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Review

Immunosensors for detection of pesticide residues

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Abstract

Immunosensors are biosensors that use antibodies or antigens as the specific sensing element and provide concentration-dependent signals. There is great potential in the applications of immunosensing technologies for rapid detection of pesticide residues in food and environment. This paper presents an overview of various transduction systems, such as electrochemical, optical, piezoelectric, and nanomechanics methods, which have been reported in the literature in the design and fabrication of immunosensors for pesticide detection. Various immobilization protocols used for formation of a biorecognition interface are also discussed. In addition, techniques of regeneration, signal amplification, miniaturization, and antibodies are evaluated for the development and applications of these immunosensors. It can be concluded that despite some limitations of the immunosensing technologies, these immuosensors for pesticide monitoring are becoming more and more relevant in environmental and food analysis.

Keywords: Immunosensor; Pesticide residues; Electrochemical; Optical; Piezoelectric

Contents

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1. Introduction

Pesticides are widely used in agriculture to protect crops and seeds before and after harvesting. They also have contributed significant health and economic benefits to society. At the same time, widespread use of pesticides has created serious concerns regarding their effect on the environment and human health (Valdez et al., 2000). The identification and quantification of pesticides are generally based on chromatographic methods, such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled with mass spectroscopy (MS). These analytical techniques have been described and reviewed extensively in the literature (Van der Hoff and van Zoonen, 1999; Núñez et al., 2005; Rial-Otero et al., 2007). These methods are very sensitive and reliable, but they are time-consuming and expensive. Moreover, they can only be performed by highly trained technicians and are not convenient for on-site or in-field detection.

Due to the great amount of pesticides currently being used, there is an increased interest for developing rapid screening systems for their detection. In recent years, enzyme-linked immunosorbent assays (ELISA) have grown rapidly as tools for pesticide measurement (Bhand et al., 2005; Kumar et al., 2006). However, they need some improvements (e.g. for continuous detection). Biosensors are potentially useful as methods to quickly detect pesticides and have been an active research area for some years (Marty et al., 1995). Biosensors have been defined as analytical devices which tightly combine biorecognition elements with physical transducers for detection of the target compounds (Guilbault et al., 2004). Many biosensors which are used for pesticide detection are based on the inhibition reaction or catalytic activity of several enzymes in the presence of pesticides (Mulchandani et al., 1999; Gogol et al., 2000; Anh et al., 2004; Deo et al., 2005; Laschi et al., 2007). Enzyme-based biosensors for pesticide determination are reviewed in the literature (Mulchandani et al., 2001; Amine et al., 2006). Since a number of pesticides have a similar mode of action affecting the activity of the same enzyme, most of enzyme-based biosensors are used for screening purposes and are unspecific for individual pesticides. They can only detect total pesticide content and do not provide specific information about a particular pesticide.

Immunosensors are biosensors that use antibodies (Ab) or antigens (Ag) as the specific sensing element and provide concentration-dependent signals. They consist of two process, a molecular recognition process, for sensing the specific Ag–Ab binding reaction at the surface of receptor, and a signal-transfer process, for responding to changes in an electrochemical, optical, spectroscopic, or electrical parameter of the receptor caused by the specific binding (Ruan et al., 2002). They appear to be appropriate for identification of a single pesticide or, in some cases, small groups of similar pesticides in environmental monitoring, as they are rapid, specific, sensitive and cost-effective analytical devices (Mallat and Barceló, 2001a). Moreover, with the development of Ab technologies, generating Ab against pesticide molecules has been relatively mature, which has provided further impetus in this area. Excellent reviews that focused on immunosensors for pesticide monitoring were described in the literatures (Suri et al., 2002; Suri, 2003). However, there is a time gap between current status in the field and the most recent reviews.

In this review, several types of immunosensors developed for their applications in pesticide analysis are highlighted. Various immobilization protocols used for formation of a biorecognition interface are also discussed. In addition, techniques of regeneration, signal amplification, miniaturization, and Ab are evaluated for the development and applications of these immunosensors.

2. Classification of immunosensors

In general, immunosensors can be distinguished from immnunoassays where the transducer is not an integral part of the analytical system. If a transduction is achieved using labeled species, the principles are similar to immunoassays. Depending on if labels are used or not, immunosensors are divided into two categories: labeled type and label-free type.

2.1. Labeled formats

This procedure involves a label to quantify the amount of Ab or analyte bound during an incubation step. Widely used labels involve enzymes (e.g. glucose oxidase, horseradish peroxidase (HRP), β-galactosidase, alkaline phosphatase), nanoparticles, and fluorescent or electrochemiluminescent probes. (Wilson et al., 1997; Keay and McNeil, 1998; Danielsson et al., 2001; Seydack, 2005; Wilson, 2005). Fig. 1 shows the schematic of labeled immunosensors. Commonly, two different formats for labeled immunosensors are available: sandwich assays and competitive assays. A sandwich assay consists of two recognition steps. In the first step, the Ab is immobilized on a transducer surface, allowing it to capture the analyte of interest. In the second step, labeled secondary Ab is added to bind with the previously captured analyte. The immunocomplexes (immobilized Ab-analyte-labeled Ab) are formed and the signals from labels

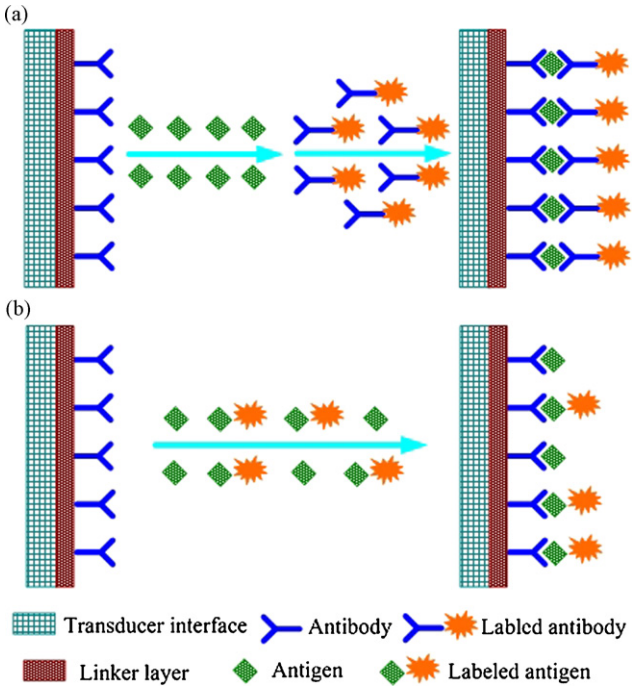

Fig. 1. Schematic of labeled immunosensors: (a) sandwich format and (b) competitive format.

increase in proportion to the analyte concentration (Sadik and Van Emon, 1996). In competitive assays, the analyte competes with labeled analyte for a limited number of antibody binding sites. As the analyte concentration increases, more labeled analyte is displaced, giving a decrease in signal if antibody-bound labeled analyte is detected (Bange et al., 2005). Some examples of immunosensors using labels for the determination of residual pesticides are presented in Table 1. Although the labeled format is usually more sensitive, labeled immunosensors are not capable of real-time monitoring of the Ab–Ag reaction and increase both development and operation costs compared to label-free immunosensors. The amount of target analyte can be inferred from the amount of labels that binds to the interface.

2.2. Label-free formats

This procedure detects the binding of pesticide and the Ab on a transducer surface without any labels. There are also two basic types in this format: direct and indirect. In the first type, the response is directly proportional to the amount of pesticides present. The vital advantage of these direct immunosensors is the simple, single-stage reagentless operation. However, such direct immunosensors are often inadequate to generate a highly sensitive signal resulting from Ab–Ag binding interactions and it is still difficult to meet the demand of sensitive detection. The second type, also based on competitive formats, is carried out as a binding inhibition test. The antigen (pesticide–protein conjugate) is first immobilized onto the surface of a transducer, and then pesticide–antibody mixtures are preincubated in solution. After being injected on the sensor surface, the antibody binding to the immobilized conjugate is inhibited by the presence of target pesticide.

It is advanced transducer technology that enables the label-free detection and quantification of the immune complex. It has been proven to be an effective method for the determination of pesticide. Some examples of label-free immunosensors for the determination of residual pesticides are presented in Table 2.

3. Transducers of immunosensors for pesticide detection

The biorecognition element determines the degree of selectivity or specificity of the biosensor, whereas the sensitivity of the biosensor is greatly influenced by the transducer (Leca-Bouvier and Blum, 2005). According to the transduction mechanism, immunosensors can be further classified into electrochemical, optical, piezoelectric and nanomechanic immunosensors as described in the following sections.

3.1. Electrochemical immunosensors

Electrochemical transducers are the oldest and most common methods used in biosensors. The principle is based on the electrical properties of the electrode or buffer that is affected by Ab–Ag interaction. They can determine the level of pesticides by measuring the change of potential, current, conductance, or impedance caused by the immunoreaction. They combine the high specificity of traditional immunoassay methods with the low detection limits and low expenses of electrochemical measurement system, and thus exhibit great advantages. They are not affected by sample turbidity, quenching, or interference from absorbing and fluorescing compounds commonly found in bio-

Table 1
Some examples of immunosensors using labels for the determination of residual pesticides

Pesticide	Detector	Label	Detection limit	Reference
Atrazine	Fluorescent	Nanoparticle	Similar to ELISA	Cummins et al. (2006)
Chlorsulfuron	Amperometric	Glucose oxidase	0.01–1 ng/mL	Dzantiev et al. (2004)
Glyphosate	Fluorescent	Glyphosate-peroxidase	0.021 ng/mL	González-Martínez et al. (2005)
Isoproturon	Fluorescent	Fluorescein	0.1 ng/mL	Mallat et al. (2001c)
Isoproturon	Fluorescent	Fluorescein	9.7 ng/L	Pulido-Tofiño et al. (2006)
Simazine	Potentiometric	HRP	3 ng/mL	Yulaev et al. (2001)
Mesotrione	Fluorescent	Fluorescein	0.04 ng/mL	Mastichiadis et al. (2002)
Hexaconazole			0.06 ng/mL	
Paraquat			0.09 ng/mL	
Diquat			0.10 ng/mL	

Table 2
Some examples of label-free immunosensors for the determination of residual pesticides

Pesticide	Detector	Detection limit	Reference
2,4-D	Impedimetric	45 nmol/L	Navrátilová and Skládal (2004)
2,4-D	SPR	0.1 ng/mL	Gobi et al. (2007)
Atrazine	Impedimetric	20 ng/mL	Hleli et al. (2006)
Atrazine	Impedimetric	8.34 ± 1.37 ng/mL	Valera et al. (2007)
Atrazine	Piezoelectric	1.5 ng/mL (direct) 0.025 ng/mL (competitive)	Přibyl et al. (2003)
Chlorpyrifos	SPR	45–64 ng/L	Mauriz et al. (2006a,b,c,d)
DDT	Nanomechanical	Below nM range	Alvarez et al. (2003)
Trifluralin	Optical waveguide	100 ng/mL (direct) 2×10^{-7} ng/mL (competitive)	Székács et al. (2003)

logical samples. However, a high-performance, cost-effective field analysis still remains the big challenge.

3.1.1. Potentiometric methods

Potentiometric immunosensors are based on measuring the changes in potential induced by the label used, which occur after the specific binding of the Ab–Ag. They measure the potential across an electrochemical cell containing the Ab or Ag, usually by measuring the activity of either a product or a reactant in the recognition reaction monitored. The measured potential is given by the Nernst equation:

$$E = \text{constant} + \frac{RT}{nF \ln a} \quad (1)$$

where E is the potential to be measured, R, T, F are constants, n is the electron transfer number, and a is the relative activity of the ion of interest.

Examples of these kinds of immunosensors for the determination of the pesticides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been reported (Dzantiev et al., 1996). The assay monitored the competitive binding of free pesticide and pesticide-peroxidase conjugate with Ab immobilized on a graphite electrode by potentiometric measurement of peroxidase activity in the immune complexes on the electrode surface. Detection limits were 40 ng/mL for 2,4-D and 50 ng/mL for 2,4,5-T. Life of the electrode extended to 60 sequential measurements. The assay optimization steps (choice of electrode, substrate mixture, regeneration regime, etc.) were characterized in another paper for herbicide simazine monitoring (Yulaev et al., 2001). The limit of simazine detection was 3 ng/mL. The operating lifetime of the sensor was 15 days. An immunosensor based on ion-selective field effect transistors (ISFET) was developed for the quick determination of herbicide simazine (Starodub et al., 2000). Two schemes of the assay were applied—competitive immune analysis and so called sequential saturation of Ab. The activity of bound peroxidase was measured by basic pH shift of ascorbic acid solution after addition of hydrogen peroxide. The limit of simazine detection by competitive method was 1.25 ng/mL and the linearity was observed in the range of 5–175 ng/mL. Sequential saturation of Ab allowed increasing the sensitivity to 0.65 ng/mL with the linearity in the range 1.25–185 ng/mL.

3.1.2. Amperometric methods

Amperometric sensors are a subclass of voltametric sensors. Amperometric immunosensors detect the concentration-dependent current generated when an electroactive species is either oxidized or reduced at the electrode surface to which Ab–Ag binds specifically (Patel, 2002). It is held at a fixed electrical potential. The current is directly proportional to specific Ab–Ag binding. The current and bulk concentration of the detecting species can be approximated as:

$$i = ZFk_{\text{m}}C^* \quad (2)$$

where i is the current to be measured, Z and F are constants, k_{m} is the mass transfer coefficient and C^* is the bulk concentration of the detection species.

While amperometric methods offer good temporal resolution, they cannot discriminate between different molecules undergoing an electrochemical reaction step at the electrode (Bond, 1980). This is important for avoiding interference of other electroactive species that could be present in the sample solution. For the simultaneous analysis of several samples using only one device, Skládal and Kaláb (1995) developed a multichannel immunosensor. The 2,4-D molecule conjugated to horseradish peroxidase was used as a tracer, which was determined amperometrically using hydrogen peroxide and hydroquinone as substrates. The detection limit for free 2,4-D in water was 0.1 ng/mL. A similar immunosensor coupled with an ELISA microtiter plate was also reported (Deng and Yang, 2007), and the detection limit of 0.072 ng/mL was achieved. The advantages of the presented electrochemical detector were high stability and sample throughput, low detection limit, the ability to be repeatedly used, and no need for regeneration. In another amperometric immunosensor (Bäumner and Schmid, 1998), hapten-tagged liposomes entrapping ascorbic acid as a marker molecule were chosen for the generation and amplification of the signal, and the sensitivity of measurements in tap water was below 1 ng/mL of atrazine and terbutylazine. A separation-free electrochemical technique was developed for the determination of atrazine (Keay and McNeil, 1998) and chlorsulfuron (Dzantiev et al.,

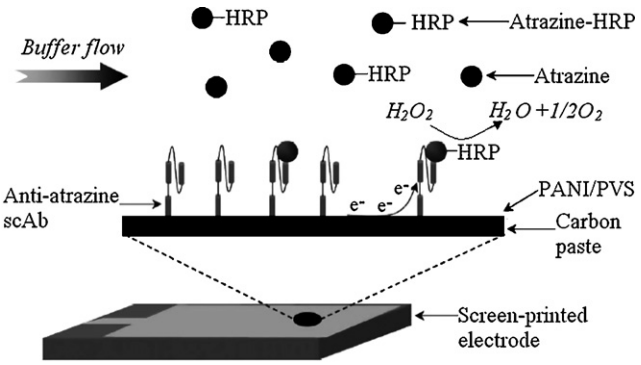

Fig. 2. Schematic of the electrochemical real-time sensing process for atrazine detection. Free and HRP-labeled atrazine compete for binding to immobilized scAb fragments (Grennan et al., 2003).

2004) in water samples. Signal detection was carried out by a screen-printed electrode, incorporating horseradish peroxidase in the carbon ink working electrode to determine glucose oxidase activity. Grennan et al. (2003) described an amperometric immunosensor for the analysis of atrazine using recombinant single-chain antibody (scAb) fragments. The sensor was based on carbon paste screen-printed electrodes incorporating the conducting polymer, which enables direct mediatorless coupling to take place between the redox centres of antigen-labeled HRP and the electrode surface (Fig. 2). The system was capable of measuring atrazine to a detection limit of 0.1 ng/mL. Killard et al. (2001) described an amperometric separation-free immunosensor for real-time environmental monitoring. It illustrated that determination of atrazine as low as 0.13 μM could be made using equilibrium incubation with an analytical range of 0.1–10 μM. Since an amperometric sensor can operate in other modes, cyclic voltammetry (CV) was performed in the analysis of the pesticide (Killard et al., 2000). An electrochemical immunosensor with paraoxon Ab loaded on the gold nanoparticles to monitor the concentration of paraoxon in aqueous samples was reported by Hu et al. (2003), and the detection limit of 12 ng/mL was achieved. To a large extent, the immunosensor can eliminate the interferences from other co-existing electroactive pollutants.

3.1.3. Capacitance/conductance/impedance methods

The concepts of impedance, conductance, capacitance, and resistance are different ways of monitoring the test system and are all inter-related (Milner et al., 1998). Impedance immunosensors measure different changes of an electrical field. Those changes could be overall electrical conductivity of the solution and capacity alteration due to the Ab–Ag interaction on the electrode surface, which also can be reflected in impedimetric response. Electric impedance spectroscopy (EIS) is a sensitive technique, which detects the electrical response of the system studied after application of a periodic small amplitude AC signal. In recent years, the electrochemical impedance immunosensors have attracted extensive interest in the sensing formation of Ag–Ab (Sadik et al., 2002; Darain et al., 2004; Yang and Li, 2005; Chen et al., 2006). EIS in connection with immunochemical methods was tested for the direct determination of the herbicide 2,4-D (Navrátilová and Skládal, 2004). The changes of impedance parameters (φ_{max} and Z_{min}) due to immunocomplex formation, which served as parameters characterizing changes on the sensing surfaces, were evaluated. It was possible to measure response to 2,4-D in a concentration range from 45 nM to 450 μM. Hleli et al. (2006) also showed that the application of this innovative way for designing pesticide biosensors was proven to be quite efficient. This immunosensor has a high sensitivity to atrazine antigen and a good linear response of membrane resistance differences ΔR_m to atrazine concentration in the range of 10–300 ng/mL with a detection limit of 20 ng/mL.

3.2. Optical immunosensors

After electrochemical approaches, optical devices are the second most commonly used transducers. A general optical sensor system consists of a light source, a number of optical components to generate a light beam with specific characteristics and to direct this light to a modulating agent, modified sensing head, and a photodetector. Optical immunosensors have been shown to be able to measure adsorbed molecular layers, which utilize the evanescent wave to form the sensing device. Different techniques can be used for creating an optical change, e.g., reflectometric interference spectroscopy, interferometry, optical waveguide lightmode spectroscopy, total internal reflection fluorescence, and surface plasmon resonance.

Optical immunosensors offer advantages of compactness, flexibility, resistance to electrical noise, and a small probe size. A label-free approach of detection is preferred and of high value. In addition, the use of very low amounts of reagents makes optical immunosensors advantageous to use. It is important to wash the surface before reading of the signal.

3.2.1. Reflectometric interference spectroscopy (RIFS)

RIFS is one of the reflectometric techniques that has been applied to direct immunosensing. A white incident light passing the interface between different refractive indices will be reflected in part. These reflected beams superimpose and build a characteristic interference spectrum. The binding of Ab to the surface changes the thickness of the toggling layer, which causes a change in the reflectance spectrum (Fig. 3). Thus, the interaction process between the Ab and the hapten derivative on the surface can be detected time-resolved (Proll et al., 2004).

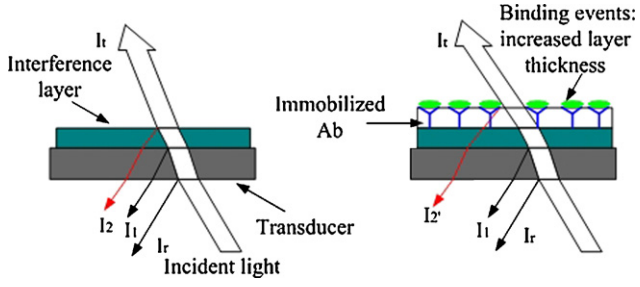

Fig. 3. Schematic of the RIFS detection system. The optical thickness of the transducer changes during binding events onto the surface.

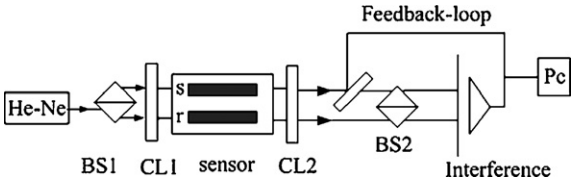

Fig. 4. Experimental set-up of interferometry. CLl, CL2 = cylindrical lens. BSl, BS2 = beam splitters (Lechuga et al., 1995).

Brecht et al. (1995) investigated a direct optical immunosensor based on RIFS for atrazine detection with surface immobilized antigen. They presented a comparison to other optical transducers and a critical discussion about the feasibility of this direct optical immunosensor in pesticide detection. The exiting RIFS transducer technology had not reached the European Union limit of 0.1 ng/mL or below it. However, several other optical transducers could reach the required limits of detection. Sample handing and physical constraints limited the device performance under practical conditions, such as the change in refractive index of the sample with temperature.

3.2.2. Interferometry

When an immunoreaction takes place on the waveguide surface, it produces a change in the refractive-index profile within the evanescent field volume; correspondingly, the effective refractive index of the waveguide system is changed. As shown in Fig. 4, in the Mach–Zehnder interferometry (MZI), an optical waveguide is split into two arms and after a certain distance they are recombined again. The sensor arm will be exposed to a variation of the refractive index due to an immunoreaction in the sensor channel. During this distance, light traveling in the sensing arm will experience a phase shift in comparison with guided light in the reference arm (Prieto et al., 2003).

Lechuga et al. (1995) discussed the feasibility of an evanescent wave interferometer immunosensor for pesticide detection. The interferometer immunosensor showed a high resolution (2×10^{-3} nm). An increase of both the receptor layer binding capacity and net response rate should result in the required increase in the sensitivity of the MZI, which made the direct detection of pesticides become feasible.

3.2.3. Optical waveguide lightmode spectroscopy (OWLS)

The OWLS technique is a new sensing technique using evanescent field for the in situ and label-free study of surface processes at molecular levels. It is based on the precise measurement of the resonance angle of linearly polarized laser light, diffracted by a grating and incoupled into a thin waveguide layer (Adányi et al., 2006). The incoupling is a resonance phenomenon that occurs at a defined angle of incidence that depends on the refractive index of the medium covering the surface of the waveguide. In the waveguide layer, light is guided by total internal reflection to the edges where it is detected by photodiodes. By varying the angle of incidence of the light, the mode spectrum is obtained from which the effective refractive indexes are calculated for both electrical and magnetic field waves (Luppa et al., 2001).

This technique can be applied for the detection of the herbicide trifluralin (Székács et al., 2003). Within the immobilized Ab based immunosensors, this method allowed the detection of trifluralin only above 100 ng/mL due to the small molecular size of Ag, while the immobilized Ag based OWLS system allowed the detection of trifluralin in the concentration range of 2×10^{-7} to 3×10^{-5} ng/mL. Trifluralin concentrations detected by the indirect OWLS sensor were correlated with those detected by ELISA and GC–MS methods.

3.2.4. Total internal reflection fluorescence (TIRF)

This technique is based on utilization of the evanescent wave of an electromagnetic field, which extends out from the interface of two different media into the medium with lower refractive index. This field is created when light from a specific angle is totally reflected due to differences in refractive index in these two different media. Molecules with fluorescence characteristics are excited in the evanescent field of the waveguide creating a fluorescent evanescent wave. This evanescent wave allows quantitative measurements of adsorbed molecules on the surface without influence of any moderate bulk concentration of the same analyte (Engström et al., 2006). The advantages of the format are its robustness, availability, versatility, low cost, and portability, which allow it to be used in the field, while its main drawback is the need for labels (González-Martínez et al., 2006). A TIRF immunosensor was shown to allow the detection of a multitude of analytes in one single test cycle (Klotz et al., 1998). Calibration curves obtained for 2,4-dichlorophenoxyacetic acid (2,4-D) and simazine had detection limits of 0.035 and 0.026 ng/mL, respectively. One limiting factor on the ability to perform more than one assay simultaneously on the same transducer was the availability of low cross-reactant Ab combined with high affinity between the antibody and the analyte. Prototype immunosensors for multi-analyte applications, such as the European RIver ANAlyzer (RIANA) based on a TIRF that takes place on an optical transducer chip, were developed in recent years and applied to monitor pesticides in water samples (Mallat et al., 2001b,c; Rodriguez-Mozaz et al., 2004a; Tschmelak et al., 2004). Detection limits of several analytes were at or below part per trillion level. Individual assays were completed within 15 min including total regeneration of the transducer.

3.2.5. Surface plasmon resonance (SPR)

SPR immunosensing involves immobilizing Ab (or Ag) by a coupling matrix to the thin gold surface deposited on the reflecting surface of a glass prism (Fig. 5). Interaction of Ag and Ab on the surface will elicit a change in the refractive index as variations in light intensity (Dutra and Kubot, 2007). The detection principle relies on detecting changes in the refractive index of the solution close to the surface of the sensor chip due to shifts in mass occurring after biomolecule binding. This is in turn directly related to the concentration of solute in the surface layer (Hock, 1997; Homola, 2003). SPR has an inherent advantage over other types of biosensors in its versatility and capability of monitoring binding interactions without the need for labeling of the biomolecules. It is versatile owing to its outstanding attributes of miniaturization, reliable portable instrumentation, and automation.

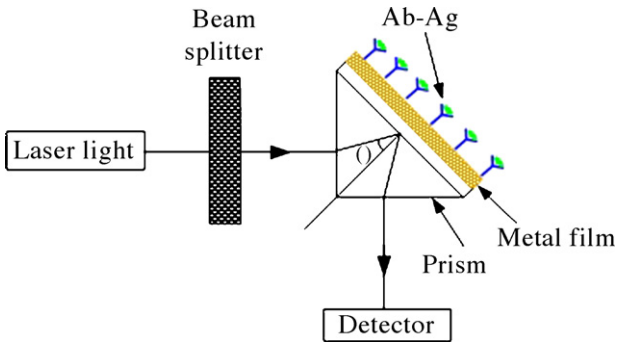

Fig. 5. Schematic of the SPR device.

Monitoring of the pesticide chlorpyrifos in water samples was performed using SPR immunosensors (Mauriz et al., 2006a). The chlorpyrifos derivative was immobilized onto the gold-coated sensing surface and competed with free chlorpyrifos for binding to the Ab, and as a result, increasing concentrations of chlorpyrifos will reduce the SPR signal. Other examples of single and multi-analyte assays for simultaneous detection of different pesticides by SPR were reported by the same research group (Mauriz et al., 2006b,c,d). This portable immunosensor based on SPR technology could provide a highly sensitive detection of pesticide analytes at nanogram per liter levels. The regeneration of the immunosurface was accomplished throughout more than 200 assay cycles without degradation of the covalently immobilized molecule. The stability of the immunosensor was proved by performing 15 series of daily measurements. Another sensitive and reusable SPR based immunosensor was developed for the determination of 2,4-D (Gobi et al., 2005). The SPR sensor was capable of detecting part per billion levels of 2,4-D in 20 min and the regeneration ability enabled the achievement of as many as 20 measurement cycles.

SPR immunosensors may suffer from disturbances caused by changes in the refractive index or temperature. The use of a reference surface makes it possible to separate signals related to binding events from signals caused by differences in refractive index between a sample and running buffer (Mullett et al., 2000).

3.2.6. Fluorescence/luminescence

Fluorescence occurs when a valence electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy (Lazcka et al., 2007). The common principle of luminescence immunosensors is that an indicator or chemical reagent placed inside or on an immmunoreactor is used as a mediator to produce an observable optical signal. Typically, conventional techniques, such as spectrometers, are employed to measure changes in the optical signal. This approach has been reviewed by Schobel et al. (2000).

A fluorometric immunosensor system was developed based on the principle of heterogeneous competitive enzyme assay using mouse monoclonal anticarbaryl Ab either in solution or immobilized for the determination of pesticides (González-Martínez et al., 1997). In the direct format, the limit of detection was 26 ng/L (11 min/assay), and the useful life of the sensor was 60–70 cycles. In the indirect format, the limit of detection was 284 ng/L (17 min/assay), and the useful life of the sensor was 160–200 cycles. Another portable miniaturized flow-injection immunosensor was designed for field analysis of environmental pollutants (Ciumasu et al., 2005). A chemiluminescence signal was inversely proportional to the concentration of target analyte in the given range of detection. The detection limit of the field prototype in the laboratory was below 0.2 ng/mL, and the temperature-controlled field prototype can run for 6 h without external power supply. However, the stability of immunoreagents needed to be improved. In an optical immunosensor, indium tin oxide (ITO) could make a good platform using fluorescent nanoparticle labels in a competitive assay format for small pesticide molecule detection (Cummins et al., 2006). However, when used in combination with fluorescent particulate labels, a highly sensitive excitation/detection system was needed. A disposable multiband optical plastic capillary fluoroimmunosensor using Ab labeled with fluorescein was exploited for the simultaneous determination of four different pesticides in the same sample (Mastichiadis et al., 2002). The detection limits of this immunosensor were below or equal to 0.1 ng/mL.

3.3. Piezoelectric immunosensors

Piezoelectric immunosensors are devices based on materials such as quartz crystals with Ag or Ab immobilized on their surface, which resonate on application of an external alternating electric field (Fig. 6). The resonant frequency has a proportional relation to the mass changes of the quartz crystal. The biospecific reaction between the two interactive molecules, one immobilized on the surface and the other free in solution or gas phase, can be followed in real time. The potential of quartz crystals microbalance (QCM) devices in chemical sensor applications was realized after Sauerbrey derived the frequency-to-mass relationship (Sauerbrey, 1959),

$$\Delta F = (-2.3 \times 10^6) f_0^2 \frac{\Delta M}{A} \tag{3}$$

where ΔF (Hz) is the change in the fundamental frequency of the coat crystal, f_0 is its resonant frequency, A (cm^2) is the area coated, and ΔM (g) is the mass deposited. This Sauerbrey equa-

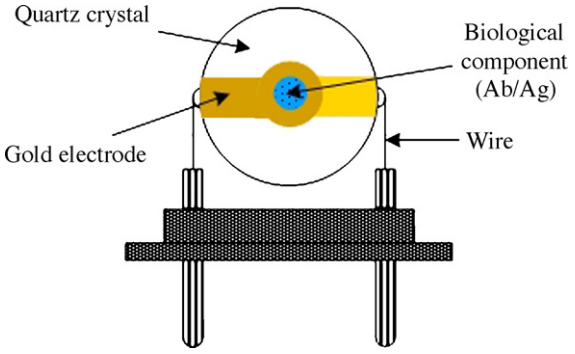

Fig. 6. Schematic of a piezoelectric immunocrystal.

tion, however, holds only for the case of rigid coated material. The frequency responses are also influenced by many factors, such as effective viscosity, conductivity, dielectric constant, electrode morphology, density and temperature of the liquid, and ionic status of a crystal/electrode interface to a water/buffer. Thus, when used practically, operating conditions should be evaluated (Yao and Zhou, 1988).

In many cases, piezoelectric immunosensors can be designed for detecting pesticides without the need of expensive or hazardous labels. They offer advantages such as real-time output, high sensitivity, simplicity of use, and cost-effectiveness. The main problem associated with these immunosensors is a change from a sample with low ionic strength to a sample with high ionic strength and vice versa. Moreover, there is a long time needed to reestablish a stable baseline due to changes in viscosity and other parameters close to the QCM surface.

Piezoelectric immunosensors have attracted research interest in recent years and served as alternatives to the conventional immunoassay tools. Improved direct piezoelectric immunosensors operating in liquid solution for the competitive label-free assay of 2,4-D were developed (Horáček and Skládal, 1997). For the Ab E2/G2 and E4/C2, the limits of detection for free 2,4-D were 0.27 and 0.24 ng/mL, respectively. Development of piezoelectric immunosensors for competitive and direct determination of atrazine (Přibyl et al., 2003) and 2,4-D (Halámek et al., 2001) were compared. In the competitive format, the mixture of object pesticide and specific Ab was preincubated in solution and passed through the flow cell with the piezoelectric crystal modified with hapten-pesticide. In the direct method, an immunosensor was constructed by oriented immobilization of Ab to activated sensing surface, followed by direct binding of analyte present in solution. The result showed that the detected concentration of a direct assay was low enough for future direct monitoring of pesticide, while the competitive assay provided much lower limit of detection. The Ag-immobilized strategy has the advantage that the regeneration process can be performed without loss of activity of the immobilized reagent. The main advantage of Ab immobilized option is the economy of expensive Ab and the reduction of assay steps. Since most pesticides are small molecules, a response resulting from binding of hapten to the immobilized Ab is typically much lower than in the opposite arrangement.

3.4. Nanomechanics immunosensors

Nanomechanics biosensors are based on the surface stress and consequently the bending of microfabricated silicon cantilevers, caused by the adsorption of molecules onto the sensor surface (Moulin et al., 2000; Raiteri et al., 2001). For the first time, it was applied for detection of dichlorodiphenyltrichloroethane (DDT) by performing a competitive assay (Alvarez et al., 2003). A synthetic hapten of the DDT conjugated with bovine serum albumin (BSA) was covalently immobilized on the gold-coated side of the cantilever by using thiol self-assembled monolayers and the cantilever was exposed to a mixed solution of the monoclonal Ab and DDT. It allowed pesticide detection below the nanomolar range without need of labels. Compared with other label-free biosensors such as SPR and QCM biosensors, the reaction area was tiny ($100\,\mu m^2$). Cantilever miniaturization combined with micro-nanofluidics could lead to femtomolar sensitivity with low reagent consumption. Kaur et al. (2004) used an atomic force microscope (AFM) to directly evaluate specific interactions between pesticides and Ab on a biosensor surface. Oriented immobilization of Ab against 2,4-D and atrazine on gold was carried out to create the active immunosensor surfaces. The study indicated that AFM could be utilized as a convenient immunosensing tool for confirming the presence and also assessing the strength of Ab–hapten interactions on biosensor surfaces. Future trends in nanomechanical biosensors will mainly address new fabrication methods to enhance cantilever response.

4. Immobilization protocols

The immobilization of Ab/Ag onto a transducer or a support matrice is a key step in optimizing the analytical performance of an immunosensor in terms of response, sensitivity, stability, and reusability. The immobilization strategies most generally employed are physical or chemical methods. In general, they mostly fall into following methodologies:

(1) *Physical adsorption*: Physical adsorption is generally based on interactions such as van der Waals forces and electrostatic interactions between the Ab/Ag and the transducer. It is especially common on hydrophobic polymer surfaces. The main advantages of this mode of immobilization are its rapidity and simplicity, while its main drawbacks are random orientation and weak attachment. An example can be found in the determination of herbicide 2,4-D. A functional sensing surface of the immunosensor is created by immobilizing an ovalbumin conjugate of 2,4-D by simple physical adsorption on an SPR thin-film gold chip (Gobi et al., 2007).
(2) *Covalent coupling*: Ab/Ag can be covalently linked to the surfaces of a transducer through formation of a stable covalent bond between functional groups of Ab and the transducer. The procedure provides increased stability of the Ab but decreases the activity of Ab/Ag and is generally poorly reproducible. Blocking steps are usually necessary to limit the non-specific binding. An example of where this approach has been exploited is illustrated by immobilization of 2,4-D on the surface of amino-silanized optical fibers (Wittmann et al., 1996).
(3) *Self-assembled monolayer* (*SAM*): A SAM may be generated by the spontaneous chemisorption of molecules onto a gold surface. It is built of long-chained *n*-alkylthiols with derivatized organic functional groups, which are easily linked to the gold film via the thiol groups (Wink et al., 1997). This technique was used to immobilize analyte derivatives onto the surface of gold-coated sensors. The immunosurface formed can be used for over 120 cycles (Farré et al., 2007).

In addition to these conventional methods, new materials, such as nanoparticles have been employed in immobilizing Ab when constructing immunosensors (Hu et al., 2003). Nanopar-

ticles are used as solid phase due to their large surface area in a small fluid volume and good biocompatibility. Biological interactions, such as biotin/streptavidin interactions can be used to easily immobilize the Ab on the surface of nanoparticales. One of the most advantageous features of this system is that although the affinity constant between avidin and biotin is rather high, the bonding is of non-covalent nature, which allows for multiple washing and reuse of the same sensing device (Lazcka et al., 2007).

5. New trends

5.1. Regeneration

For monitoring purposes, an immunosensor should be easy to regenerate after making a measurement. Two different strategies may be followed to achieve the renewal of the sensing surface: (1) breakage of the Ab–Ag bond and reusing the immunologic reagent immobilized in the solid phase; and (2) elimination of the Ag–Ab complex from the solid support and immobilization of fresh immunologic material (Santandreu et al., 1998). In the first strategy, a careful selection of the dissociating agent must be made for efficiently dissociating the Ag–Ab complex without affecting association bonds between the support matrix and Ab. On the development of an immunosensor for the organophosphorus pesticide ethyl parathion (EP) using EP Ab, different dissociating agents were used (Kandimalla et al., 2004). The results reported in this investigation indicated that glycine–HCl (pH 2.3) buffer containing 1% dimethyl sulphoxide is a highly efficient dissociation buffer. In the second alternative, complete removal of the proteic material from the surface is achieved when using several regeneration solutions with extreme pH values and/or high salt concentrations (Sardinha et al., 2002; Mauriz et al., 2007). In addition to these conventional methods, some elegant regeneration protocols were reported. For example, an assay using the reversible interaction between the lectin concanavalin A and a-D-glucose covalently bound on the chip surface was developed for the determination of the pesticide 2,4-D (Švitel et al., 2000).

5.2. Signal amplification

Signal amplification is crucial for obtaining low detection limits in biosensors. Most amplification schemes for immunosensors rely on target labeling. Thus, amplification techniques lay outside the domain of label-free immunosensors. To amplify the immunoreaction signal, many methods have been proposed. Generally, methods for amplifying the immunoreaction signal are based on the detection of electroactive species, which are generated in the presence of tracer coupled to the Ag or Ab. To monitor trichloropyridinol, a potential insecticide biomarker, the electrochemical signal of the enzymatic product was greatly enhanced by dual accumulation events: magnetic accumulation of enzyme tracers bound magnetic beads and constant potential accumulation of enzymatic product. Under the optimal conditions the detection limit was 5 ng/L (Liu et al., 2006).

On the other hand, precipitation of an insoluble product on the transducer was also applied to amplify the immunoreaction (Chen et al., 2005). Kurosawa et al. (2006) found that immunosensors were able to amplify their analytical sensitivity and extend the detection limits through the introduction of the anti-bisphenol-A (BPA) Ab conjugated polymeric nanoparticles as a new material in the second immunoreaction (the signal-enhancing step).

5.3. Miniaturization

Miniaturization is expected to have a marked impact in the development and applications of immunosensors. Miniaturization of an immunosensor not only reduces the size of detection device and sample volume, but also integrates all steps of the analytical process into a single-sensor device. Thus, it results in reduction of both the time and cost of analysis. Moreover, it is expected to lead to a further portability for in vivo sensing and in-field applications. The miniaturization trend involves adaptation of microfabrication and nanofabrication techniques, such as microelectro mechanical systems (MEMS). MEMS combined with microelectronic circuitry are sometimes referred to as “smart sensor systems”, which in turn can be configured into highly portable and inexpensive handheld instrumentation (Mcglennen, 2001).

Some challenges still remain to be overcome before miniaturized sensors can be commercially applied. For instance, miniaturization coupled with higher detection sensitivity places serious demands on the process and methodology inherent in Ab/Ag immobilization. In addition, the practical application of miniaturization requires the use of complete analysis systems for sample handling, such as pumping, filtering, valving, and sample conditioning (Rodriguez-Mozaz et al., 2004b).

To the best of our knowledge, immunosensors based on SPR are now the most well known immunosensors due to their miniaturization and portability. The combination of SPR immunosensor with flow-injection analysis (FIA) techniques offers great potential in future devices for pesticide monitoring.

5.4. Antibody evaluation

Ab may be polyclonal or monoclonal depending on the way they are prepared. Polyclonal Ab are produced by using traditional immunization procedures, namely in rabbits, goats, sheep and pigs. A certain drawback of the method lies in the fact that it is not possible to produce identical Ab specificity even in two animals of the same species (Franek and Hruska, 2005). They also suffer from potential lack of specificity and abundance. The monoclonal Ab, although less sensitive, are also less ‘cross-reactive’ as compared to the polyclonal Ab. In the course of selecting a monoclonal Ab, specificity is a prime consideration and usually chosen at the expense of sensitivity or binding affinity (Iqbal et al., 2000). The hybridoma technology guarantees the unlimited production of monoclonal Ab with constant characteristics (Hock et al., 1995).

Since new developments in protein engineering can contribute to the improvement of Ab, novel Ab in a more cost-effective manner, such as antibody fragments, can be represented. Antibody fragments represent the next generation of immunochemical reagents extending options of poly- and monoclonal Ab for application to clinical, environmental and food analysis. Production of scFv recombinant fragments against 2,4-D hapten using a naïve phage library were developed (Brichta et al., 2003). A key advantage of using scFv antibody fragments is that the recognition element can be inexpensively produced in a 'ready to use' form. In literature, another example of using antibody fragments can be found in the development of an electrochemical immunosensor for the analysis of atrazine associated to biotinylated-Fab fragment K47 antibody (Hleli et al., 2006).

6. Conclusions

Immunosensors based on Ab–Ag complexation are strong candidates for screening pesticide residues and they become more and more relevant in environmental and food analysis. Compared to chromatography and other methods, the strengths of immunosensors can be described as follows:

- They are very selective and sensitive.
- They can be carried out for use in the field.
- They can work with complete automation and give the results after a short period of time.

In particular, methods with electrochemical, optical, and piezoelectric transducers have the potential to achieve the low limits of detection imposed by legislation. Despite the promise of immunosensors, they do have certain limitations. For example, few immunosensors are commercially available at the present time and are yet to be established as research or routine tools, due to a lack of validated protocols for a wide range of sample matrices. On the other hand, several problems, such as biomolecule deactivation or leaking and high diffusion resistance of the substrate/biocomponent are also key factors in the development of immunosensors that can be successfully applied to pesticide detection.

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