteins (Choi and Chae 2010). Although many research groups have reported methods to reduce NSA in the biosensing devices by using various functionally modified chemicals at the biosensing surface (Wang et al. 2010; Chang et al. 2010; Masson et al. 2006), NSA is still an enduring problem in commercializing biosensors. Besides, NSA becomes much more severe when the biosensors are integrated with microfluidics which typically uses a hydrophobic material, PDMS (Polydimethylsiloxane) (Wong and Ho 2009). This is because NSA easily occurs on the PDMS walls, leading to masking samples, which consequently force analysis useless. For example, adsorption of fluorescent markers can cause a drift in the background fluorescence intensity, failing optical analysis (Munson et al. 2004). Prior-reported methods to reduce NSA have been implemented in the microfluidic channels such as chemical surface modification (PEG, OH-SAM, and zwitterionic) and
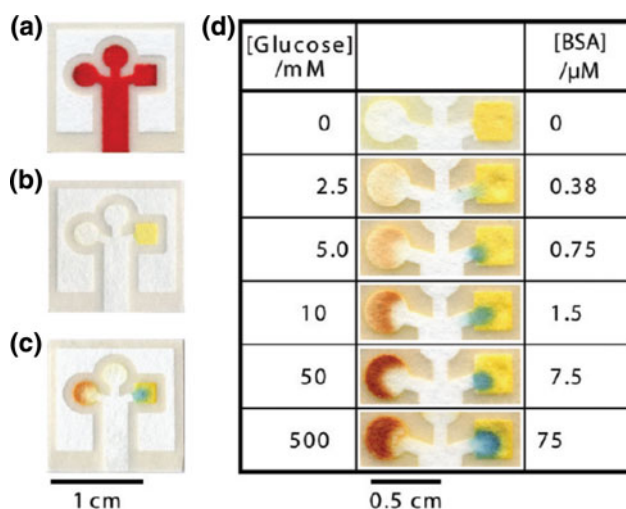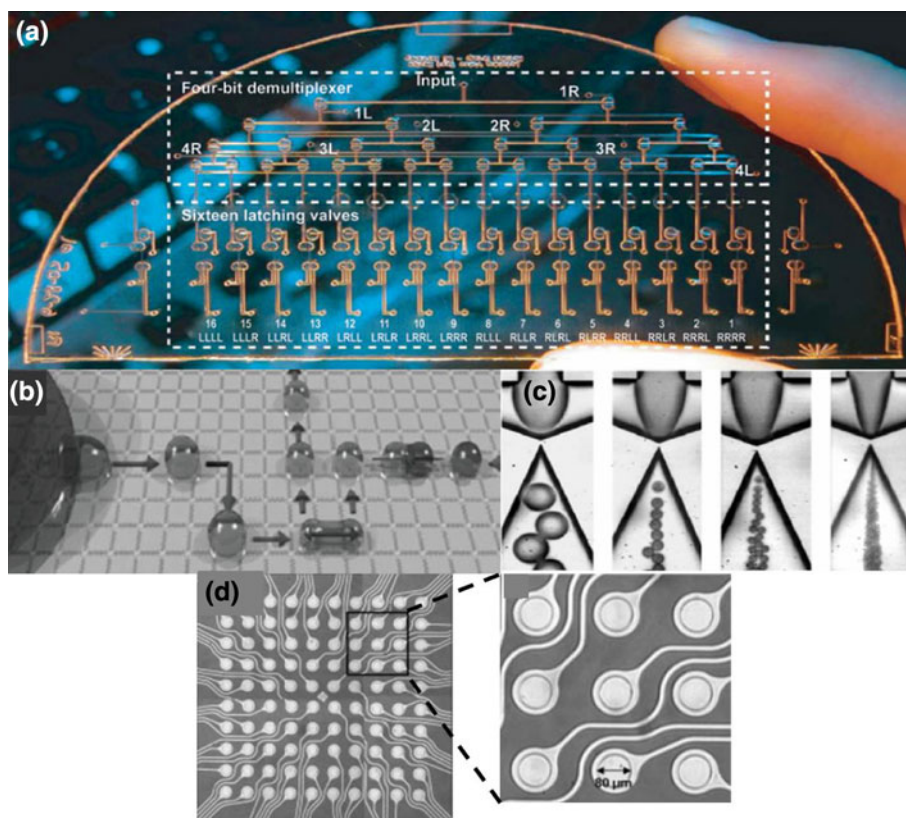
Fig. 8 µPADs for analysis of glucose and protein in urine. **a** Patterned paper after distributing 5 µl of red ink to show the integrity of the hydrophilic channel. **b** Complete µPADs after spotting the reagents. **c** Positive assays for glucose and protein using 5 µl of a solution that contained glucose and BSA in an artificial urine solution. **d** Results of paper-based glucose and protein assays using a range of concentrations of glucose and BSA in artificial urine (Martinez et al. 2010)

physical adsorption (BSA). However, NSA in microfluidic-based biosensors remains to be addressed for clinical POC diagnostics.

3.2 System integration

Another challenge in POC diagnostics is to develop a fully integrated biochemical analysis system that is capable of performing all procedures on a single platform. Fluid delivery, mixing, separation, and concentration are some of the fundamental sample preparation steps often required in a typical biomedical assay. The requirement of bulky supporting equipments, the difficulty in miniaturization of the fluidic operation modules, and most importantly the integration of these modules present key technological challenges that hinder the integration of sample preparation modules into a POC device (Mariella 2008). Recently, several microfluidic platforms are emerging for effective integration of multiple sample preparation modules toward fully automated biomedical analysis. Multilayer soft lithography (Fig. 9a), digital microfluidics (Fig 9b), multiphase flow systems (Fig. 9c), and electrokinetic microelectrode arrays (Fig. 9d) are some of the promising platforms for POC diagnostics. Multilayer soft lithography is a technique extended from soft lithography, in which devices consisting of multiple layers are fabricated by bonding layers of elastomeric materials (Unger et al. 2000). The deformability of the elastomeric materials allows a large actuation of the PDMS membrane with pneumatic control, which allows several fundamental microfluidic operations, such as on–off valves, peristaltic pumps, and

Fig. 9 **a** An integrated microfluidic platform fabricated by multilayer soft lithography (Grover et al. 2006). **b** Schematic of the digital microfluidic platform (Lienemann et al. 2006). **c** Formation of water-in-silicone oil droplets with an embedded circular orifice (Yobas et al. 2006). **d** A microelectrode array device (Krishnan et al. 2008; Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission)

mixers, for integrating large-scale microfluidic networks (Melin and Quake 2007). However, to implement the pneumatic-controlled microfluidic network at POC, external pneumatic sources, and multiplexed gas valves are required to be integrated into the final system, which could present a technological hurdle for system integration. Digital microfluidics is another promising technology for developing microfluidic systems with precise and active control of the fluid in the form of droplet (Lawi et al. 2009). Droplet motion in digital microfluidics can be driven by the electrowetting mechanism, which is a result of the surface energy change due to the applied potential. However, surface fouling presents a challenge in the operation and a large array of microelectrodes needs to be independently addressed for high-throughput applications. Multi-phase flow of immiscible fluids, or droplet microfluidics, represents another promising technique for implementing the necessary sample preparation procedures. Droplets or bioreactors in sub-nanoliter volume can be formed spontaneously in microchannels when two immiscible fluid streams merge (Griffiths and Tawfik 2006).

Various droplet processes such as formation, sorting, storage, fusion, and manipulation have been demonstrated toward biomedical analysis (Teh et al. 2008). To implement droplet-based bioreactors, precise controls over the surface properties of the channel are required as the wetting property of the fluid with respect to the channel wall is important in determining the droplet production (Dreyfus et al. 2003). Finally, microelectrode arrays for AC or DC electrokinetic sample preparation is an emerging lab-on-a-chip platform for POC diagnostic (Wong et al. 2004a). Electrokinetic sample preparation is especially suitable for POC diagnostic application, since electrokinetics biochips require only low-power electronic interfaces, which can be integrated and implemented effectively in portable systems. In particular, microfluidic operations such as mixing, pumping, concentration, and separation based on AC electrokinetic can be performed with low applied AC potential (<10 Vpp) (Sigurdson et al. 2005; Feldman et al. 2007; Gregersen et al. 2007; Wong et al. 2004b; Sin et al. 2009a, b). The low voltage requirement of AC electrokinetics not only prevents electrolysis but also facilitate the implementation at POC. However, electrokinetic phenomena are sensitive to buffer conductivity, which may results in large performance fluctuations for the POC devices. A built-in impedance measurement device for testing the buffer conductivity is required to optimize the performance of electrokinetics sample preparation systems.

A large number of ongoing research work focuses on developing new research tools for biomedical or pharmaceutical applications by means of microfluidics. In addition to the above-mentioned technology, other promising approaches, such as acoustic actuation, inertial microfluidic devices, and optoelectronic manipulation, are also emerging and could play an essential role in the future POC diagnostic systems (Chiou et al. 2005; Shah et al. 2009; Chung and Cho 2008; Shi et al. 2009; Hur et al. 2010; Williams et al. 2008). The stringent requirements of POC diagnostics represent unique challenges for sample preparation. As one of the ultimate goals is to develop fully automated POC devices that enable medical diagnostics to be performed, low-cost, effective system integration strategies will likely become a key area of microfluidic development in the future.

4 Conclusion

This review presents an overview on recent advances in the development and the application of microfluidic-based biosensors; nucleic acid and protein sensors. Such devices are extremely useful for delivering clinically relevant information in a simple, fast- and low-cost fashion, and are thus uniquely qualified for meeting the demands of POC testing. One of the challenges is to avoid NSA which causes false response errors and decrease sensitivity. Another issue to be addressed is the integration and automation of the technology as well as development of appropriate sample preparation methods. Moreover, successful development of POC systems will require continued improvement and validation of biomarkers and development of bioreceptors for those biomarkers. In short, while there is still a long way to go for POC testing, microfluidic biosensors will eventually become one of the strongest candidates for a real-world tool.

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