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CHAPTER 9

Aspects of recent development of immunosensors

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9.1 INTRODUCTION

9.1.1 General working principle of immunosensors

Immunosensors are affinity ligand-based biosensing devices that involve the coupling of immunochemical reactions to appropriate transducers. In recent decades, immunosensors have received rapid development and wide applications with various detection formats [1–2]. The general working principle of the immunosensors is based on the fact that the specific immunochemical recognition of antibodies (antigens) immobilized on a transducer to antigens (antibodies) in the sample media can produce analytical signals dynamically varying with the concentrations of analytes of interest. Here, the highly specific reaction between the variable regions of an antibody and the epitopes of an antigen involves different types of bonding, basically hydrophobic and...
electrostatic interactions, van der Waals force, and hydrogen bonding. The antigen–antibody reaction is reversible and, owing to the relative weakness of the forces holding the antibody and antigen together, the complex formed would dissociate in dependence upon the reaction environment (e.g. pH and ion strength). The strength of the binding of an antibody to an antigen could be characterized by its affinity constant \((K)\), which is of the order between \(5 \times 10^4\) and \(1 \times 10^{12}\) \(\text{L mol}^{-1}\). The high affinity and specificity of this antigen–antibody binding reaction defines the unique immunosensor characteristics.

The general immunosensor design consists of three individual parts in close contact: a biological recognition element, a physicochemical transducer, and an electronic part. Antibodies or antibody derivatives (antigens or haptens) usually serve as the biological recognition elements, which are either integrated within or intimately associated with a physicochemical transducer. This recognition reaction defines the high selectivity and sensitivity of the transducer device. The electronic part is used to amplify and digitalize the physicochemical output signal from the transducer devices such as electrochemical (potentiometric, conductometric, capacitative, impedance, amperometric), optical (fluorescence, luminescence, refractive index), and microgravimetric devices. Gizeli and Lowe [3] suggested that an ideal immunosensor design should possess the following specifications: the ability to detect and quantify the antigens (antibodies), the capacity to transform the binding event without externally added reagents, the ability to repeat the measurement on the same device, and the capacity to detect the specific binding of the antigens (antibodies) in real samples. All of these specifications have been the main issues to pursue in developing immunosensors applied in various fields.

9.1.2 Main performance characteristics of immunosensors in clinical analysis

As an important branch of immunoassay techniques, immunosensors possess all essential performance characteristics of immunoassays. They show high selectivity, sensitivity, reversibility and efficient reagent usage. At the same time, the immunosensors are generally simple to operate, and easy to realize automation, digitization, and miniaturization. They may bypass some inherent problems of traditional analytical methods. Therefore, immunosensors have been the subject of expanding interest in the immunochemical studies with enormous potential in clinical diagnosis [1–2, 4], environmental analysis [5–6], and biological process monitoring [7]. As for the medical diagnosis of some diseases, herein considerable efforts have been devoted to the development of precise, rapid, sensitive, and selective immunosensors by measurement of the markers or pathogenic microorganisms responsible for the diseases, such as proteins, enzymes, viruses, bacteria, and hormones [1, 8–9]. Chagas’ disease, an American trypanosomiasis caused by the hemoflagellate *Trypanosoma cruzi*, is an example. An amperometric immunosensor has been recently proposed to probe the presence of antibodies against *T. cruzi* in blood donors, and to follow the antibody decay during treatment of chagasic patients with the available drugs [10]. Yuan *et al.* reported a novel potentiometric immunosensor for detection of hepatitis B surface antigen by immobilizing
hepatitis B surface antibody on a platinum electrode [11]. A piezoelectric immunosensor was developed for the on-line detection of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) in sputum in the gas phase. Compared to other SARS detection techniques, this method can rapidly test SARS-CoV at low cost [12]. Moreover, the determination of some tumor markers plays an important role in diagnosing, screening, and determining the prognosis of a cancer disease. Such tumor markers to be detected are often found in abnormally high amounts in the blood, urine, or tissue of patients with certain types of cancers. The examples include carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), carcinoma antigen 125 (CA125), alpha-fetoprotein (AFP), prostate specific antigen (PSA), CA15-3 and human chorionic gonadotropin (HCG) [13–15]. Wilson proposed an electrochemical immunosensor for the simultaneous detection of two tumor markers of CEA and AFP [15]. An increasing number of immunosensors have been utilized to analyze a series of biochemical targets for diagnosing infectious diseases, although there are still problems concerning the assay of analytes in real sample matrixes [1].

### 9.2 Immobilization of Immunoactive Elements

Since immunosensors usually measure the signals resulting from the specific immunoreactions between the analytes and the antibodies or antigens immobilized, it is clear that the immobilization procedures of the antibodies (antigens) on the surfaces of base transducers should play an important role in the construction of immunosensors. Numerous immobilization procedures have been employed for diverse immunosensors, such as electrostatic adsorption, entrapment, cross-linking, and covalent bonding procedures. They may be appropriately divided into two kinds of non-covalent interaction-based and covalent interaction-based immobilization procedures.

#### 9.2.1 Non-covalent interaction-based immobilization procedures

This type of immobilization of immunoactive entities is based on the non-covalent interactions between the antibody or antigen molecules and the transducer substrates, and usually refers to hydrophobic interaction, electrostatic interaction, van der Waals force, and hydrogen bonding. One notices that besides pure physical adsorption, some weak chemical interactions are also involved here. The non-covalent interactions may vary from the different substrates of transducers. For a non-polarity sensing substrate, the antibody or antigen molecules can be adsorbed through the hydrophobic interaction and van der Waals force. Wenmeyer et al. attached anti-digoxin antibodies at the surfaces of polystyrene microtubes by direct adsorption interaction, achieving the determination of digoxin with a detection limit of 50 pg mL$^{-1}$ [16]. While for the charged substrates, the non-covalent interactions are mainly associated with the electrostatic interactions. The most typical layer-by-layer technique of self-assembly has attracted considerable attention in biomolecular immobilizations [17–21]. Caruso and coworkers assembled polyallylamine hydrochloride/polystyrene sulfonate layers on the self-assembled...
monolayer of mercaptopropionic acid, providing a charged polyelectrolyte layer on the transducer surface [19]. The biomolecules of avidin and anti-immunoglobulin (IgG) antibodies were then well immobilized through electrostatic interaction. A novel biosensing interfacial design strategy has been developed for immobilizing the antibodies onto the positively charged surfaces of plasma-polymerized film (PPF) via electrostatic interaction through a polyelectrolyte-mediated layer [20]. The immunosensors so prepared exhibited excellent response sensitivity due to the low disturbance of the electrostatic adsorption immobilization to the activity of antibody. The PPF surfaces can be regenerated repetitively by changing the pH of the buffer solutions to remove the polyelectrolyte-mediated layer.

Moreover, antibodies or antigens may be physically entrapped into the films of organic high polymers or inorganic materials (e.g. sol-gel, graphite powder) with stereo meshy structures. Of these entrapment immobilizations, the sol-gel-based immobilizations have recently attracted much attention due to their ability to encapsulate biomolecules at low temperature, as well as the physical tenability, optical transparency, mechanical rigidity, and low chemical reactivity [22–23]. Most applications of the sol-gel-based immobilizations have been primarily directed to the optical immunosensors [14, 22, 24] and the electrochemical immunosensors [23, 25–28]. Martínez-Fàbregas et al. proposed a polishable entrapment immobilization based on rigid biocomposite materials consisting of graphite powder, rabbit IgG, and methacrylate (or epoxy resins) [28]. The surface of the immunosensor can be regenerated by simply polishing to obtain a fresh layer of immunocomposite ready for next immunoassay. The aforementioned physical interaction-based immobilization procedures are demonstrated to be operated simply and rapidly. However, their immobilization stability might be influenced by the bulk metal surfaces and environmental factors such as temperature, pH, and ion strength of solution, resulting in a loss of bioactivity or denaturation of the proteins. Moreover, the gradual elution of proteins physically adsorbed may occur during the analytical performances, which may in turn bring about some problems associated with loss of detection sensitivity and low reproducibility of the sensors.

In recent years, nanomaterials (e.g. noble metals, magnetic oxides, and carbon nanoparticles or nanotubes) with unique physical and chemical properties have been successfully applied to modify immunosensing interfaces to achieve greatly improved immobilization of antibodies or antigens [29–31]. Some pioneering works have shown that the assembly of the gold nanoparticle layer on an electrode would lead to substantially increased electrode surface areas available for direct adsorption of biological entities, thus offering the possibility of the great enhancement of analytical sensitivity [11, 32–36]. For example, a new immobilization procedure of antibodies for capacitive immunosensor has been recently proposed using thiol compound and gold nanoparticles [36]. It was here demonstrated that the proposed immobilization procedure could retain the high biological activity of immobilized entities and provide favorable sensing performances. Moreover, magnetic nanoparticles as special carriers for immobilizing biomolecules have also been the current hot subject of a series of investigations for the construction of different immunosensors [37–39]. The easy localization of magnetic beads was used to generate a sensing layer at the surface of a piezoelectric
sensor, where the magnetic beads bearing antibodies were immobilized with the help of a permanent magnet at the surface of the crystal [37]. More recently, an amperometric immunosensor has been developed by employing a kind of core–shell magnetic nanoparticle of (CdFe₂O₄–SiO₂) to immobilize antibody onto the electrode surface with a magnet field [38]. Additionally, magnetic beads may be applied to label or attach antibodies (antigens) for the magneto-detection of the immune complex based on the perturbation of a magnetic field, which could be quantified using a suitable electronic device [39]. Compared with the conventional immobilization methods, these magnetism-driven immobilization procedures may have some merits such as simple manipulation, easy biomolecule modification, low cost, and repeatable regeneration.

### 9.2.2 Covalent interaction-based immobilization procedures

The covalent interaction procedures, typically the cross-linking methods, are the most popular immobilization manipulation for fabricating various immunosensors. Due to the lack of an amount of active covalently binding sites at some transducer substrates (e.g. metals, semiconductor, or optical fibers), the precoatings of the base transducers with thin films are generally necessary for covalently binding the antibodies or antigens by using the functional reagents such as glutaraldehyde, carbodiimide succinimide ester, maleimide, and periodate. Many traditional coating materials, such as polyethyleneimine [40–41], (γ-aminopropyl)trimethoxysilane [42–43], and copolymer of hydroxyethyl- and methyl-methacrylate [44], are often used as the mediate layers for immunoactive molecule immobilization. In recent decades, however, some new coating or functionalized film techniques (materials) have been introduced into this field.

Self-assembled monolayers (SAMs) offer promising functionalized films for the immobilization of antibodies or antigens [45–46]. Since sulfur donor atoms strongly coordinate on noble metal substrates (e.g. Au, Ag, and Pt), various sulfur-containing molecules such as disulfides (R-SS-R), sulfides (R-S-R), and thiols can form various functionalized SAMs of highly organized and compact construction. The applications of the SAM technique in the immobilization of biomolecules have been widely documented [47–49]. Knoll and coworkers presented a versatile biotin-functionalized SAM, on which the biotinylated antibodies can be readily immobilized through an avidin mediator [48]. Mixed SAMs composed of long-chain thiols with carboxylic and hydroxyl groups are also used to attain a specific and stable affinity interface of immunosensors [50–51]. Langmuir-Blodgett (LB) films are other useful alternatives to traditional mediate layers [52–54]. LB films, which are usually prepared by transferring a monolayer on a solid substrate, have great potential in helping to control the orientation and surface density of the antibodies. Hirata et al. [52] successfully prepared the lipid-tagged antibody/phospholipid monolayers with high immobilization properties using the LB technique. Vikholm et al. demonstrated the incorporation of lipid-tagged single-chain antibodies into lipid monolayers obtaining desirable retention of antibody activities [53–54]. Moreover, recent years witness a newly emerged ultra-thin polymer film, plasma-polymerized film (PPF), which is reported with successful applications in various immunosensor designs [19, 55–57]. PPFs, which are generally prepared by
using glow discharge or plasma of organic vapors, are extremely thin, homogeneous, mechanically and chemically stable, with strong adhesion to the substrates. Karube’s group first reported the application of PPF to QCM immunosensors [55]. They verified that the resultant sensors were more reproducible from batch to batch, and might have lower noise and higher sensitivity than sensors using some conventional organic coatings (e.g. polyethylenimine). This kind of functionalized film may offer promising alternatives in interfacial design of immunosensors of various transducers.

In recent years, various nanomaterials are found to be skillfully applied in combination with the covalent interaction-based immobilization procedures for immunosensors. Carbon nanotubes (CNTs), for example, have been recognized as the quintessential nano-sized materials since their discovery in 1991 [58]. These nanotubes are now chemically functionalized for the immobilization of biological entities for different biosensors, i.e. electrochemical devices [59–61]. Pantarotto et al. successfully bound a model pentapeptide and a virus epitope of foot-and-mouth disease onto single-walled CNTs [61]. They found that the CNTs-loaded peptide might retain the structural integrity to be well recognized by monoclonal or polyclonal antibodies, indicating the potential applications for diagnostic purposes and vaccine delivery. A silica nanoparticles-based immobilization strategy was also proposed by Wang et al. for direct immunosensing determination of Toxoplasma gondii-specific IgG [62]. Herein, the preparation strategy could allow for antigens covalently bound with higher loading amount and better retained immunoactivity compared to the commonly applied cross-linking methods.

The aforementioned covalent interaction-based procedures may usually allow for the immunoactive proteins immobilized with high stability and repeatability, and the robust covalent bonds may favor the low noise of detection. Nevertheless, problems associated with these covalent bond immobilizations are the decrease of binding capacity of antibodies (antigens) in the immobilization process. Such a phenomenon may be presumably contributed to the partial loss of the immunoactive sites and the random orientation of antibody molecules bound on the transducer surfaces. In addition, cross-linking can produce a three-dimensional multilayer matrix that creates diffusion barriers and transport limitations, resulting in long immunoreaction time and low sensitivity [63]. It is established that the oriented immobilization of antibodies has low influence on their immunological activities to a certain degree [64–68], which antigen binding capacity was demonstrated with a factor of 2–8 higher than that of antibodies randomly immobilized [68]. Therefore, special interest has been given to the development of the orientation-controlled immobilization techniques for antibodies, i.e. mostly through proteins A or G to specifically bind the antibody Fc fragment, or by directly binding the chemical groups at antibody Fc region [64, 69–71].

Lee et al. utilized the self-assembled layer of thiol group-modified protein A for the oriented immobilization of antibodies [64]. An increased binding capacity was further observed. As another illustrative instance, a protein A-based orientation-controlled immobilization strategy for antibodies was proposed for the fabrication of a QCM immunosensor using nanometer-sized gold particles and amine-terminated PPF [65]. Moreover, in recent years, there has emerged another oriented immobilization
methodology for antibodies through their native thiol (-SH) groups, which were liberated after the splitting of the intact IgG into two antibody fragments [72–73]. Karyakin et al. reported a site-oriented immobilization strategy of antibodies on the gold electrode surfaces by use of native sulfide groups of IgG fragments obtained by reduction of intact IgG [72]. They found that antibodies immobilized by this procedure showed an antigen binding capacity 20–30 times higher than that of non-specifically adsorbed intact ones traditionally used.

In general, an ideal immobilization should have the following characteristics: (i) a sufficient loading amount of active antigens or antibodies at the transducer surface; (ii) the immobilized antigens or antibodies staying stable during the measurement process; (iii) the immobilization process having no influence to the sensing behavior of the transducer; and (v) the ability of sensor regeneration. An effective dissociation of the antigen and regeneration of antibody, i.e. by using Gly-HCl buffer (pH 2.3), for cost effectiveness is of practical interest in real immunosensor applications [74].

### 9.3 MAJOR TYPES OF IMMUNOSENSORS

There are mainly three types of transducers used in immunosensors: electrochemical, optical, and microgravimetric transducers. The immunosensors may operate either as direct immunosensors or as indirect ones. For direct immunosensors, the transducers directly detect the physical or chemical effects resulting from the immunocomplex formation at the interfaces, with no additional labels used. The direct immunosensors detect the analytes in real time. For indirect immunosensors, one or multiple labeled bio-reagents are commonly used during the detection processes, and the transducers should detect the signals from the labels. These indirect detections used to need several washing and separation steps and are sometimes called immunoassays. Compared with the direct immunosensors, the indirect immunosensors may have higher sensitivity and better ability to defend interference from non-specific adsorption.

#### 9.3.1 Electrochemical immunosensors

The majority of known immunosensor devices belong to the group of electrochemical immunosensors. Electrochemical immunosensors may possess several advantages, for example high sensitivity, low cost, and portable design. The principle of their operation is based on the electrochemical detection of the labeled immunoagents or markers such as enzymes, metal ions, or other electroactive compounds, thus providing an opportunity to analyze complex multicomponent mixtures for diagnosing diseases or monitoring the status of patients [75]. The kinds of detection transducer for electrochemical immunosensors can be mainly subdivided into potentiometric, conductometric, capacitive, impeditive, and amperometric (metal and graphite electrodes) devices.

**Potentiometric transducers** now belong to the most mature transducers with numerous commercial products. For potentiometric transducers, a local equilibrium is established at the transducer interface at near-zero current flow, where the change
in electrode or membrane potential is logarithmically proportional to the specific ion activity. The relationship of logarithmic proportionality constitutes the fundamental principle of all potentiometric transducers such as the ion-selective electrodes (ISE).

The groups of biosensors are characterized as simple in preparation, robust in operation, and moderately selective in analytical performance [76–81]. Janata first proposed a potentiometric transducer for immunosensing and named it “immuno-electrode” [78], using the immuno-electrode to detect Concanavalin A through covalent attachment to the surface of a PVC membrane deposited on a platinum electrode. The incorporation of ISEs, pH electrodes or gas-sensing electrodes into potentiometric immunosensors to improve their assay sensitivity has been extensively investigated by Rechnitz and coworkers, i.e. for immunochemical measurements of digoxin and human IgG [79–80]. D’Orazlo et al. reported the indirect measurements of immunoagents using ion-selective electrodes [81]. A potentiometric immunosensor based on a molecularly imprinted polymer was prepared as a detecting element in micro total analysis systems with the intent of providing easy clinical analysis [82]. Moreover, the ion-selective field-effect transistor (ISFET) as a semiconductor device is generally constructed by substituting an ion-sensing membrane for the metal gate of a field-effect transistor (FET) [83]. The ISFET is able to respond to the surface potential change resulting from the specific immunochemical reaction between the immobilized antibodies and the free antigens.

The pH-sensitive ISFETs, as the most widely used sensor of this type, are fabricated with a large range of possible insulators (i.e. SiO₂, Si₃N₄, and Al₂O₃) and enzyme labels (i.e. urease, peroxidase, and glucose oxidase) [84–85]. Nevertheless, only a few examples of ISFET-based immunosensors could be found in the literature [86–88]. For example, Zayats et al. report the impedance measurements on an ISFET device that can be used to detect antigen–antibody interactions on the gate surface [88]. In the meantime, they performed complementary surface plasmon resonance (SPR; see above) experiments to illustrate that the ISFET impedance measurements and the SPR reveal comparable sensitivities.

Conductometric transducers, as the oldest electrochemical devices, seem not to enjoy wide applications due to their poor selectivity. For example, Yagiuda et al. proposed a conductometric immunosensor for the determination of methamphetamine (MA) in urine [89]. The decrease in the conductivity between a pair of platinum electrodes might result from the direct attachment of MA onto the anti-MA antibodies immobilized on the electrode surface. The system was claimed to be a useful detection technique of MA in comparison with a gas chromatography–mass spectrometry method.

Capacitance and impedance transducers with high sensitivity are widely employed for various immunosensing assays [90–102]. The capacitance sensors are essentially based on the principle that the electrolyte capacitance of an electrode depends on the thickness and dielectric behavior of the dielectric layer on the electrode surface and the solid/solution interface. Dijksma et al. designed an immunosensor for the direct detection of interferon-γ at the attomolar level by using the AC impedance approach [90]. The immobilization processes of antibodies (antigens) play an important role in these immunosensors, and the sensitivity of a capacitive immunosensor increases with the decreasing thickness of the insulating layer. Shen and coworkers fabricated
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a heterostructure of Au/o-aminobenzenethiol layer/covalent-coupling antibody/electrode for the direct detection of the antibody–antigen interaction by capacitance measurements [91]. A capacitive immunoassay based on antibody-embedded ultra-thin alumina sol-gel films (~20 to 40 nm) was reported and used for direct determination of antigens with a detection limit as low as ~1 ng mL⁻¹ [92]. Fernandez-Sanchez et al. reported a successful integration of the lateral flow immunoassay format and impedance detection for prostate-specific antigen of tumor marker, where the electrochemical transducer was coated with a pH-sensitive polymer layer [93].

Although capacitance and impedance immunosensors can directly be utilized to investigate the antibody–antigen interaction without the need of other reagents and a separation step, their analytical sensitivity is limited in clinical applications [14]. In order to amplify the capacitance or impedance response to immunoreaction for the sensitive detection of various clinical markers, different labels have been used including enzymes, fluorophores, and metal chelates [103–104]. Ruan et al. developed an immunosensor based on enzyme-stimulated precipitation for the detection of Escherichia coli O157:H7 using an electrochemical impedance spectroscopy [103]. Another illustrative example was the sensitized immunosensor proposed by Chen et al. [104]. In their study, a receptor protein was directly adsorbed on a porous nanostructure gold film to perform a sandwich immunoreaction with the precipitation of insoluble product on the electrode. The impedance signals so amplified showed good linearity with the content of IgG in the range 0.011–11 ng mL⁻¹ with a detection limit of 0.009 ng mL⁻¹. A new strategy of signal amplification was also introduced for highly sensitive impedance measurements using biotin-labeled protein–streptavidin network complex [105].

Amperometric immunosensors, as the most popular immunoassaying formats, are based on the measurement of the currents resulting from the electrochemical oxidation or reduction of electroactive species at a certain constant voltage. This kind of immunosensor usually uses a complex three-electrode measuring system consisting of a working electrode (e.g. gold, glassy carbon, or carbon paste), a reference electrode (e.g. Ag/AgCl), and a conducting auxiliary electrode (e.g. platinum). Since most antibodies and antigens are not electrochemically active, there are only a few applications available for direct amperometric sensing. Therefore, most amperometric immunosensors are indirect ones which can detect mainly the redox currents associated with electroactive or catalytic labels [25–26, 28, 106–116]. Aizawa et al. first developed an amperometric immunosensor for the determination of human chorionic gonadotropin using an amperometric oxygen electrode [106]. Among the labels used, enzymes are the most popular ones in different types of immunoassays, such as horseradish peroxidase (HRP) or glucose oxidase. An immunosensor was designed for determining isopentenyl adenosine based on the electro-polymerization of polypyrrole and poly(m-phenylenediamine) entrapped with HRP on the glassy carbon electrode [108]. A design strategy of reagentless immunosensor was reported for the detection of carcinoma antigen-125 antibodies by direct HRP-labeled electrochemistry [109]. Due to the high sensitivity inherent in these transducers by enzymatic catalysis, amperometric immunosensors can obtain a much higher sensitivity than the classical ELISA. For the immunosensors used in clinical applications, their surfaces should be capable of renewal. Yu et al. developed
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a renewable amperometric immunosensor for the determination of *Schistosoma japonicum* (Sj) antibody by using the paraffin graphite–Sj antigen biocomposite paste electrodes which might be regenerated by polishing the surface [25]. Ionescu and his collaborators have developed two similar amperometric immunosensors for cholera antitoxin immunoglobulins, where the cholera toxin biorecognition entities were bound to a biotinylated polypyrrole film or pyrrole–biotin and lactitobionamide electropolymerized copolymer [117–118]. Moreover, nano-sized particles or sol-gel matrixes have also been increasingly employed in the design of amperometric immunosensors with enhanced analytical performance [23, 119–120]. For example, an electrochemical immunosensor has been developed for probing complement III (C3) by use of nanogold particle monolayer as the sensing interface [119]. With the coupling of sol-gel and screen-printing technologies, a sensitive thick film immunosensor was fabricated by dispersion of rabbit immunoglobulin G, graphite powder, and a binder in the sol-gel solution [23]. A new HRP-labeled amperometric immunosensor for determination of chorionic gonadotrophin in human serum was constructed by immobilizing HCG within titania sol-gel on a glassy carbon electrode [120]. Anodic stripping voltammetry as an electrochemical assay technique has been well adopted for sensitive measurements of heavy metals such as copper and silver, which may also offer an attractive way of sensitive immunosensor development [121–122]. An immunosensor was designed by coupling immunoassay with the square wave anodic stripping voltammetry technique involving copper ion-labeled antigen in the competitive immunoreaction [121]. This immunosensor might allow rapid, accurate, and inexpensive detection of gibberellin acid with a concentration as low as 1 µg mL⁻¹. Chu et al. designed a silver-enhanced colloidal gold metalloimmunoassay for the determination of *Schistosoma japonicum* antibody (SjAb) in rabbit serum [122]. In their study, after the immunoreaction of SjAb target with immobilized Sj antigens, colloidal gold-labeled secondary antibody was introduced to favor the silver enhancement process. An acidic solution was further used to dissolve silver metal atoms, followed by the sensitive determination of dissolved silver ions using anodic stripping voltammetry. In addition, many immunoreaction signal-amplified methods or processes have also been adopted for the development of sensitive amperometric immunosensors. Willner’s group reported an amplified immunoassaying scheme of chronopotentiometry and Faradaic impedance spectroscopy by way of a bio-catalyzed precipitation of the insoluble product onto the gold electrode [123]. They also designed a variation of this scheme with signal amplification by employing liposomes labeled with biotin and HRP as a probe to amplify the sensing of antigen–antibody interactions [124]. In this case, the electrode with the antigen–antibody complex was exposed to the biotinylated anti-IgG antibody, and further the biotin-labeled HRP-liposomes through an avidin bridge to achieve the biocatalyzed precipitation of an insoluble product on the conductive support.

### 9.3.2 Optical immunosensors

Since almost all optical phenomena at sensing surfaces (e.g. adsorption, fluorescence, luminescence, scatter or refractive index, etc.) can be used for biochemical sensing
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Designs, optical immunosensors are considered as one of the most promising alternatives to the traditional immunoassays in clinic diagnosis and environmental analysis. In recent years, there has been an increased trend in the use of optical transduction techniques in immunosensor technologies due to the advantages of applying visible radiation, non-destructive operation mode, and the rapid signal generation and reading [1, 125–126]. The optical immunosensors may be divided into two types of approaches: direct optical immunosensors and indirect immunosensors depending upon the use of labeled signaling molecules.

Surface plasmon resonance (SPR) as a direct and reliable optical transducer is commonly based on the evanescent wave, in which a thin gold layer is generally deposited on a prism serving as an optically rarer medium [127–128]. Not requiring additional labels and separation steps, the direct SPR immunosensors have been proven to be powerful analytical tools for rapid real-time monitoring the immunological targets. Schofield and Dimmock developed a SPR system in combination with the flow system for detection of influenza virus by use of carboxylated dextran polymer matrix to couple monoclonal antibody of HC10 [129]. In order to validate the feasibility of SPR immunosensor as a tool for diagnosing type I diabetes, Choi et al. modified mixed SAMs onto the optical substrate achieving the immuno-response detection for monoclonal antibodies of anti-glutamic acid decarboxylase [130]. Moreover, the fatty acid-binding protein assay has an application potential in clinical analysis for diagnosis of myocardial infarction. A direct optical immunosensor based on SPR was developed for detecting the human heart-type fatty acid binding protein with a detection limit of 200 ng mL$^{-1}$ [131]. Highly sensitive SPR-based immunosensors using self-assembled protein G have also been successfully applied for the detection of microbes such as Salmonella typhimurium and Legionella pneumophila [132–133]. More importantly, several instrument systems using SPR technology have been commercially available, such as the BIAcore™ system from Pharmacia Biosensor, the Iasys™ system from Affinity Sensors, and so on. Nevertheless, at present, there are still some unsolved problems for these SPR devices, such as non-specific adsorption and poor analytical sensitivity to analytes of low molecular weight.

Fluorescence immunosensors, as the total internal reflection fluorescence devices, continue to prove themselves as another promising type of sensitive and selective optical immunoassay technique, in which labels are sometimes used [134]. When the fluorescence-labeled antibodies or antigens are attached to the transducer surface and enter the evanescent field, the incident light will excite fluorescent molecules producing a fluorescent evanescent wave signal to be detected. The optic-fiber immunosensor system by fluorescence enhancement or quenching is separation-free, reagentless and applicable to the determination of various proteins by antigen–antibody reactions [134–138]. Maragos et al. described the development of a fluorescence polarization-based competition immunoassay for fumonisins in maize using fumonisin-specific monoclonal antibodies [135]. A fluorescence-based immunosensor array for simultaneous determination of multiple clinical analytes was developed by Rowe et al. [137]. In their study, the patterned array of recognition elements was immobilized onto the planar waveguide to “capture” the analytes from the samples to be quantified by means
of fluorescent detector molecules. Moreover, in recent years, quantum dots as the most suitable fluorescence labels have received increasing applications for developing fluorescence immunosensors due to their high fluorescence quantum yield and sensitivity to environmental changes upon binding proteins. Aoyagi et al. proposed a reagentless, regenerable, and portable optic immunosensor for the ultra-sensitive detection of a model sample of IgG based on changes in fluorescent intensity of fluorescent quantum dot-labeled protein A [138]. An antibody for leukemia cell recognition was attached to the luminophore-doped nanoparticle through silica chemistry, yielding an optical microscopy imaging technique for the identification of leukemia cells [139]. Experimental results in this report showed that the new technique using the antibody-coated luminophore nanoparticles could allow leukemia cells to be easily and clearly identified with high efficiency.

Chemiluminescence sensors have also been extensively applied in routine clinical analysis as well as biomedical research due to the advantages of no radioactive wastes, simple instrumentation, low detection limit, and wide dynamic range [14, 140–144]. A chemiluminescent immunosensor for carbohydrate antigen 19-9 (CA19-9) was described by Lin et al., with CA19-9 immobilized on the cross-linked chitosan membrane [141]. The decrease of the immunosensor chemiluminescent signal was proportional to the CA19-9 concentration in the range 2.0–25 U mL$^{-1}$, with the detection limit of 1.0 U mL$^{-1}$. Pandian et al. developed an automated chemiluminometric immunoassay for the measurement of HCG [142]. It was demonstrated that the immunoassay might facilitate exploration of HCG utility for Down syndrome screening, early pregnancy detection, and differentiation of invasive from non-invasive trophoblastic disease. An optical microbiosensor has been newly designed for the diagnosis of hepatitis C virus (HCV) by using a novel photo-immobilization methodology based on a photo-activable electro-generated polymer film [143]. Herein, the immunosensor using optical fiber photochemically modified was tested for the determination of anti-E$_2$ protein antibodies through chemiluminescence reaction. Another published study presented the use of electrogenerated luminol chemiluminescence in a homogeneous immunosensor, where digoxin was labeled with luminol through a luminol-BSA-digoxin conjugate [144]. The prepared chemiluminescence immunosensor in a competitive format was shown allowing for the detection of free digoxin with the concentration as low as 0.3 µg L$^{-1}$.

9.3.3 Microgravimetric immunosensors

Microgravimetric immunosensors may incorporate high sensitivity of piezoelectric response and high specificity of antibody–antigen immunoreaction. The detection principle of these devices is generally based on adsorbate recognition where the selective binding may cause the changes in mass loading and interfacial properties (i.e. viscoelasticity and surface roughness), which can be recognized by a corresponding shift in the oscillation frequency [145–148]. Outstanding features of these sensors include low cost, simple usage, high sensitivity, and real-time output. Microgravimetric immunosensors have two kinds of sensing formats, gas phase and solution phase sensing. The sensitivity to the mass change in air on the transducer surface is about 1 Hz ng$^{-1}$.
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for a bulk acoustic wave device with 9 MHz of fundamental frequency, which can be described by the Sauerbrey equation [145]. The microgravimetric transducer is thus mainly known as the quartz crystal microbalance (QCM). Microgravimetric immunosensors in solution phase sensing were used for the quantification of a number of biological targets [42, 65, 146–153]. Nüsslein’s group reported a QCM assay for bacteria using a cell-selective polymer film, with desirably low detection limit and no need for prior sample treatment [149]. Wang and coworkers initially developed an integrated QCM immunosensor array composed of four kinds of leukemic lineage-associated probes to explore the differentiated leukocyte antigens for immunophenotyping of acute leukemia [150]. In their study, the probes (crystals) of the array were immobilized separately with Fab fragments of leukemic lineage-associated monoclonal antibodies (markers). The developed immunosensor array was demonstrated to be able to rapidly identify normal cells from leukemic blasts and define the leukemic blasts within certain phenotypic groups (lineages). Recently, a QCM immunosensor using protein A for antibody immobilization has been described for the detection of Salmonella typhimurium in chicken meat sample by simultaneous measurements of the resonant frequency and motional resistance [152]. Based on the modification of mixed SAMs on gold electrodes for covalently binding antigens, another piezoelectric immunosensor has been recently developed to detect antisperm antibody [153]. The analytical results for evaluating several clinical specimens by the developed method were found to be in satisfactory agreement with those given by the classical ELISA.

Despite many salient successes, the use of QCM-based immunosensors for trace biological target detection is still challenged by its relatively low intrinsic sensitivity. Kim et al. incorporated the immunomagnetic separation with the QCM-based impedance technique achieving a new immunoassay for quantifying Salmonella typhimurium with very high sensitivity of cell detection [154]. Herein, antibodies immobilized on magnetic particles were delivered into the sample medium to capture the targets. The resultant immunocomplex was further magnetically collected onto the piezoelectric crystal to be quantified with impedance spectroscopy. Through the enzyme-catalyzed formation of a precipitate on the QCM surface, a mass-amplified microgravimetric immunosensor was proposed in combination with a sandwich enzyme-linked immunoassay [155]. Su and his coworkers successfully used QCM for detection of dengue virus [156]. The authors immobilized two monoclonal antibodies on the crystal that act specifically against the dengue virus envelope protein and non-structural protein. The sensitivity reported for the fabricated piezoelectric immuno-chip was 100-fold greater than the conventional sandwich ELISA method. A highly sensitive microgravimetric biosensor has been developed incorporating noble metal particle-amplified sandwiched immunoassay and silver enhancement reaction [157]. Upon the formation of the sandwiched immunocomplex, the sensor surfaces were coated with gold nanoparticles serving as the nucleation sites to catalyze silver ion reduction. The silver metal deposition would result in a large change in frequency responses, achieving approximately two orders of magnitude improvement in human IgG quantification.

Moreover, there is another important type of microgravimetric immunosensor which is based on the immunological agglutination events. The agglutination immunoreaction
of antibody-bearing suspensoids such as polymers, microbeads, and nanoparticles may induce a corresponding change in the solution parameters (i.e. density and viscosity) and the interfacial properties of the crystal monitored by the QCM device [158–161]. In contrast to the common conventional piezoelectric assays, the QCM sensing format offers a unique advantage in that the immobilization of antibodies or antigens on the crystal is not necessary. The kind of QCM-sensing methods are widely recognized to be simple, sensitive, and feasible for detecting relevant targets responsible for many clinical diseases [158–163]. Kurosawa et al. first developed an agglutination-based piezoelectric immunoassay using antibody-bearing latex, termed as the latex piezoelectric immunoassay (LPEIA), for detecting C-reactive protein [158]. Recently, it has been demonstrated that the LPEIA could be greatly improved by using gold nanoparticles as replacements for latex particles, resulting in a novel agglutination-based piezoelectric immunoassay for directly detecting anti- \textit{T. gondii} immunoglobulins in infected rabbit sera and bloods [159].

### 9.3.4 Other kinds of immunosensors

In recent years, considerable efforts have been devoted to the development of cantilever-based immunosensors with unique enantio-selective antibodies [164–165]. These devices are mainly used for quality and process control, and diagnostic biosensing for medical analysis. They may have fast responses and high sensitivity and are suitable for mass production. Lee et al. fabricated a piezoelectric nanomechanical cantilever by a novel electrical measurement. They found that this technique might allow for the label-free detection of a prostate-specific antigen (PSA) with a detection sensitivity as low as 10 pg mL$^{-1}$ [164]. A microfabricated cantilever was utilized to perform the direct (label-free) stereo-selective detection of trace amounts of an important class of chiral analytes, the r-amino acids, based on immunomechanical responses involving nanoscale bending of the cantilever. The major advantages of the microcantilever sensors over more traditional scale transducers such as the QCM reside in the superior sensitivity to minute quantities of analytes and the ability to micro-fabricate compact arrays of cantilevers to facilitate simultaneous and high throughput measurements [165].

Moreover, mass-sensitive magnetoelastic immunosensors are exploited to design extraordinarily versatile and useful sensor platforms [166]. Magnetoelastic sensors are well established and benefit from mass sensitivity compared to that of a surface acoustic wave (SAW) sensor. However, they may cost much less and are much smaller in size than SAW devices. Ruan et al. proposed a mass-sensitive magnetoelastic immunosensor based on the immobilization of affinity-purified antibodies on the surface of a micrometer-scale magnetoelastic cantilever achieving the highly sensitive detection of \textit{Escherichia coli} O157:H7 [167]. In addition, imaging ellipsometry (IE) has also been developed as a new kind of immunosensor, i.e. for the detection of pathogens of \textit{Yersinia enterocolitica} [168]. As another example, a label-free multi-sensing immunosensor based on the combination of IE and the protein chip was reported to be able to detect multiple analytes simultaneously, and even to monitor multiple biological interaction processes in situ and in real-time conditions [169].
9.4 CONCLUSION AND FUTURE TRENDS

Immunosensors incorporate the specific immunochemical reaction with the modern transducers including electrochemical (potentiometric, conductometric, capacitative, impedance, amperometric), optical (fluorescence, luminescence, refractive index), and microgravimetric transducers, etc. [1]. These immunosensor devices with dramatic improvements in the sensitivity and selectivity possess the abilities to investigate the reaction dynamics of antibody–antigen binding and the potential to revolutionize conventional immunoassay techniques. With the rapid development of immunological reagents and detection equipments, immunosensors have allowed an increasing range of analytes to be identified and quantified. In particular, simple-to-use, inexpensive and reliable immunosensing systems have been developed to bring immunoassay technology to much more diverse areas, such as outpatient monitoring, large screening programs, and remote environmental surveillance [9]. However, there are still some unsolved problems associated with the immobilization of immunoactive entities, non-specific adsorption from sample backgrounds (e.g. blood, serum, plasma, urine, and saliva) and practical applications of various transducer devices.

The current development of new immunosensors should aim at solving the problems of clinical analysis in medicine and of chemical analysis in the food industry and biotechnology. The development trends of immunosensors are likely to be primarily driven by the requirements of analytical practice on the improvement in sensitivity, selectivity, rapidity, and especially efficiency of assays (i.e. immunoassaying array or microfluidic system). Immunosensors with lowered detection limits and increased sensitivities have been developed in various fields, particularly in clinical analysis. For example, the sandwich immunoassay using enzyme-functionalized liposomes as the catalytic label is proposed to obtain the substantially improved assay sensitivity, validated in the immunoassay of cholera toxin [170]. Meanwhile, as the latest paradigm of development topic, nanomaterials with unique chemical and physical properties should continue to be exploited to offer important possibilities for new immunosensor designs [29]. A noticeable development trend is also observed in the development of immunosensors combining with other techniques such as flow injection analysis (FIA) or capillary electrophoretic (CE) analysis, to complement and improve the present immunoassay methods [171–172]. Moreover, the miniaturization and automation of immunosensing devices should be another important intention of development to facilitate the significantly shortened analysis time and simplified analytical procedure (i.e. one-step analysis). Of note, protein and antibody array technologies are envisaged to have potential for biomedical and diagnostic applications in recent years [173–177]. Belov et al. have proposed a novel immunophenotyping method for leukemias using a cluster of differentiation antibody microarray [174]. A microarray of enzyme-linked immunosorbent assay has been developed for autoimmune diagnosis of systematic rheumatic disease, where the high titers of antinuclear antibodies against various nuclear proteins and nucleoprotein complexes might be detected with high throughput [177]. At the same time, the screen-printing techniques may also appear to be the most promising technology for immunosensor array to be commercialized on a large
scale and widely applied in clinical diagnosis. Moreover, there have been increasing reports focusing on the development of microfluidic immunosensor systems for proteomics and drug discovery in recent years [178]. Microfluidic system integrating multiple processes in a single device generally seeks to improve analytical performance by reducing the reagent consumption and the analysis time, and increasing reliability and sensitivity through automation. The micro total analysis systems (µTAS) are already under development and should represent the future of high throughput immuno-tests [179]. In addition, with the development of protein engineering technology and molecular biology techniques, more flexible antibodies suitable for immunosensing applications may be expected. For example, the recombinant or fusion approach is powerful in the production of antibodies and antibody derivates. Use of various new generations of antibodies should lead to the enhancement of activity and stability of the immobilized bio-species and even the improvement of the regeneration and sensitivity of the immunosensors. As an inspiring illustrative instance, aptamers are beginning to emerge as a class of synthetic oligonucleotides or molecules that rival antibodies in both therapeutic and diagnostic applications [180–182]. Baldrich and coworkers first demonstrated the exploitation of an aptamer in an extremely rapid and highly sensitive displacement assay, the displacement enzyme-linked aptamer assay, using enzyme-labeled target as a suboptimal displaceable molecule [182].

To sum up, immunosensors are now becoming one of the most widely used analytical techniques, embracing a vast repertoire of analytes that are detected by a diverse range of transducer devices. The enormous potential of immunosensors in clinical diagnosis, environmental analysis, and biological process monitoring has been widely accepted and increasing efforts have been devoted to these fields. In particular, with the continual development of transducer technology, laser technology, nano-sized material technology, and antibody engineering technology, immunosensors based on the application of these technologies should be inevitably powerful tools in increasingly wide analytical areas [9].

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