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Biosensors in Microfluidic Chips

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Abstract A biosensor is a sensing device that incorporates a biological sensing element and a transducer to produce electrochemical, optical, mass, or other signals in proportion to quantitative information about the analytes in the given samples. The microfluidic chip is an attractive miniaturized platform with valuable advantages, e.g., low cost analysis requiring low reagent consumption, reduced sample volume, and shortened processing time. Combination of biosensors and microfluidic chips enhances analytical capability so as to widen the scope of possible applications. This review provides an overview of recent research activities in the field of biosensors integrated on microfluidic chips, focusing on the working principles, characteristics, and applicability of the biosensors. Theoretical background and applications in chemical, biological, and clinical analysis are summarized and discussed.

Keywords Biosensor · Electrochemical · Mass · Microfluidic chip · Optical · Transducer

Contents

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Abbreviations

PSA Prostate-specific antigen
TFT Thin film transistor

1 Introduction

Microfluidic technology for chemical or bioanalytical purposes concerns the precise control of fluids in a limited space, which may be intentionally patterned on chips, because a number of valuable benefits are expected from such systems [1, 2]. As many articles and reviews have pointed out, the alleged advantages include reduced reagent consumption, short analysis time, a small-sized scale, low cost, and high sensitivity. Over the last two decades, there has been an explosive development of miniaturized analytical systems and related techniques based on microfluidics for chemical analysis, bioanalysis, clinical diagnostics, and other applications [3–15].

In spite of the impressive opportunities, the use of microfluidics for chemical and biological analysis involves considerable challenges as well. The analytes in the fluids of microsystems are exposed to unusual physical conditions, such as high surface tension and high surface-to-volume ratio, which could be the reason why analytical information from the microfluidic systems might be significantly different from that predicted from conventional methods [16–19]. In many cases,

pretreatment and handling of the samples for analysis in microfluidic systems are not merely a matter of scale-down. Analysis using microfluidic analyzers often faces troubles in adsorption, injection, mixing, and so on, which are not significant in conventional analysis in a large scale. Potential solutions for the problems that may occur in the microfluidic systems have been suggested in numerous papers, but many of them are still inadequate.

Another approach is omission or simplification of pretreatment and handling processes. This may be a preferable strategy for more effective analysis. However, better sensing parts are required to possibly share the burden by playing more roles than a simple detector, without any or negligible loss of the analytical quality. Biological functional units such as antibodies or enzymes can endow a detector with selective recognition ability so that the corresponding microfluidic analyzer should work without complicated and strict pretreatment prior to detection.

Traditionally, a biosensor is created by combining a biological component with a physiochemical detector [20–23]. Biosensors exploit biochemical molecular recognition properties for a selective analysis. The general structure of biosensors consists of analyte recognition, signal transduction, and readout, as shown in Fig. 1. The representative example of commercial biosensors is the blood glucose sensor. Since the first classical type from Clark and Lyons was introduced in 1962 for detecting glucose levels in blood, it has been followed by many different biosensors [24]. However, only a limited number of the biosensors fulfill the stringent requirements for commercialization, e.g., practical demand in terms of market size, and functional reliability in the real environments in which they are expected to operate. As informational technology becomes more widespread and more convenient applications are pursued, biosensors are increasingly demanded in modern life. On-site analysis at low cost and within a short time is undoubtedly important, not only for ordinary people but also for researchers working in laboratories.

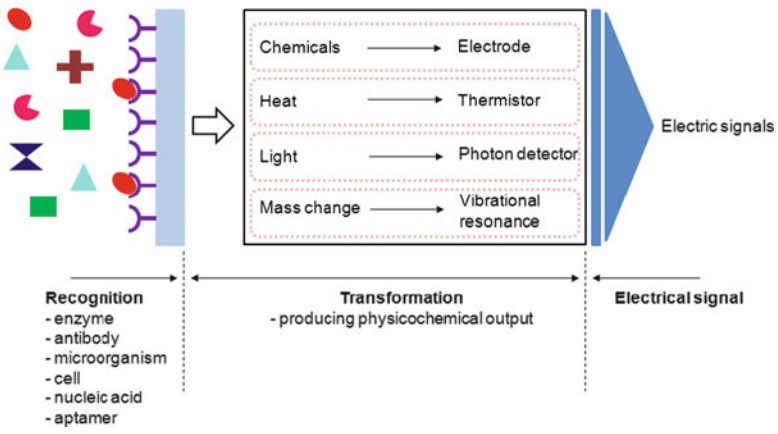

Fig. 1 Configuration of a biosensor

Generally, biosensors are designed to extract analytical information about certain analytes in given samples without pretreatment. Nonetheless, combination with microfluidic systems could provide conventional biosensors with opportunities for improvement in terms of analytical power or in applicability for a wider range of applications. For example, bioassays could benefit from automatic sequential separation and chemical synthesis that are linked to the detection part of a miniaturized system [25, 26]. Drug discovery, for instance, needs innovative bioassay technology that uses as small a volume as possible to minimize expensive, difficult, and inefficient processes. The lab-on-a-chip is a big target in the fields of medicine, biotechnology, and pharmacology and is one of the key motivations for integrating micro- or submicroscale biosensors into microfluidic systems.

Microfluidic-based biosensors have advanced greatly in various fields over the last few decades [8, 27]. The fundamental concept underlying the microfluidic biosensors that have been reported is to integrate the analytical functions necessary for biochemical analysis onto a single chip, including sample preparation, pretreatment, detection, and sometimes molecular separation or sorting.

This review summarizes the research achievements concerning integration of biosensors into microfluidic chips, which have been reported mostly since 2005. We discuss the latest developments by categorizing them on the basis of four different aspects; detection principles, analytes, functional receptors, and microfluidic components. Based on a balanced view of recent trends, the review is concluded by looking at the future perspectives in this field.

2 Detection Principles

The detection principles for biosensors integrated on microfluidic chips are classified into several types, including optical, electrochemical, and mass-sensitive methods. The trend in the development of detectors has been to constantly pursue two key virtues: sensitivity and selectivity. In this regard, the research issues on the detectors for microfluidic systems are not significantly different to those for conventional biosensors.

2.1 Optical Methods

Most optical detection methods for biosensors are based on ultra-violet (UV) absorption spectrometry, emission spectroscopic measurement of fluorescence and luminescence, and Raman spectroscopy. However, surface plasmon resonance (SPR) has quickly been widely adopted as a nonlabeling technique that provides attractive advantages. Fueled by numerous new nanomaterials, their unique, SPR-based or related detection techniques are increasingly being investigated [28–31].

The conventional methods for optical detection need various filters, lenses, light sources, and photomultiplier tubes (PMTs), which are essential for microfluidic systems as well. However, the functional components for optical detection in microfluidic analytical systems are seldom miniaturized, and necessarily sophisticated or expensive in many cases. Considering that microfluidic chips are basically intended to conduct handy, cheap, user-friendly, and on-site analysis, it is obvious that the crucial issue in the optical setup for microfluidic systems is how to overcome such a problem. To cope with this fundamental challenge, many researchers have made efforts to miniaturize waveguides, filters, and lenses so that they are tiny and simple enough to be integrated on a chip. Such optoelectronic components include laser diodes, light-emitting diodes (LEDs), charge coupled devices (CCDs), and complementary metal oxide semiconductors (CMOSs), which replace the conventional optical elements [32, 33]. Frank et al. provided a broad overview of current research and development in point-of-care diagnostic devices utilizing optical detectors in microfluidic platforms [13]. Various optical detections of biological samples covering absorbance, fluorescence, luminescence, and SPR are summarized in Table 1.

Absorption spectrometry is a classic detection method for chemical analysis. It requires a long path length of incident light through a sample at lower concentration. This is critical for absorbance measurements in microfluidic chips because the narrow microfluidic channels are obviously problematic for sensitive absorbance measurement. That is why there are not many cases employing absorbance detection. A few peptides have been separated by on-chip electrochromatography and detected by UV absorbance [34]. Optical fibers are normally used to enhance efficient absorbance in the microfluidic chips. For instance, the incident light from a deuterium–tungsten light source follows a optical fiber to reach the sample and then goes to a CCD array detector through the bottom fiber [13]. Incident light can be focused by integrating a microlens in a complex three-layer microfluidic chip [54]. This system has slit channels filled with "black ink" to absorb any scattered light, and also a cylindrical microlens in the polydimethylsiloxane (PDMS) layer on the chip. This creative design enabled detection of the peptide separation with enhanced sensitivity. Llobera et al. reported an optical air mirror that utilized the refractive index difference between PDMS and air [49]. Ro et al. showed efficient and sensitive absorbance detection using a light collimating system on a PDMS microchip for capillary electrophoresis (CE) [35]. This collimating setup consisted of various optical components features and gave significant reduction of stray light. Steigert et al. proposed an interesting concept of a centrifugal compact disk (CD)-based system that allowed a fast colorimetric assay of alcohol in a single droplet of whole blood [55]. In this optical system, the laser was illuminated perpendicular to the flat surface of the cyclic olefin copolymer disk, and the optical path length increased by one order of magnitude in comparison to the conventional direct incidence. Caglar et al. designed a glass–PDMS microfluidic chip for determination of calcium ions in samples by introducing an optical fiber and a ball lens [36].

Fluorescence detection involves molecular light absorption, which triggers the emission with longer wavelengths than for excitation. This method is often

Table 1 Optical detection

Method	Sensing parts	Analytes	LOD	References
Absorbance	Optical fiber, CCD detector	Thiourea	167 μM	[34]
	Optical fiber, microlens, slits, PMT detector	Fluorescenin	S/N = 3	[35]
	Optical fiber, a ball lens, CCD detector	Ca^{2+} ions	0.027 mM	[36]
	Optical fiber, PMT detector	Ammonia	2 ppm	[37]
Fluorescence	Microscope-based PMT detector	Protein	12 μg/mL	[38]
	Optical path length, CCD detector	Flavin	1 mM	[39]
	Optical fiber, pinhole filter, PMT detector	FITC	10^{-7} M	[40]
	Spherical lens, filter, PMT detector	Fluorescence	0.01 nM	[41]
	LED, SSPM	Fluorescence	N/A	[42]
Chemiluminescence	VersaDoc	CRP	0.1 mg/L	[43]
	Multianode-photomutiplier array	SEB	0.1 ng/L	[44]
		E. coli	10^4 cfu/mL	
		M13	10^5 pfu/mL	
	TFT photosensor	DNA, horseradish peroxidase	0.5 nM	[45]
	PMT detector	Cytochrome c	~250 nM	[46]
		Myoglobin	~160 nM	
		Horseradish peroxidase	~240 nM	
Bioluminescence	Microscope, PMT detector	ATP	~μM	[47]
Electrochemiluminescence	ITO electrode, PMT detector	Proline	1.2 μM	[48]
Internal reflection spectroscopy	LED, optical fiber, air mirror	Phosphate buffer	41 nM	[49]
Surface plasmon resonance	Direct capture	Cowpea mosaic virus	12.5 μg/mL	[50]
	Nucleotide hybridization	*Fusarium culmorum*	0.06 pg	[51]
	Substractive inhibition	*Puccinia striiformis*	10^5 sp/mL	[52]
	Substractive inhibition	*Phythothora infestans*	10^6 sp/mL	[53]

CCD charge-coupled device, *CRP* C-reactive protein, *FITC* Fluorescein-5-isothiocyanate, *ITO* indium tin oxide, *LED* light-emitting diode, *LOD* limit of detection, *PMT* photomultiplier tubes, *SEB* Staphylococcal enterotoxin B, *S/N* signal-to-noise ratio, *SSPM* solid-state photomultiplier, *TFT* thin film transistor

employed for sensing in microfluidic chips because of its superior sensitivity and selectivity for a variety of samples, in addition to its compatibility with micro-devices [56, 57]. However, the fluorescence of microfluidic systems has some drawbacks, e.g., autofluorescence and nonspecific emission from biomolecules in the sample. Hofmann et al. showed that sensitivity was improved by monolithically integrating an optical long-pass filter on a PDMS microfluidic chip [58]. Schmidt et al. developed a chip-sized spectrometer by combining a linear variable band-pass filter with a CMOS camera [59]. A liquid–liquid waveguide was introduced to the fluorescence detection system on a microfluidic chip by Vesenov et al. [60]. Balslev et al. made a monolithic optoelectronic hybrid platform including an on-chip optically pumped liquid dye laser, waveguides, and microchannels with passive diffusive mixers [32]. Pais et al. reported a disposable lab-on-a chip system equipped with a thin-film organic LED excitation source and an organic photodiode detector for on-chip fluorescence analysis [33]. On the other hand, a portable fluorescence biosensor with quantum dots (QDs) was developed for quantitative analysis and rapid screening of trace amounts of proteins [61]. Schulze et al. realized native fluorescence detection of the proteins separated by microchip electrophoresis using a deep UV neodymium-doped yttrium aluminum garnet (Nd:YAG) laser at 266 nm and a PMT detector [38]. Quin et al. utilized a pulsed nitrogen laser, a fluorescence emission guide, and a CCD detector for flavin metabolites detection [39]. Li et al. proposed a microfluidic chip into which the one end of an optical fiber was introduced, and the other terminal was coupled to a blue diode-pumped laser [40]. Renzi et al. demonstrated a hand-held microchip-based analytical instrument for detection of proteins [41]. Recently, a portable microfluidic flow cytometer with simultaneously detection of fluorescence and impedance was reported for cell analysis [42]. This system exploited an LED for excitation and detected fluorescent emission with a solid-state photomultiplier (SSPM).

Luminescence detection involves light emission as a result of chemical reaction, which may be from a living organism or artificial system like a luminol-labeled biosensor. Since chemiluminescence needs no light source, the system becomes even simpler than fluorescence methods while still producing sensitive responses. In many cases, detection based on luminescence is a very attractive choice for sensitive analysis because it is conducted in the complete absence of background light. QDs are a conspicuous example that show how powerful luminescence detection is. They emit strong luminescence and thus are increasingly being used for bioanalytical applications. Huang et al. reported that QDs have much higher photoluminescence quantum efficiency than their bulk materials [62]. In their study, a urea-sensing system composed of water-soluble mercaptosuccinic acid (MSA)–QDs and urease was prepared by a simple procedure and provided sensitive responses to urea. Hatakeyama et al. employed a DNA-arrayed thin film transistor (TFT) as a DNA chip platform and light detector [45]. The limit of detection (LOD) of their system was 0.5 nM, which was a much lower concentration than detected by a conventional device. A DNA chip-based protocol for clinical diagnostics was realized on a plastic card including an optoelectronic DNA chip and pyrotechnic microvalves for electrical fluid handling [63]. A plastic microfluidic immunosensor

for C-reactive protein (CRP), a cardiac and inflammation marker, is another example of a sensing system that uses luminescence detection strategy [43]. This sensor gave an even better LOD than conventional enzyme-linked immunosorbent assay (ELISA) analysis. Multiplexing is believed to be the general trend of modern analytical techniques and not an exception for immunoassay based on luminescence. A multiplexed chemiluminescent capillary enzyme immunoassay was shown to be capable of simultaneously detecting toxins, bacteria, and viruses [44]. The LOD was 0.1 ng/mL for the toxin Staphylococcal enterotoxin B (SEB), 10^4 cfu/mL for *Escherichia coli* O157: H7, and 5×10^5 pfu/mL for the bacteriophage M13. Bioluminescence (BL) is the light emission from a living organism. Liu et al. integrated CE and BL detection into a chip for ATP analysis [47]. Electrochemiluminescence (ECL) is a kind of luminescence produced during electrochemical reactions. Qiu et al. presented a $Ru(bpy)_3^{2+}$ ECL detector for CE on a microfluidic chip [48].

Internal reflection spectroscopy (IRS) is another promising optical detection for immunoassay [64]. Llobera et al. reported PDMS-based multiple internal reflection systems comprised of self-alignment systems, lenses, microfluidic channels, and mirrors. It is noteworthy that the LOD was lowered down to 41 nM using an "air mirror" [49].

Raman spectroscopy has great potential in terms of analytical applications because it gives characteristic spectra in aqueous phase. Its critical problem is weak signals, and there have been ceaseless attempts to overcome this by exploiting surface-enhanced Raman scattering (SERS) [65]. The enhancement of Raman scattering is a phenomenon that is sensitive to the substrate surface. The enhanced signals by SERS come from the Raman-active bonds of the molecules adsorbed on rough surfaces [66, 67], nanoparticles [68–70], nanoshells [71], or extremely narrow gaps ("hot spots") [72, 73] of some metals such as Ag, Au, Pt, or Cu [74]. SERS-based detection is also advantageous because it is nondestructive [75]. Recently, Lee et al. reported a SERS decoding using thin microgold shells modified with Raman tags within a microfluidic chip [76].

SPR is a representative physical phenomenon that is widely utilized for label-free characterization of molecules on thin metal films. The basic principle and operation of SPR has been described in more detail in several review articles [77, 78]. The reports on SPR-based immune sensors have steeply increased for detection of analytes with low molecular weights in recent years. SPR detection in microfluidic systems can provide various advantages. Immunoreactions are completed within a short time due to small sample volumes down to the nanolitre scale. Kim et al. developed a simple and versatile miniaturized SPR immunosensor enabling parallel analyses of multiple analytes [79]. Their SPR sensor was claimed to exhibit good stability and reusability for 40 cycles and more than 35 days. Feltis et al. demonstrated a low-cost handheld SPR-based immunosensor for the toxin Ricin [80]. Springer et al. reported a dispersion-free microfluidic system with a four-channel SPR sensor platform, which considerably improved the response time and sensitivity [81]. The sensor was able to detect short sequences of nucleic acids down to a femtomole level for 4 min. Waswa et al. demonstrated the immunological detection of *E. coli* O157:H7 in milk, apple juice, and meat juice extracted from

ground beef [82]. Wei et al. detected *Campylobacter jejuni* in poultry meat [83]. A palm-sized SPR biosensor has been developed in which the light source is modulated by a rotating mirror [84]. This system introduced a folded light path and a diode laser. The same group devised another briefcase-style sensor that is capable of simultaneously detecting small molecules, proteins, viruses, bacteria, and spores [80].

Nanostructures provide new opportunities for SPR sensing. It was shown that both sensitivity and selectivity can be improved by introducing an integrated array of nanoholes serving as substrate for SPR detection [85]. Huang et al. exploited the localized SPR properties of gold nanoparticles and successfully applied them for label-free monitoring of biomolecular interactions in real time [86]. They also discussed the unique localized surface plasmon resonance (LSPR) property of gold nanoparticles, which provides a basis for measuring the molecular adsorption onto the surface of metal nanoparticles [87]. LSPR is a phenomenon that is observed in noble metallic nanoparticles (silver, gold) and is caused by the collective oscillations of conductive electrons [88, 89]. Recently, the numerical simulation of LSPR microfluidic chips with grooved optical fibers was proposed to optimize the sensor [90]. Luo et al. fabricated a PDMS microfluidic device containing an array of gold spots onto which antigens or antibodies of interest could be attached [91]. The gold spots of this system are suitable for carrying out immunoreactions with SPR detection. Reportedly, the refractive index change caused by the molecules bound to the surfaces of the gold spots allows SPR imaging through real-time monitoring of immune reactions [92]. Pang et al. studied surface plasmon polariton dispersion related to a 2D nanohole array [93]. In this work, the sensitivity of the nanohole array sensor was attributed to the periodicity of the array and the excited surface plasmon polariton effect. Plant pathogen biosensors based on SPR were also reported [50–53].

2.2 *Electrochemical and Electronic Methods*

Electrochemical detection has been regarded as particularly appropriate strategy for microfluidic chip systems. Electrochemical biosensors in microfluidic chips enable high sensitivity, low detection limits, reusability, and long-term stability. And the detection mechanism and instrumentation for realization are simple and cost-effective. These valuable features have made electrochemical devices receive considerable attention [20, 94, 95]. The electrochemical detectors are commercially available for a variety of analyses [96]. The review written by Wang summarized the principles of electrochemical biosensors, important issues, and the state-of-the-art [97]. Lad et al. described recent developments in detecting creatinine by using electrochemical techniques [98].

In general, electroanalytical detection principles can be divided into three: potentiometry, amperometry, and conductometry (or impedometry). Potentiostats used for electrochemical biosensors are mostly equipped with amperometric and

potentiometric modules. Potentiometry is used to measure the cell potential difference at virtually zero current (open circuit voltage). The representative potentiometric sensors are ion-selective electrodes (ISEs), which include the conventional glass electrodes widely used for pH determination. Bobacka et al. gave an overview of electrochemical theory related to potentiometric principles and nonequilibrium models for ISE [99]. Feridbod et al. reviewed potentiometric sensors that employ conducting polymers [100]. In amperometry, the current associated with the reduction or oxidation at working electrode is measured under a constant potential difference versus the reference electrode. Conductometry is used to measure the electrical conductance in solutions, in which the charge carriers are cations and anions. Applying an AC electrical field between two electrodes, the conductometer or impedance analyzer acquires the output data of changes in amplitude and/or phase angle. Amplitude change provides serial resistance value in the equivalent circuit, whereas phase angle shift carries capacitive and inductive components that are mostly involved in the electrochemical interface between the electrode surface and the solution. DC input signals give pure resistance and require even simpler instruments, which are favorable for miniaturized systems like microfluidic chips. Durand et al. used ionic conductance in a nanochannel for label-free determination of protein surface interaction kinetics [101]. However, DC input in conductometry out of microchannels or capillaries is normally avoided because the applied potential difference is concentrated on the electric double layer on the electrode surface rather than the solution.

With regard to biosensors and analytical chip systems, challenging problems of electrochemical detection strategy are deterioration in selectivity and stability of biological functional objects like enzymes and chronic passivation of the underlying electrodes. Insufficient selectivity or specificity is an intrinsic limitation of electrochemical detection, which has been addressed by combining chemically or biologically specific receptors.

A variety of electrode materials have recently been tried for electrochemical analysis, including nanostructured metals, carbon nanotubes, nanoparticles, and many more. Park et al. showed that the nanoporous platinum electrode offers the unprecedented advantage of very low polarizability due to the high exchange current density (i_0) of the electrode surface [102]. This technique also demonstrated near-Nernstian behavior with ignorable hysteresis and very short response time. Henry et al. suggested pretreated gold electrodes in a microfluidic cell to detect cancer markers [103]. Wang et al. analyzed amino acids on a PDMS device coated with titanium dioxide nanoparticles by indirect amperometry [95]. An interdigitated ultramicroelectrode array (IDUA) was introduced to enable highly sensitive detection of nucleic acid, giving 1 fmol of LOD and dynamic range of 1–50 fmol [104]. Boehm et al. identified and quantified bacteria in a microfluidic chip by simple and rapid impedometry (conductometry) [105]. By coupling an impedance-based detection system with monoclonal antibodies immobilized onto the surface in a microfluidic chip, they detected bacteria in a sample within a few minutes. Swensen et al. performed continuous, direct, and real-time detection of a small molecule analyte through combination between aptamer-based sensing and microfluidic sample

handling technology [106]. A number of researchers have sought new ways to multiplex assay by using aptamer-based electrochemical biosensors that exhibit subpicomolar detection limits [107]. Javanmard et al. proposed the use of a microchannel with electrodes, on which functional receptors to target biomarkers are immobilized [108]. This attempt accomplished the detection of the anti-hCG antibody at a concentration of 1 ng/mL in a dynamic range of three orders of magnitude in less than 1 h.

Electronic or electric detection is hardly distinguished from electrochemical detection because it is linked to chemical or biological interaction in the vicinity of a conductor or semiconductor in which an electric current flows. In the sense that it extracts information from predominantly electric signals without directly involving an electrochemical reaction, it may be discussed separately from electrochemical detection. The working principle underlying an ion-sensitive field-effect transistor (ISFET) is similar to that of a metal oxide semiconductor field effect transistor (MOSFET). In ISFET, change in the ion concentration leads to the corresponding response in current between source and drain. A pH-sensitive field effect transistor (pH-FET) is a typical ISFET working sensitively and reliably. It provides a versatile basis for various chemical sensors, e.g., polymer membrane-coated ISFETs, enzyme-immobilized (ENFETs), and gas-sensing FETs coupled with gas-permeable membranes, because a lot of reactions are accompanied by stoichiometric generation or consumption of protons. The underlying electrode surface and the enzyme immobilization method are important factors that determine the sensitivity, stability, and selectivity. Masadome et al. devised a cationic surfactant ISFET using polystyrene plates and stainless wires as a template for fabricating the channel [109]. Truman et al. reported a single silicon thin film FET, which was designed to monitor transport and chemical properties of liquids in microfluidic systems [110]. A single polypyrrole nanowire-based FET sensor for real-time pH monitoring is another example [111]. In this system, polypyrrole nanowires were fabricated by electrochemical deposition inside the pores of an anodized aluminum oxide template, providing higher sensitivity and selective performance toward pH variation. Kim et al. demonstrated an extended gate FET (EGFET)-based biosensor combined with a silicon microfluidic channel for the electronic detection of streptavidin–biotin protein complexes [112]. The EGFET device was also used for the electronic detection of nucleic acids [113].

2.3 Mass-Sensitive Methods

Mass-sensitive sensors involve piezoelectric effects and surface acoustic waves. The piezoelectric effect was discovered in 1880. Piezoelectricity is the ability of some materials, mostly crystals and ceramics, to generate an electric potential in response to mechanical stress. The piezoelectric effect was mainly utilized for immunosensors and nucleic acid sensors because antigen–antibody association and DNA hybridization cause relatively large changes in mass. Mass-sensitive

sensors may exploit surface acoustic waves. There have been reported a number of sensors based on quartz resonators: electrochemical quartz crystal microbalance (EQCM), surface acoustic wave (SAW), thickness shear mode (TSM), flexural plate wave (FPW), and shear-horizontal acoustic plate mode (SH-APM). In EQCM, both sides of a quartz disk are covered with thin film electrodes. SAW-based biosensors in microfluidic platforms have been applied to immunoassays and the detection of DNA, bacteria, and small molecules [114]. QCM sensors consist of a thin quartz disk and two electrodes. When an oscillating electric field is applied across the disk, an acoustic wave is induced at certain resonance frequency. Lee et al. showed that antibodies immobilized on the self-assembled monolayer of thiosalicylic acid on QCM allowed detection of *Bacillus cereus* and *Listeria monocytogenes* at 104 cells/mL and 450 spores, respectively [115]. Cooper et al. prepared QCM immunosensors that are potentially suited for detection of pathogens [116]. Actually, mass-sensitive sensors commonly suffer from difficulty in discriminating between mass changes arising from the binding of authentic target molecules and from the nonspecific adsorption of contaminants. A TSM sensor is a piezoelectric-based sensor in which perturbation in mechanical shear strain induces change in AC voltage and vice versa. Ergezen et al. monitored adhesion and aggregation of platelets by a TSM sensor in real time basis [117]. FPW sensors attracted interest due to their high sensitivity, high accuracy, and low operating frequency in clinical, environmental, and biological fields. A representative example is the allergy sensor based on a very high mass-sensitivity FPW detector [118]. SH-APM sensors involve leaky waves, where the wave is only partially confined to the surface. Rocha–Gaso reviewed the SH-APM sensor and other biosensors that exploit surface-generated acoustic waves for the detection of pathogens [119].

An important tool for mass detection is atomic force microscopy (AFM), which utilizes cantilevers for surface characterization. Adsorption onto the sensing electrode, composed of two chemically different materials, produces a differential stress between the surfaces, resulting in bending. Molecules adsorbed on a cantilever also bring about vibration frequency changes. Microfabricated cantilever functionalized with antibodies has been sought as a new detection device for biosensors. The sensors based on this physical phenomenon are summarized in Table 2. Philip et al. reviewed the technologies and recent developments in micro-/nanoscale cantilevers, which have been applied to the detection of relatively small analytes [128]. Recently, nanomechanical motion induced by protein–protein interactions on a microfabricated cantilever was reported to allow detection of prostate-specific antigen (PSA) [120, 121]. Cherian et al. investigated the adsorption characteristics of calcium ion on gold and silicon surfaces of a cantilever through variation in resonance frequency and bending [122]. Watari et al. confirmed the capability of multiple cantilever arrays to monitor bending forces induced by the ionization reactions in aqueous samples [123]. A cantilever was also used to measure the surface stress on micromechanical structures produced by DNA hybridization or biomolecular reaction [124]. Arntz et al. showed continuous label-free detection of two cardiac markers, creatine kinase and myoglobin, using an array of microfabricated cantilevers on which anticreatine kinases or antimyoglobin

Table 2 Mass-sensitive methods based on microcantilvers

Analyte	Receptors or sensing bodies	LOD	Typical stress (mN/m)	References
Prostate-specific antigen	SiN_x with Au coating	0.2 ng/mL	–	[120]
Prostate-specific antigen	Ta/Pt/PZT/Pt/Si O_2	10 pg/mL	–	[121]
Ca^{2+} ions	Bare silicon/Au cantilever	1 mM	1–450	[122]
pH	Thiol-modified cantilever	pH 4.5–9	1–30	[123]
DNA	Oligonucleotide	100 pM	1–30	[124]
Protein	Antibody	20 μg/mL	1–6	[125]
Protein	Oligonucleotide aptamers	100 pg/mL	1–10	[126]
Peptide	Antibody fragments	20 ng/mL	1–10	[127]

antibodies were immobilized [125]. DNA aptamer and single-chain antibody were also employed as receptor molecules [126, 127].

2.4 Others

Temperature is a useful variable that reflects analytical information by associating with biological functional elements. An enzyme thermistor utilizing a commercialized thermistor is a kind of resistor that detects resistance changes as a function of the ambient temperature. In this system, the molar enthalpies of enzyme-catalyzed reactions are sensed by the thermal detector.

3 Analytes

3.1 Small Molecules

Small molecules in this review are defined as organic compounds with low molecular weights. Small molecules can have a variety of biological functions. For instance, cell signaling, which may be triggered by small molecules, has a great importance for drug discovery, environmental analysis, mass production processes, and so on. Another example is bacteria modalities, which are becoming increasingly crucial. Boehm et al. demonstrated an on-chip microfluidic biosensor for impedometric bacterial detection and identification in the presence of monoclonal antibodies immobilized in the microfluidic system [105].

Label-free detection based on silicon nanowire FET devices was used to identify small molecule inhibitors [129]. High-throughput screening of small molecules is undoubtedly one of the hot issues in pharmaceutical drug discovery. Chan et al. quantified small molecule aggregation in a high throughput fashion without using

any label, but a photonic crystal [130]. Wang et al. exploited optical extinction for probing interactions between nucleic acids and small molecules [131].

3.2 Proteins

Proteomics on a large scale have emerged as one of the major themes in modern biomedical research. Recently, proteomics based on the concept of protein analysis on biochip was reviewed [132]. Jonkheijm et al. summarized and discussed chemical, biological, and nanotechnological strategies that involve protein biochips as a new kind of tool [133]. Concerning detection of proteins and polypeptides, we should note the meaning and potential of multiplex analysis. Diercks et al. proposed a multiplexed protein detection strategy, which was expected to eliminate the difficulties in directly functionalizing PDMS of microfluidic devices by using prefunctionalized microparticles [134]. Osterfeld et al. accomplished multiplex protein detection of potential cancer markers at subpicomolar concentration levels with a dynamic range over four decades by introducing magnetic nanotags [135]. Fluorescence resonance energy transfer (FRET) was proved to be a powerful method for detection of protein–protein interactions, enzyme activities, and the presence of small molecules in the intercellular milieu [136]. Li et al. discussed the theoretical basis of FRET focusing on the key parameters responsible for the sensitivity of FRET biosensors [137]. In addition, Gales et al. used a bioluminescence resonance energy transfer (BRET) to directly monitor interactions between receptor and G protein in living mammalian cells [138]. A protein biosensor array based on SPR differential phase imaging was reported that worked by detecting a time-modulated differential phase [139].

Label-free protein biosensors witnessed great advance in recent years. An impedometric label-free detection was proposed to conduct immunoassay with high sensitivity and specificity [140]. Aptamers that were immobilized on solid substrates received appreciable attention because they have high permanent charge density, which is possibly exploited for label-free detection such electrochemical impedance spectroscopy (EIS) [141]. There are many cases of aptamers-based sensors, which are separately described in Sect. 4.4.

Siwy et al. demonstrated the utility of a single conically shaped gold nanotube that was embedded in a mechanically and chemically robust polymeric membrane [142]. They reported biofunctionalized conical Au nanotubes, which are potentially useful for obtaining highly sensitive and selective protein biosensors. So et al. introduced a single walled carbon nanotube field effect transistor (SWNT-FET) combined with aptamers as an alternative to the corresponding antibody [143].

Overall, current microfluidics is mature enough to provide the technology for protein analysis, including pretreatment and separation. Rather, it seems that biosensors with biological functional elements need significant progress to make contribution to practical protein research and practical applications.

3.3 Electrolytes and Gases

Most electrolyte sensors are ISEs that involve functional membranes containing appropriate ionopores and measure the concentration of ions in chemical, biological, or clinical samples. Especially for clinical uses, rapid and cost-effective analysis of sodium, potassium, chloride, calcium, and magnesium ions in physiological fluids including human blood are essential. ISEs are strongly advantageous in these aspects and thus widely used for quantitative analysis of electrolytes in chemical and clinical laboratories. Faridbod et al. reviewed applications of conducting polymers for potentiometric sensing [100]. For further improvement, much effort has been made for miniaturization. Fueled by the development of MEMS technology, many fabrication methods were introduced to address analytical concerns like LOD, biocompatibility, and sensor stability [144]. Wang et al. reviewed electrochemical biosensors for point-of-care testing of electrolytes and gases, and for immunoassays [95, 145].

4 Functional Receptors Integrated in Microfluidic Systems

4.1 Enzyme Electrodes

Enzymes integrated in a biosensor system catalyze the conversion of metabolite molecules to consume or produce detectable species. The change in concentration of the species resulting from enzyme reaction is detected by a corresponding signal transducer. Thus, the analytical performance of these biosensors should critically depend on the activity and stability of the immobilized enzymes. In many cases, the enzyme immobilization is the most important step that determines whether or not it is successful to develop reliable biosensors. In this respect, it is no wonder that a number of new immobilization schemes and materials have been proposed to improve the analytical capabilities of biosensors.

The targets of enzyme-based biosensors that have been reported are mostly small molecules that are involved in human metabolism or environmental cycles, e.g., glucose, urea, lactate, and many others [95, 97, 98, 146, 147]. The most popular strategy is the amperometric glucose sensor, in which glucose-specific enzymes like glucose oxidase are immobilized onto electrodes [148]. Besides electrochemical detection, optical signal transformation is also widely used with the enzyme-based biosensors. Whatever the signal conversion technique applied for enzymatic biosensors, the most valuable benefit obtained by using enzymes is excellent selectivity. The biological specificity of enzymes provides artificial systems with invaluable opportunities.

However, most enzymes may lose their activity when they are immobilized on substrates like electrodes or optical fibers. This is the core issue of enzymatic biosensors. Continuous glucose monitoring in the subcutaneous layer for diabetes

mellitus is the example that shows how critical this issue is. Once a glucose sensor is implanted, it is not easy to calibrate its sensitivity. As the clotting and immune reactions proceed under the skin, the environment to which the implanted sensor is exposed keeps changing and possibly affects enzymatic activity in unexpected manner. Since the real-time information from such a continuous glucose sensor is more crucial to the patient than that of a disposable strip, variance in enzyme activity is of great concern. As a result, a variety of immobilization methods, including adsorption, capsulation, entrapment, crosslinking, and covalent bonding, have been developed to formulate an enzyme layer with minimal damage in enzyme activity and long term stability.

In addition to the enzyme activity, charge transfer rate in electrochemical types is another key issue in enzymatic biosensors. Charge transfer rate from the active site to the electrode is closely related to the overpotential that is applied. Lower overpotential reduces the probability of interference by redox active species. Facilitated charge transfer may also help maintaining the condition of diffusion limiting even when significant loss of enzyme activity deteriorates sensitivity.

The bananatrode reported by Sidwell et al. is interesting in that a banana slice was combined with gas-permeable dialysis membrane and acted as a membrane to detect dopamine in a biological sample [149]. Polymers still provide a lot of opportunities for better protocols of enzyme immobilization. Rauf et al. exploited cellulose acetate and poly(methyl methacrylate) (PMMA) for reducing enzyme leakage and enhancing the stability by 94% during 1 month of shelf life [150]. An array of hydrogel-entrapped enzymes in microfluidic channels was employed for simultaneous quantitative analysis of biological samples [151]. The hydrogel microarray was allegedly easy to fabricate in the microfluidic channels, which eliminated the potential cross-talk among the trapped enzymes. As for lactate biosensors, most of them are based on lactate dehydrogenase (LDH) or lactate oxidase [152, 153]. Lactate in serum was analyzed without pretreatment using screen printed carbon on which an LDH layer was coated [154]. Romero et al. reported an amperometric lactate sensor with a protective Nafion membrane, linearly responding to lactate concentrations between 2 and 1,000 μM [153]. Elevated levels of urea indicate possible problems in kidney and liver function. Nikoleli et al. used a urea sensor based on an air-stable lipid film that contained urease and was stabilized on a glass filter by photopolymerization [155]. Kuralay et al. immobilized urease in the poly(vinylferrocenium) (PVF^{+}) matrix [147] and Gutierrez et al. showed a potentiometric bioelectric tongue for the analysis of urea that employed PVC and carboxylate PVC [156]. In this work, the calibration process based on an artificial neural network and partial least squares was introduced to determine the concentration of urea without compensating endogenous ammonia or chemically rejecting the alkaline interferences.

Nanomaterials are a new class of research material that have been tested for enzyme immobilization. Sawicka et al. measured urea concentration by using nanocomposite fibers of urease and polyvinylpyrrolidone (PVP) [146]. Biocomposite nanofibers were prepared by electrospinning a solution in which urease and PVP were dissolved, leading to improvement in response time and sensitivity.

Barhoumi et al. discussed a ZnAl-based enzyme nanohybrid system for a urea biosensor, in which ureases were entrapped within layered double hydrides [157]. Tsai et al. employed a sol–gel method to fabricate an enzymatic optical biosensor array for the analysis of multiple samples [152].

4.2 Antibodies

Immunoglobulins (Igs) are gamma-globulin proteins that are present in blood and play important roles in identifying and neutralizing foreign objects such as bacteria and viruses in the immune system. An antibody is a kind of Ig composed of heavy (above 150 kDa) globular plasma proteins such as IgA, IgD, IgE, IgG, and IgM. An antigen is a molecule or pathogen that is capable of eliciting an immune response. Immunoassays make use of the sensitivity and specificity of the antibody–antigen interaction. There are direct and indirect methods that have been adopted for immunosensors. Direct methods acquire electrochemical, optical, or electrical signals resulting from the event of immunochemical complex formation without any labeling or secondary reactions, as shown Fig. 2 [158, 159].

Indirect methods utilize labeling. Typical labels for immunoassays are enzymes, fluorescent or radioactive molecules, nanoparticles, chemiluminescent probes, metal tags, and so on [158, 159]. By labeling, the immune reaction can be detected more sensitively through amplifying the corresponding electrochemical, optical, or other physical responses. That is why most conventional immunoassay protocols adopt labeling methods. Sandwich-type immunoassays are the most widely used

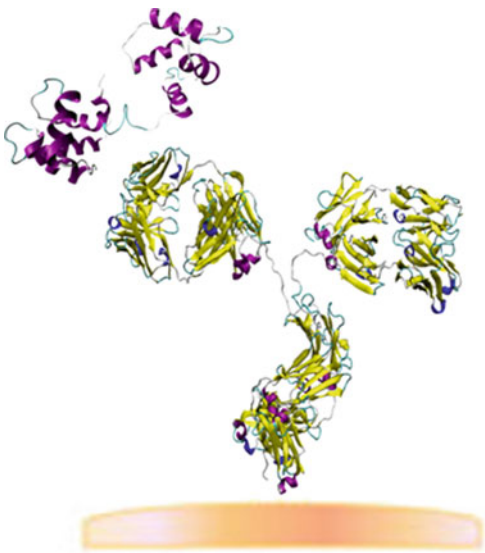

Fig. 2 Schematic of direct immunoassay

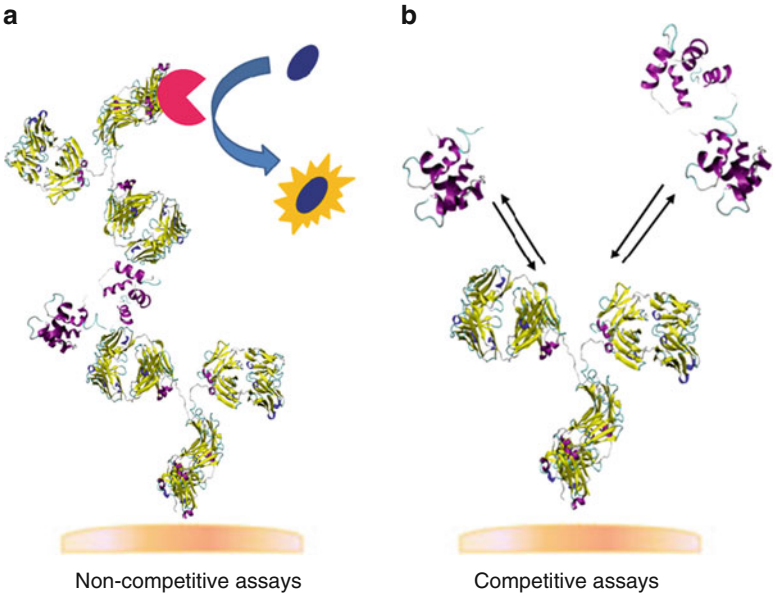

Fig. 3 Schematic of indirect immunoassay

method, which utilize secondary antibody with label. The general scheme of sandwich-type immunoassays is illustrated in Fig. 3a. A representative example is the ELISA, also known as an enzyme immunoassay (EIA), which is a fundamental technique in bioanalysis.

However, we should take into account the fact that labeling processes may increase cost and elongate the time of analysis because a few more steps in the analytical procedure are required. More importantly, the sandwich-type immune assay is not a good choice for small molecule analysis. If a target analyte is too small to be bound with another primary antibody, it is difficult to expect additional association events. In such cases, competitive immunoassays are often considered [160]. Unlabeled antigens in the sample are allowed to compete with labeled ones that used to be bound to antibody, as illustrated in Fig. 3b. The antigens in the sample replace the labeled antigens, which are released to the solution and measured. Higher concentration of unlabeled antigens (analytes) result in smaller amount of the labeled antigens that remain bound to the antibodies.

To avoid labeling hassle, label-free methods have been suggested. Some examples of label-free approaches are back-scattering interferometry [161], impedometric immunosensor for the stroke marker detection [162], and impedometry coupled with magnetic nanoparticle immunoseparation for direct detection of pathogenic bacterial cells in food samples [163]. Immunoassays in microfluidic devices employing SPR have also been reported. Luo et al. developed a PDMS microfluidic device with an array of gold spots onto which antigens or antibodies of interest were attached [91]. Recently, a promising strategy is to immobilize

antibodies on mobile substrates instead of planar substrates. Such mobile platforms could be polymer-based microspheres such as latex beads or nanoparticles, which provide fundamental basis for multiplex immunoassay. Highly carboxylated polystyrene/polyacrylic acid latex microspheres coated with QDs is a good example [164]. The LOD was successfully enhanced as low as 50 ng/mL on a microfluidic chip. The immobilization of biological receptors like antibodies onto the solid supports is one of the core issues because it is closely related to how to maximize the efficiency of the recognizing ability with minimal damage to their activities. This stimulated intensive studies on the orientation-controlled immobilization of intact proteins [133, 165, 166].

4.3 Molecular Receptors

Valdes et al. suggested the utility of various ligands as molecular receptors for biosensors [167]. Molecular receptors have been used as substitutes for antibodies for specific binding and detection of pathogens or toxins, including carbohydrate derivatives, proteins, bacteriophages, and lysozymes. Koo et al. showed an efficient capture of target analytes for a specific detection of pathogenic microorganisms [168]. Jen et al. harnessed fiber-optic localized plasma resonance (FO-LPR) and the characteristic optical properties of self-assembled gold nanoparticles on the surface modified with a receptor to increase the sensitivity and shorten the response time on microfluidic chips [169].

4.4 Aptamers

Aptamers are artificial nucleic acids that specifically bind to target molecules. The aptamers with optimal sequences are obtained through a combinational selection process such as systematic evolution of ligands by exponential enrichment (SELEX) from random RNA or DNA libraries [170, 171]. Recently, aptamers biosensors based on fluorescence, electrochemistry, QCM, and infrared spectroscopy measurements have been actively studied [106, 107, 172–179]. Swensen et al. improved LOD and response time [106], and Xiao et al. constructed an electronic aptamer-based sensor by covalently attaching a methylene blue (MB)-labeled thrombin-binding DNA aptamer to a gold electrode. [176]. Gokulrangan et al. addressed a rapid aptamer-based bioanalysis for the sensitive detection of IgE using fluorescence polarization [172]. Hansen et al. presented a multianalyte electrochemical aptamer biosensors that exhibited subpicomolar (attomole) level LOD employing QD semiconductor nanocrystals [107]. This is an impressive achievement in regard to highly sensitive and selective simultaneous bioelectronic detection of several protein targets. Bang et al. used beacon-type aptamers based on intercalation of MB as an electrochemical marker and achieved an LOD of 11 nM

thrombine [173]. Liao et al. took advantage of Fourier transform infrared attenuated total reflection (FTIR-ATR) and surface modification techniques [175]. Aptamer-modified carbon nanotube FETs were utilized for the label-free detection of IgE [178]. Centi et al. simplified the target capturing step by using aptamer-functionalized magnetic beads for sandwich electrochemical assay of thrombin [179]. Non-Faradic impedance spectroscopy was shown to possibly monitor the capacitance change induced by aptamer–protein association, with the aim of in vivo cytokine sensor development for chronic neural prostheses [180]. Wang et al. reviewed nanoparticle-based electrochemical bioassays by using aptamer-based immunoassays and nanoparticles [145]. Aptamer applications for the detection of a variety of samples such as thrombin, cytokines, and protein were also discussed [181, 182].

4.5 Miscellaneous

Beside enzyme, antibody, molecular ligands, and aptamers, significant research achievements have been made with the molecular imprinting method. The strategy of molecular imprinting is that the sites remember the local environment to selectively bind the analytes, even after the previously bound analytes are released [183, 184]. In recent years, the molecular imprinting method is widely studied because of its cost-effectiveness and high specificity. Alexander et al. reviewed the development of molecular imprinting science and technology [185]. Janiak et al. summarized molecularly imprinted polymers that had been synthesized by crosslinking of functional monomers in the presence of a template molecule. This review dealt with the molecular imprinting of peptides and proteins in aqueous media [186]. Eroz et al. exploited QCM with molecular imprinted layers to detect a variety of analytes, including glucose [187]. Xie et al. presented the molecular imprinting on walls of silica nanotubes for 2,4,6-trinitrotoluene (TNT) [188]. Hansen reviewed the molecular imprinting of proteins [189].

5 Microfluidic Components

5.1 Pretreatment

With regard to microfluidic-based integrated biochip technology, a number of laboratories have pursued devices that are capable of handling and analyzing real biological samples. Human whole blood is a typical example that has a variety of ingredients including blood cells, platelets, proteins, and many molecular species such as glucose, mineral ions, hormones, carbon dioxide, etc. For this reason, sample pretreatment is indispensable in many cases and thereby has been a critical issue in

clinical, biological, and biochemical analysis on microfluidic chips. Traditional protocols of separating blood cells from whole blood involve labor-, cost-, and time-consuming processes that normally involve centrifuging steps. Therefore, separation technology for sample preparation is an urgent demand, to which rapid and efficient microfluidic technology could make the biggest contribution. Various technologies have been suggested as alternatives to centrifugal separation. For instance, plasma separation from whole blood was conducted by many kinds of membrane filters, which include a planar microfilter, bent channel structure, T-shaped channel, and micropost array [190]. Jaggi et al. observed the depletion of red blood cells (RBCs) from whole blood at high volume flow rates [191]. In this study, a high-aspect ratio channel bifurcation was used to separate RBCs. Moreover, particle density in a suspension was able to be tuned and the samples were filtered through microstructures such as micropillar arrays and membranes with circular, hexagonal, and rectangular holes. Chun et al. realized rapid and selective RBC lysis by quickly mixing the whole blood sample with a lysing reagent between a pair of salt bridges that were integrated on a microfluidic chip, and applying an AC electric field at low frequency [192].

5.2 Surface Modifications

Surface modification is crucial for improving the sensitivity of biosensors, which should minimize nonspecific binding. There have been a number of ways to modify the surfaces, e.g., wet chemical, organosilanization, ionized gas treatments, and UV irradiation [193]. Surface modification methods that have been published to date can be categorized into chemical and physical methods. Chemical modification immobilizes functional molecules to create surface properties desired so that the surfaces become passivated or activated with chemicals. On the other hand, physical modification may change the surface roughness, grain size, and grain boundary by exposure to lasers, plasmas, heat, and polishing. To achieve better sensitivity and reliable performance, a sufficient amount of analytes and functional molecules need to be immobilized onto the surface of interest in the microfluidic biosensors. A good example is the surface modification of protein-modified microcantilevers that were prepared from multiple surface conjugations [194]. Diao et al. tried a direct silanization with aminopropyltriethoxysilane and activation with glutaraldehyde [195]. Biofouling of the surfaces has been one of the major problems in biological analysis. Several variables like wettability, biocompatibility, and nonspecific adsorption were addressed to tackle the biofouling problem. Poly(ethylene glycol) (PEG) is well known for its protein-resistant property [196]. Bi et al. reported a simple method for the deposition of PEG brush on PMMA microfluidic channel surfaces [197]. The fabrication of PEG-coated microfluidic chips would provide a biocompatible surface for complex biological analysis. Prakash et al. showed covalent attachment of various polymers fused with silica or silica-coated silicon in glass [198]. The PDMS layer

was adhered to the surface of another PDMS by gaseous plasma [199]. Lee et al. generated a protein micropattern on the PEG hydrogels by surface graft polymerization and photolithography [200]. It was reported that PEG layers were able to reduce nonspecific interactions [201].

5.3 Beads

Rapid and reliable quantitative analysis of cells is increasingly important. Although utility of beads are not limited to biological applications, cellular measurements such as cell counting, antibody-mediated agglutination, and diagnostics using cultured cells attract exceptional interest as they could benefit immensely from beads. Bead-based analysis for cell research and development can be discussed from two points of views: molecular/immune assay and flow cytometry. Since the multiplex bead array assay (MBAA) was introduced by Horan et al. in 1977 [202], polymeric microparticles have been considered and tried as solid supports for molecular/immunoassay. Such beads can reduce the volumes of reagents as well as samples by providing a high surface-to-volume ratio that is significantly enlarged in comparison to conventional microarray. Saunders et al. reported a competitive binding assay using beads on which antibodies were anchored and associated with nonlabeled antigens prior to exposure [203]. Holmes et al. presented a microfluidic rapid analysis of polymer beads labeled with fluorescent dye in small volumes [204]. This research showed the possibility that bead-based immunoassay could induce a substantial advance in sensitivity, precision, and accuracy by employing microfluidic chips. Gao et al. enlarged the reaction chambers for CD4-positive T lymphocyte capture to raise cell capture efficiency compared with conventional methods based on CD4 separation [205]. More importantly, it was demonstrated that bead-based immunoassay is a promising alternative to ELISA based on plates, and offers several valuable advantages [206]. Concerning the MBAA, there are two key issues. One is functionalization of the bead surfaces as desired and the other is recognition of individual beads that are suspended in the solution. The former issue has been intensively addressed by research and development in industry as well as academia. Bead-based multiplexing systems are commercially available from Luminex, Illumina, and BioArray Solutions, as summarized in Table 3. Beads are dispersed in the solution to have sufficient chances to interact with potential analytes and then need to be collected before rinsing or other subsequent processes. To successfully enable active dispersion and recollection, magnetic nanoparticles were impregnated in polymeric microbeads for immunoassay [207].

The bead surface critically affects nonspecific binding, which is particularly serious in the presence of physiological fluids, e.g., human whole blood. Conventional polymeric surfaces need chemical coupling processes to immobilize functional molecules and are vulnerable to contamination originating from nonspecific adsorption of proteins that are not analytes, like albumins in blood. Polymers including ethylene glycol oligomers have been tried in an effort to

Table 3 Bead-based multiplex technologies and companies

Company	Model	Analysis targets	Related methods
Luminex	xMAP	Cardiac markers	Gene expression profiling
		Cancer markers	Nuclear receptors
		Metabolic markers	HLA testing
		Neurobiology	Infectious disease
		Cytokines	–
		Chemokines	Cystic fibrosis
		Growth factor	–
Illumina	Sentrix	SNP genotyping	Gene expression
BioArray Solutions	BeadChip	Human erythrocyte antigen genotyping	Ashkenazi genetic disease genotyping

HLA Human leukocyte antigen, *SNP* Single nucleotide polymorphism

reduce nonspecific adsorption [201, 208], but it is still challenging. A thin gold shell on a polymeric microsphere was proposed as a new platform to tackle a couple of issues that were involved in bead surfaces [76, 209]. This approach is still at an early stage, but seems to deserve watching to see what will happen in the future.

As for identification of the suspended beads, it is necessary to achieve not only cost-effective and reliable encoding technology but also high throughput and a facile decoding strategy. Unlike microarrays, the positions of sensing sites are not fixed in the suspension array system. So, beads dispersed in solution should be recognized individually to reveal what kind of receptors is immobilized on their surfaces. Microfluidic devices are expected to provide important opportunities of higher throughput and better reliability to encoding as well as decoding technologies, eventually making multiplex analysis possible. The encoding–decoding methods that have been reported up to now are based on fluorescence, size, or shape [210–212]. The most popular way to encode microbeads is to incorporate fluorescent dyes and QDs into the beads [213, 214]. To read out the barcodes of the beads, flow cytometry is widely used due to its multifunctional capabilities of counting, characterizing, and sorting. It can count and characterize a variety of small particles, including cells, beads, and microparticles. The fluorescence-activated cell sorter (FACS) is a sophisticated readout device that allows fast decoding and sorting [215, 216]. However, the FACS system is normally a large and expensive instrument so that it deters rapid expansion of applicability to multiplex suspension arrays at low cost. Recently, research effort has been directed to microfluidic chip-based flow cytometers, which require small sample volume, less reagent consumption, and precise control of particles [217]. A miniaturized flow cytometer was proposed that used a unique polyelectrolytic salt bridge for accurate discriminative detection of cells as a function of their size and fluorescence [42, 218]. Fan et al. reported a magnetic-bead-based chemiluminescent immunoassay in microfluidic chips [219]. Antibody-conjugated magnetic beads were shown to be useful for detecting viruses, with the assistance of sample pretreatment devices for purification and preconcentration [220].

5.4 Droplets

Droplet-based microfluidics is expected to solve the problems of mixing and reagent diffusion in laminar flow-based microfluidics. Micro- and nanoscale emulsions of immiscible liquid droplets were applied in various applications such as DNA encapsulation [221] and drug delivery systems [222]. Microfluidic droplets have a few parameters of droplet size, frequency, composition, and so on. Properties of droplets are very sensitive to their physical size and its distribution. The conventional way to fabricate the droplets is to use macroscale instruments such as high-pressure homogenizers, high-power generators, and ultrasonic generators. However, it is very difficult to control the size of droplets using traditional methods. For these reasons, microfluidic techniques have been sought for producing droplets. Liu et al. chose dichloroethane as the continuous organic phase to create droplets inside a hydrodynamic focusing chip [223]. By this method, uniform droplets were made and their size was under control by varying the flow rates of the organic and water phases.

Rapid and reliable characterization of droplets in microfluidic environments has been widely addressed. Electrochemical methods can offer a simpler route to that goal than traditional optical imaging. Monpichar et al. reported a droplet-based microfluidic platform to study streptavidin–biotin binding kinetics with millisecond time resolution [224]. In this study, a droplet microfluidic platform was integrated with a confocal fluorescence detection system. Several microfluidic chips were reported to make droplets with better emulsion quality [225–227]. Recently, separation and collection of emulsion microdroplets have been explored. For example, a passive and active satellite droplet filtration system was demonstrated [227]. There were a few reports on a microfluidic chip that was capable of generating uniform and tunable sizes of emulsion droplets by using microchoppers [228].

5.5 Pumping and Valves

Microfluidic analytical systems require active or passive micropumps to control the reagent and sample delivery. A large number of micropumps have been devised since the microfluidic system was first introduced. Several mechanisms have been suggested for transporting the fluids in microfluidic systems, and can be discussed in two categories: displacement and dynamic pumping. Displacement pumps exert pressure forces on the fluid through one or more moving boundaries. Micropumps are based on periodic displacement involve reciprocating or rotary actuations [229–232], while periodic type micropumps have piezoelectric [233–238], peristaltic [239, 240], thermopneumatic [241, 242], electrostatic [243, 244], pneumatic [245], and electromagnetic moving units [246]. A number of reports have been published on membrane- or diaphragm-based displacement micropumps. Peristaltic pumps are reciprocating displacement micropumps that have check or active

valves [247]. Cho et al. reported a fully integrated pumping system using centrifugal microfluidics on a polymer-based CD [248].

Dynamic pumps infuse energy to the fluid in a manner that increases either its momentum (centrifugal pumps) or its pressure (electroosmotic and electrohydrodynamic pumps) as shown in Table 4. They involve centrifugal or hydrodynamic actuations and, more specifically, are driven by electro-/magneto-hydrodynamic [257], electroosmotic [254–256], electrokinetic [251–253], electrowetting [258], and acoustic forces [259]. Centrifugal pumps are typically less effective for fluids with low Reynolds numbers and have limitation in miniaturization.

Sometimes electromagnetic field and acoustic-wave are regarded as suitable means for creating pressure to make flow. The field-free micropump driven by electroosmotic flow is an interesting example of swift electromagnetic manipulation of field-sensitive biological objects [260]. The design of the proposed system was a "Y"-shaped channel network with two arms, which have positively and negatively charged surfaces on the inner walls by depositing polyelectrolytes on the respective surfaces. The fluid on this chip is under quick and precise control by a programmed external electric field, while the designated region, in which biological elements are being handled, is free from electromagnetic field. On other hand, microelectromechanical systems technology was introduced to a micropump that needs no external power source [261]. This work showed a new actuation mechanism for the self-generated peristaltic motion of cascaded actuators that employ the fluidic circuit of an elastic tube. A self-priming low-power micropump based on a polypyrrole actuator can be fabricated without microfabrication equipment [262].

Microvalves are sometimes regarded as a part of micropumps in many reviews [263]. In recent years, new methods for the valves have been reported, as summarized in Table 5. A magnetic hydrogel nanocomposite valve was utilized for remote

Table 4 Classification of micropumps

Micropump type	Operating principle	Key factors	References
Mechanical	Diaphragm	Piezoelectric	[233–238]
		Thermopneumatic	[241, 242]
		Electrostatic	[243, 244]
		Pneumatic	[245]
		Peristaltic	[239, 240]
Displacement	Rotary	Rotating gear	[229, 230]
		Viscous force	[231, 232]
	Fluid	Ferrofluid	[249]
		Phase change	[250]
	Electromagnetic	Magnet	[246]
Dynamic	Centrifugal	Momentum	[248]
	Electrokinetic	Induction	[251, 252]
		Injection	[253]
	Electroosmotic	DC	[254, 255]
		AC	[256]
	Electro-/magneto-hydrodynamic	Lorentz force	[257]
	Electrowetting	Electric	[258]
	Acoustic	Waves	[259]

Table 5 Classification of microvalves

Microvalve type	Operating principle	Operating principle	Key factors	References
Active	Mechanical	Magnetic	External magnetic fields	[264]
			Integrated magnetic inductors	[265]
		Electric	Electrostatic	[266]
			Electrokinetic	[246]
		Piezoelectric	Frequency	[267]
		Thermal	Bimetallic, thermopneumatic	[268]
	Nonmechanical	Electrochemical	–	[269]
		Phase change	Hydrogel, sol–gel, paraffin	[270–272]
		Rheological	Electro-rheological, ferrofluids	[273]
	External	Modular	Built-in, rotary	[274]
		Pneumatic	Membrane, in-line	[275, 276]
Passive	Mechanical	Check valve	Flap	[277]
			Membrane	[278]
			Ball	[249]
			In-line mobile structure	[279]
	Nonmechanical	Capillary	Hydrophobic valve, abrupt	[280]

control of the flow in a microfluidic device by applying alternating magnetic fields [281]. This approach chose temperature control to regulate the collapse of a nanocomposite, leading to opening of the valves. Yoo et al. suggested a paraffin-actuated microvalve in which phase transition of the paraffin from solid to liquid is triggered by a thermal cue and results in volume change [282]. This system is practical for controlling the mass transport of reagents and samples in a microfluidic chip precisely and efficiently. Many valving principles have been introduced to steer the flow by a centrifugation protocol. Honda et al. reported a rotary disk driven by centrifugal force for highly integrated immunoassay [283]. They demonstrated that the CD based on centrifugal force successfully handled small volumes of samples and reagents with sufficiently good precision. With respect to flow control in more complicated microfluidic networks, Hisamoto et al. harnessed the surface wettability of the inner walls of microchannels, which were coated with a thermo-responsive polymer [284].

5.6 Peripherals

The micromixer is an essential microfluidic component for specific applications. Compared with conventional macroscale mixers, micromixers need a somewhat different approach to working principle, specification, and design. First of all, it is hard to make turbulence in such a limited volume [285, 286]. And, more than two solutions in a microfluidic system are necessarily mixed as rapidly as possible for

many applications, e.g., studies on the kinetics of enzyme reactions, fast liquid reactions, discriminative cell lysis with minimal side effects, etc.

There are two types of micromixers: passive and active micromixers. Passive micromixers operate without an external driving force so that the mixing process depends on diffusion and chaotic advection. In order to increase the contact interface between the liquids, various attempts have been made, including multilayer lamination [287], fluid injection [288], and 2D or 3D structures [289]. Most passive mixers rely on the chaotic advection principle to enhance mass transport by incorporating complex structure [290, 291], grooves in the channel well [292, 293], or zig-zag-shaped channels [294]. Hardt et al. discussed passive micromixers for applications in microfluidics [295]. Active micromixers involve the disturbance caused by external forces for the mixing process. External forces that have been suggested for active micromixers are pressure [296], electrodynamic [297], dielectrophoretic [298], electrokinetic [299], and magneto hydrodisturbance [300]. Chun et al. used polyelectrolytic salt bridges to obtain an efficient active micromixer operating by low voltage and straight/smooth surfaces [192]. In this system, a pair of positively charged polyelectrolytic gel electrodes, which face to each other, play the key role of inducing ion depletion/enrichment phenomenon. Chang et al. reviewed the recent progress in micromixing phenomenon based on electrowetting-on-dielectric, dielectrophoresis, and electroosmosis [301]. Hessel et al. reviewed the mixing principles of passive and active micromixers [302].

6 Perspectives

Biosensors and microfluidic analysis systems possess an intrinsic feature that is common to both. This is the fact that chemical and bioanalyses involving biosensors and microfluidic systems could be conducted by conventional methods in the laboratory as well, or even better. The reason why these systems are needed is not to enable chemical or bioanalysis of target analytes that conventional methods have been unable to analyze. They were developed to make the analytical system better for specific purposes through size reduction, and thereby widen their range of use. It is no wonder that tremendous research activities in this area have been concentrated on finding solutions to the problems that come from miniaturization or integration on a chip, and not on seeking new analytical principles or methodologies.

In this regard, the future direction of biosensors and microfluidic analytical systems is predicted to be towards better efficiency, convenience, cost-effectiveness, compatibility, and reliability. Label-free technology is a particularly good example that deserves special attention. It would provide valuable advantages in efficiency, convenience, and cost-effectiveness by simplifying the procedure, requiring less reagents, and cutting the cost and time of analysis. However, label-free detection still has its own challenges, and subsequent research efforts must be made to accomplish the ultimate goal, as argued by Fan et al. [303].

Another example is multiplex suspension analysis. As more and more knowledge about nature becomes available, the demand for chemical and bioanalyses increases correspondingly. The revolutionary advance in biotechnology is accelerating the growth of analytical needs. New analytes that need to be analyzed are ceaselessly appearing and some of them may be extremely important in terms of clinical, economical, industrial, or environmental concerns. Moreover, analytes with a very low population, such as circulating tumor cells, need at least a few milliliters of sample, otherwise analysis is impossible. According to the conventional concept of chemical and bioanalysis, an analyte requires a sample for its own analysis. This leads to research efforts to improve analytical methods so that they consume as small a sample as possible. However, minimizing the sample volume cannot ultimately solve this fundamental problem of conventional methodology. This situation unequivocally indicates the request of multiplex analysis techniques. 2D microarrays or biosensor arrays integrated on a chip or a strip are examples of ideas on the ways to cope with these new analytical demands. Noting that the entire surface of the 2D arrays should be covered by the sample solution, we should consider the microsuspension array as a promising alternative to conventional arrays on flat substrates. Microbeads are expected to offer a useful platform for multiplex suspension array. A large number of microbeads would provide sufficient amount of data to ensure reliability. The current trend in modern bioanalysis strongly indicates that efficient barcoding technology and a handy readout device for rapid decoding/detecting the beads will make a breakthrough towards ubiquitous chemical and bioanalysis using small but powerful analytical equipment that is compatible with home appliances and mobile smart phones.

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