



Immunosensors: technology and opportunities in laboratory medicine

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An immunosensor is a device comprising an antigen or antibody species coupled to a signal transducer, which detects the binding of the complementary species. An indirect immunosensor uses a separate labeled species that is detected after binding by, e.g., fluorescence or luminescence (i.e., a heterogeneous immunoassay). A direct device detects the binding by a change in potential difference, current, resistance, mass, heat, or optical properties (i.e., a homogeneous immunoassay). Although indirect sensors may encounter fewer problems due to nonspecific binding effects, the direct sensors are capable of real-time monitoring of the antigen-antibody reaction. A wide range of molecules can be detected with detection limits ranging between 10^{-9} and 10^{-13} mol/L. However, there are only a few successful commercial applications of direct immunosensors, these being of the optical type. This review describes the principles underlying the technologies, their merits, limitations, and applications.

INDEXING TERMS: immunoassays • electrochemical detection • ion-selective electrodes • ellipsometry • surface plasmon resonance • refractive index • optrode • fluorescence • chemiluminescence • reflectance spectrometry • difference interferometry

Antibodies (and antigens) have been used for many years for the specific detection of their complementary partners; however, only in the late 1950s and early 1960s were antibodies used for quantification of an antigen [1, 2]. Today, immunoassay is the predominant analytical technique for quantitative measurements, being used over a wide concentration range, in many different biological matrices, and in a range of delivery formats. The unique feature of immunoassay that provides the desired specificity is the complementary reaction (both in chemical binding and spatial orientation of reactive groups) between antigen and antibody.

Apart from the obvious unique contribution of an antibody

to the performance characteristics of an immunoassay, a key feature of many assays is the use of a solid phase to which is coupled either the antibody or antigen, depending on the style of assay. The solid phase facilitates the separation and washing steps required to differentiate bound and free fractions of the label. The other important feature of an immunoassay is the choice of label; the first assays used radioisotopes as labels and many assays still use this approach today. However, the advent of fluorescent [3, 4], luminescent [5], light-scattering [6, 7], and particularly enzyme labels [8, 9] has led to an explosion in the techniques available [10]. For many of the nonisotopic labels, the reagents have been designed such that binding of labeled antigen to antibody in some way modulates the "activity" of the label, resulting in a homogeneous immunoassay without the need for a separation step. The use of nonisotopic labels has also facilitated the extension of the analytical range of assays by virtue of lower detection limits, successful automation of heterogeneous assays, and the development of encapsulated disposable devices that require only addition of sample (and maybe a buffer) to initiate the reaction [11]. All these developments have led to an increase in the utility of assays, higher and faster throughput as a result of automation, and the possibility of immunoassays being performed close to the patient, i.e., at the bedside.

The advent of biosensor technology, with the possibility of direct monitoring of immuno reactions, provides opportunity to gain new insight into antigen-antibody reaction kinetics and to create rapid assay devices having wide applications.

Biosensor Technology

A biosensor has two major components: a biological detector or sensor molecule, and a signal transducer that provides an indication (or signal) that the ligand has bound to the receptor (sensor) molecule (Fig. 1). A variety of receptor molecules can be used, including enzyme, antibody, affinity ligand (e.g., lectin), receptor, peptide, oligonucleotide, organelle, organism, or tissue slice [12].

The transducer detects a change in one or more physicochemical properties as a ligand binds to its receptor; the interaction may result in a change of pH, electron transfer, refractive index (RI), heat transfer, or uptake or release of gases

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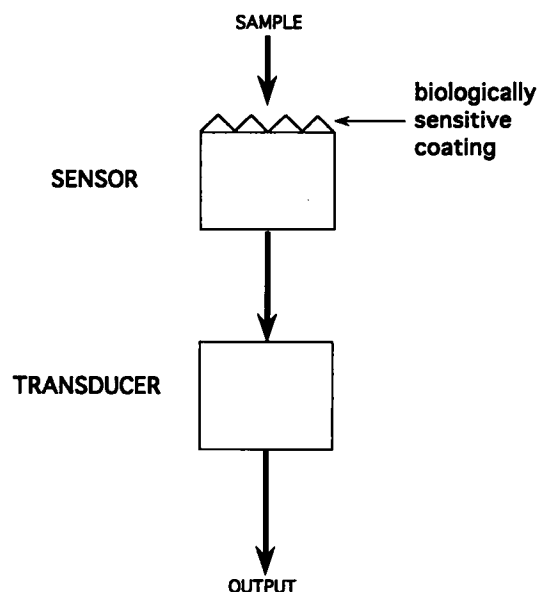


Fig. 1. Structure of a biosensor, illustrating its two main components: a sensor and a transducer.

The sensor is coated with a biologically sensitive material that interacts with a biomolecule present in a sample solution. Resulting changes in physicochemical variables, such as electron transfer, heat, or refractive index are detected by the transducer. The transducer converts these properties into a quantifiable and processable electrical signal, in response to changes in the concentration of the biomolecule.

or specific ions.¹ Amplification and processing of the signal change provides the opportunity for quantification of the ligand–receptor binding. There are many examples of sensors in routine use, including ion-selective and gas electrodes; however, fewer kinds of biosensors are in routine use, although a large number of approaches for doing so have been described. The most successful biosensor to date is probably that using an enzyme as the biological detector molecule with a range of transducers; several such biosensors have been described for measurement of glucose [13]. Fewer examples of immunosensors, both in principle or application, have been described, but the burgeoning literature in this field clearly marks it as a fertile area for development.

The term biosensor has been used here fairly literally and includes what could be described as direct and indirect sensors. For the purpose of this review, a *direct* biosensor is one in which there is direct detection of the antigen–antibody reaction in real time, with or without the use of a label; this is a true biosensor. An *indirect* biosensor is one in which a preliminary “biological” reaction takes place in a reaction tube and the products of that reaction are detected with a sensor; this is *not* a biosensor in our view and will not be considered in detail in this review.

Four main types of transducer have been used in biosensor technology; these exploit changes in electrochemical (potentio-

¹ Nonstandard abbreviations: RI, refractive index; ISE, ion-selective electrode; SPR, surface plasmon resonance; FET, field effect transistor; ISFET, ion-sensitive FET; hCG, human chorionic gonadotropin; FCFD, fluorescence capillary fill device; IRS, internal reflection spectroscopy; IO, integrated optics; TE, electrical field; and TM, magnetic field.

Table 1. Types of biosensors.

Transducer system	Measurement	Applications
Ion-selective electrodes (ISE)	Potentiometric	Ions (in biological media), enzyme electrodes, and immunosensors
Gas-sensing electrodes	Potentiometric	Gases, enzyme/organelle/cell/tissue electrodes for substrates and inhibitors, enzyme immunoelectrodes
Field-effect transistors (FET)	Potentiometric	Ions, gases, enzyme substrates, immunological analytes
Enzyme electrodes	Amperometric	Enzyme substrates, immunological systems
Conductimeter	Conductance	Enzyme substrates
Piezoelectric crystals, acoustic waves	Mass change	Volatile gases and vapors, immunosensors
Thermistors	Calorimetric	Enzyme/organelle/whole cell/tissue sensors for products/substrates/inhibitors, gases, pollutants, antibiotics, vitamins; immunosensors (TELISA)
Luminescence, fluorescence, SPR, waveguides	Optical	pH, enzyme substrates, immunological analytes

metric, amperometric, or conductimetric), mass (piezoelectric or acoustic wave), heat (calorimetric), or optical [luminescent, fluorescent, reflective, ellipsometric, surface plasmon resonance (SPR), and waveguide] properties (Table 1). The principles of these transduction systems will be described in broad terms and then as applied to the design of immunosensors.

Immunosensors

Biosensors concerned with monitoring solely antibody–antigen interactions can also be termed immunosensors [14]. Similarly to conventional immunoassays, these devices are based on the principles of solid-phase immunoassay, with either antibody or antigen immobilized at the sensor surface. An extensive range of analytes can be detected and measured by immunosensors, e.g., medical diagnostic markers such as hormones (steroids and pituitary hormones), drugs (therapeutic and abused), and bacteria, and environmental pollutants such as pesticides. However, the advantages of an absence of labeling requirements and the ability to investigate the reaction dynamics of antibody–antigen binding have given these devices the potential to revolutionize conventional immunoassay techniques. Of course, there are differences between conventional solid-phase immunoassays and immunosensors. In the former, reagents are generally used only once (albeit in small quantities), whereas immunosensors can facilitate the regeneration of the immobilized component; by thus using the reversibility of the antibody–antigen reaction, immunosensors can maximize reagent usage. However, a particularly high affinity constant and a labile immobilized ligand may make regeneration of the surface difficult.

The sensitivity and specificity of an immunosensor are

determined by the same characteristics as in other solid-phase immunoassays, namely, the affinity and specificity of the binding agent and the background noise of the detection system (transducer). In some cases, the use of labels has been incorporated into immunosensor design to enhance sensitivity, although these indirect-sensing devices can no longer be said to monitor the antibody-antigen reaction directly. A great deal of work in the development and design of immunosensors has been expended over the past decade, making use of different transducer systems as well as exploring the benefits of miniaturization and large-scale fabrication to ensure their commercial success/viability. The basic principles of immunosensors with regard to the different transducer systems will be discussed here.

Electrochemical Immunosensors

POTENTIOMETRIC TRANSDUCERS

Potentiometric transducers are based on the principle of the accumulation of a membrane potential as a result of the selective binding of ions to a sensing membrane (Fig. 2). The change in potential is measured, there being a logarithmic relationship between potential and concentration. The first description of the use of potentiometric transducers for monitoring an immunochemical reaction was published in 1975 [15]. The principle of this immunoassay was based on the measurement of the change in potential when either antibody or antigen bound to its specific partner, which was immobilized on the electrode. Later studies showed that the poor sensitivity of this system was due to nonspecific binding, and it had little success.

The development of ion-selective electrodes (ISE), in which ions present in the sample become selectively bound to the surface of the sensor and set up a charge separation between the sample and sensor surface, has proved successful (see Fig. 2). The potential generated is measured against a reference electrode maintained at zero current flow, and is proportional to the activity of ions present in the solution. Potentiometric transducers based on ISEs have been commercially successful for numerous applications [16] and have been used to measure a wide range of ions in solution. A gas-sensing electrode has also been

developed for the detection of gases such as carbon dioxide; this consists of a gas-permeable membrane and a hydrogen-sensitive pH electrode, separated by a layer of electrolyte solution. Dissolved gas diffuses through the membrane to the electrolyte, altering its pH. The pH electrode monitors these changes, and the gas concentration is thus determined.

The incorporation of ISEs, pH electrodes, or gas-sensing electrodes into potentiometric immunosensors to improve sensitivity has been extensively investigated by Rechnitz and co-workers. Examples include the use of carbon dioxide gas-sensing electrodes for immunochemical measurements of antibiotics [17, 18], human IgG [19], and digoxin [20]. In this approach a decarboxylating enzyme is immobilized at the tip of a gas-sensing probe, and the production of carbon dioxide measured is proportional to the concentration of analyte in the sample. ISE-based immunosensors have also been developed for prostaglandins [21], cortisol [22], and various other antibody-antigen measurements [23]. Antibody-sensitive immunosensors such as these measure changes in potential caused by the alteration in ionophoric properties when antibody selectively binds to immobilized antigen. The antigen is incorporated in a membrane at the electrode—either previously covalently bonded to an ionophore [24] or, if ionophoric itself, applied directly to the membrane [25]. Alternatively, immunosensors can be constructed for antigen monitoring by immobilizing monoclonal antibodies at the membrane [26]. Enzymes have also been applied to ISE-based potentiometric transducers; an IgG-detecting immunosensor described involves the “channeling” of two or more enzymes [27].

The incorporation of solid-state electronics, in the form of a field effect transistor (FET), has become increasingly popular in potentiometric sensing [28]. The FET monitors any charge build-up on the surface of the electrode and converts it into a measurable response without draining either the current or the electrode. The main advantage of FET devices is their small size; FET-based biosensors require only a very small amount of analyte, making them ideally suited for medical applications. Integrating the ion-sensitive membrane of the ISE with the FET results in the more advanced ion-sensitive FET (ISFET) system, first reported in 1970 [29] for use as a hydrogen ion-sensitive pH sensor. The device reduces background interference of the signal, a problem often encountered by conventional ISEs, and the absence of large cables makes this small instrument particularly useful for in vivo or near-patient in vitro monitoring of electrolytes. The ISFET has since been developed for a wider range of applications [30], including penicillin determination [31]. ISFET technology has also been adapted for immunosensing; one novel example detects the changes in charge densities and isoelectric points that take place upon formation of an antibody-antigen complex [32] with a detection limit in the range $1-10 \times 10^{-8}$ mol/L.

A different approach to the construction of potentiometric immunosensors, in which an electro-catalytically active biocatalyst is used as a label, has been more recently described [33]. In this indirect detection technique, insulin is immobilized at the electrode, to which monoclonal anti-insulin antibodies labeled with the enzyme lactase may bind. Lactase catalyzes the elec-

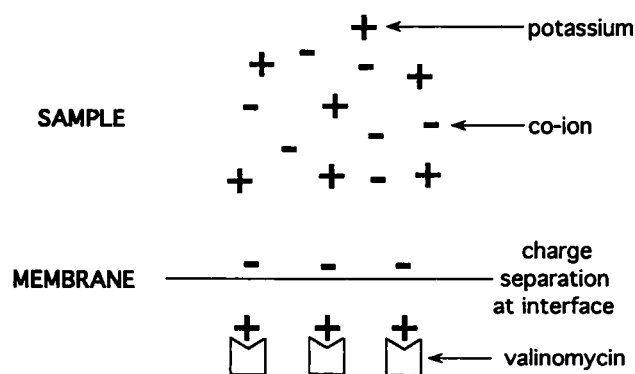


Fig. 2. Principle of potentiometric sensing, illustrated with the potassium ISE, in which the antibiotic valinomycin selectively binds potassium ions within the membrane, leaving behind the co-ions in the sample; the resulting charge separation, which is established across the sample/membrane interface, generates a potential that is related to the concentration of potassium ions in the sample.

troreduction of oxygen, causing a rapid increase in the electrode potential that is proportional to the concentration of free antigen (insulin) in solution. Although this immunosensor does not claim to have high sensitivity for antigen concentration determinations, its speed of operation and large changes in electrode potential facilitate kinetic analysis of the reaction to be performed.

Despite the developments described, however, potentiometric immunosensing has experienced several problems. The signal-to-noise ratio is low because the charge density on most biomolecules is low compared with background interferences (e.g., ions), and there is a marked dependence of signal response on such conditions as pH and ionic strength [14]. One significant problem associated with ion-selective potentiometric transducers is that the measured potential is related only to the activity of the ion; although for concentrations up to $\sim 10^{-4}$ mol/L, activity parallels concentration, above this value the activity gradually declines, which can lead to ambiguous results for highly concentrated samples. Furthermore, ISEs are vulnerable to interferences from other ions, which also reduces the specificity of the sensor.

Although FET-based devices offer improvements to potentiometric monitoring, with distinct advantages such as their small size and their ability to be adapted for multianalyte sensing, several problems associated with these devices have hampered their development as well. These include poor reliability (i.e., degradation and contamination caused by adsorption of unknown species to the device), operating limitations (light sensitivity of the materials used in their construction, and poor reproducibility and selectivity), and problems associated with fabrication of the device (e.g., labor-intensive production, which makes them more expensive than electrochemical sensors). Thus, although potentiometric sensing is applicable to the detection of immunochemical reactions, it is not always the most sensitive and specific technique, given these problems.

AMPEROMETRIC TRANSDUCERS

Amperometric transducers constitute a highly developed area in biosensing, and several are currently commercially available [34]. These devices measure current flow through an electrochemical cell held at a constant voltage. The current generated by the redox reaction of the analyte at the sensing electrode is directly proportional to the analyte concentration at the electrode surface [35]. Most often these sensors contain oxygen or hydrogen peroxide electrodes, and the current produced is directly proportional to the amount of oxygen or hydrogen peroxide reduced or oxidized at the electrode. The system has the advantages of high sensitivity and linear concentration dependence (compared with a logarithmic relationship in potentiometric systems), and high selectivity (specificity) can be obtained by altering the electrode potential independently of the sample buffer capacity. This makes the system well suited for immunochemical sensing. However, many molecules (e.g., proteins) are not intrinsically electroactive and cannot be directly detected this way. Therefore, enzymes are incorporated as labels to catalyze redox reactions that facilitate the production of

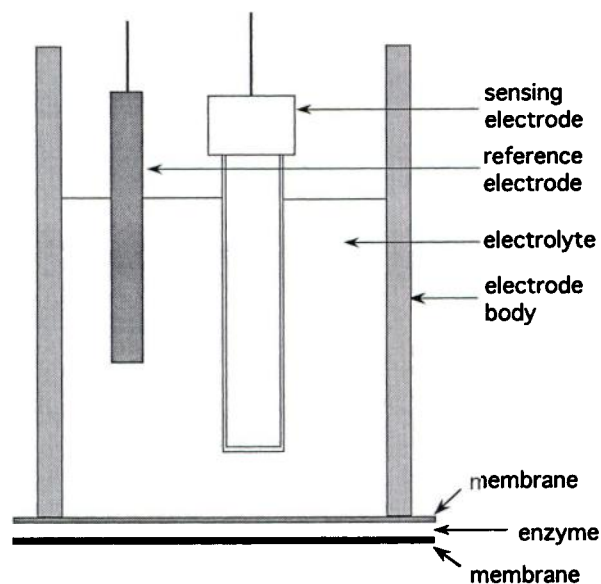


Fig. 3. The Clark and Lyons enzyme electrode [36].

In this first example of an enzyme sensor, a suitable enzyme was immobilized close to the surface of an electrode. The enzymatic reaction leading to consumption or production of a specific ion (for example, O_2/O_2^-) was monitored as a function of analyte concentration.

electroactive species, which then can be determined electrochemically.

The first generation of amperometric devices included the Clark and Lyons electrode (Fig. 3) [36], developed for glucose determination. By immobilizing the enzyme glucose oxidase at the surface of an electrical detector, the first enzyme electrode was developed. This system measures either the decrease in oxygen or the production of hydrogen peroxide that results from the conversion of glucose to gluconic acid.

Second-generation enzyme electrodes were designed by replacing oxygen with an electron transfer mediator such as ferrocene derivatives (for glucose-measuring systems) [37], benzoquinone, polyviologen, chloranil, and methylene blue. The mediator is a low-molecular-mass redox couple that reacts with and regenerates the enzyme. This eliminates problems caused by oxygen- or pH-dependency, and the electrode can be used at a lower potential. In a further modification, the mediator can be immobilized at the electrode surface along with the appropriate enzyme so that the device needs no membrane. In general, because the analyte is consumed during the redox reaction, all amperometric transducers depend on the rate of transport of analyte to the electrode surface, a limitation that can be difficult to overcome in their design.

Amperometric sensing of immunochemical reactions was first reported in 1979 with the construction of an ELISA for determining human chorionic gonadotropin (hCG) [38]. Monoclonal antibody to hCG was immobilized on the membrane of the oxygen electrode, to which hCG in the sample and catalase-labeled hCG could compete for binding. The detection limit of this assay was 20 IU/L. The use of catalase labels in amperometric immunoassays was also reported for theophylline determination [39]. Alkaline phosphatase has also been used in

amperometric assays to label antibody for the determination of Factor VIII-related antigen [40], α_1 -acid glycoprotein [41], and other serum antigens [42]; a typical detection limit for α_1 -acid glycoprotein was $1 \mu\text{g/L}$, and measurement ranges of 31 – $1000 \mu\text{g/L}$ for thyroxine-binding globulin and 1 – $20 \times 10^{-7} \text{ mol/L}$ for cortisol were obtained in serum samples. The development of amperometric immunosensors utilizing different labeling systems has recently been documented; examples include a disposable sensor for detection of herbicides [43] and an immunosensor for detecting apolipoprotein E in serum [44]. A multianalyte amperometric immunosensor has been developed for measuring the human gonadotropin hormones follicle-stimulating hormone and luteinizing hormone, achieving detection limits of 1.8 and 2.1 IU/L , respectively; the device uses two horseradish peroxidase-labeled antibodies in a ferrocene-mediated system [45]. Novel electrochemically active compounds such as *o*-aminophenol and its derivatives and polyaniline have also been used as labels in amperometric immunosensors for detecting immunoglobulins [46]. As described here, the amperometric immunosensors designed so far rely mostly on labeling, and few label-free (direct) amperometric immunosensors have been reported.

Amperometric immunosensors have shown more promising results at the early stages of design than other types of electrochemical system. The microfabrication of label-free amperometric sensors has an obvious advantage for marketability, and microamperometric devices are considered likely to be more successful than the potentiometric FET-based sensors because of improved sensitivity.

CONDUCTIMETRIC TRANSDUCERS

Conductimetric measurements are widely applicable to chemical systems, given that many chemical reactions produce or consume ionic species and therefore alter the overall electrical conductivity of a solution. Biosensors can be designed by immobilizing a suitable enzyme over a set of electrodes made of noble metal (e.g., gold, silver, copper, nickel, or chromium) and measuring the change in conductance of a solution containing the analyte of interest when an electric field is applied. For example, when urea is converted to its ionic product NH_4^+ by the enzyme urease, the increase in solution conductance measured is proportional to urea concentration [47]. With use of the appropriate enzymes, the concentrations of acetaminophen (paracetamol), creatinine, L-asparagine, penicillin G, and glucose, among others, can be determined, sometimes with multi-analyte detecting devices [48]. In another example, a conductive polymer-based immunosensor, composed of an ELISA with an electroconductive solid support, has been developed for pesticide detection [49]. Variations in ionic strength and buffer capacity of measured samples have caused problems with this type of biosensor in the past, but these drawbacks have been overcome with more recent designs [50]. Conductimetric sensors may also have problems with nonspecificity of measurements, because the resistance of a solution is determined by the migration of all ions present. To date, the development of immunosensors based on conductance has hardly been ex-

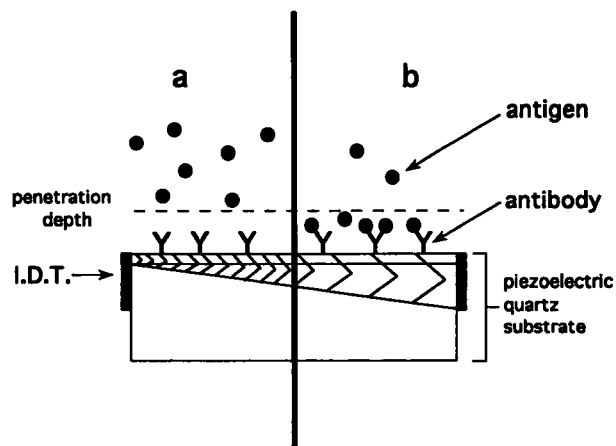


Fig. 4. Surface acoustic wave device.

An acoustic wave is generated (shown here in one direction only) between two interdigital transducers (IDT), along the surface of the quartz substrate. Mass deposition at the surface (e.g., antigen binding to immobilized antibody) alters the frequency of the wave as it passes from a to b.

ploited, and it will be interesting to see if this technology progresses in the future.

Mass-Detecting Immunosensors

An alternative property used in immunosensor design is mass change, which is measured with the use of piezoelectric crystals and acoustic wave techniques [51, 52]. The first generation of these devices were expensive to produce, lacked sensitivity, and had a high failure rate in manufacture. However, they have since improved dramatically and are now viable commercial products.

PIEZOELECTRIC CRYSTALS

The phenomenon of piezoelectricity was discovered in 1880 [53]. Electric dipoles generated in anisotropic natural crystals (with no center of symmetry) were subjected to mechanical stress, thus causing them to oscillate at frequencies between 9 and 14 MHz . There are ~ 20 naturally abundant piezoelectric crystals, including quartz (SiO_2), lithium niobate (LiNbO_3), zinc oxide (ZnO), gallium arsenide (GaAs), cadmium sulfide (CdS), lithium tantalate (LiTaO_3), tourmaline, and Rochelle salt [54]; some manmade ceramics and polymers also have piezoelectric properties. Quartz is the most commonly used piezoelectric material because of its chemical stability in aqueous solutions and resistance to high temperatures without loss of piezoelectric properties. In piezoelectric biosensors, the crystals are coated with an adsorbent that selectively interacts with the analyte of interest; subsequent binding increases the mass of the coated crystal and alters its basic frequency of oscillation (Fig. 4 [55]). Monitoring the oscillation frequency allows determination of the change in mass, which is proportional to analyte concentration. This technique has had much success in the analysis of gaseous environmental pollutants [56–58]. Piezoelectric crystal sensors have also been investigated for monitoring antibody–antigen interactions; in this procedure, the crystals are coated with antibody and the amount of antigen bound is determined, or vice versa. One example demonstrated a detection limit for IgG of 43 mg/L [59]. Other published examples include the detection of herbicides in drinking water [60, 61],

stimulating drugs in human urine [62], bovine hemoglobin [63], viruses and bacteria [64], and IgM antibodies [65]. Piezoelectric immunosensors have also been used to detect a range of human cell types such as erythrocytes [66], granulocytes [67], and T-lymphocytes [68] and viruses such as human herpes [69] and hepatitis [70]. The measurement of HIV-specific antibodies in serum has also been reported, involving piezoelectric quartz crystals coated with peptides to HIV [71, 72], although this was complicated by non-specific binding of serum proteins in the sample. In fact, use of piezoelectric immunosensors is greatly affected by nonspecific binding to the adsorbed substrate on the crystal.

ACOUSTIC WAVES

Acoustic wave technology, e.g., surface acoustic wave measurements, has also attracted interest in biosensor design. In this method, the oscillation of the piezoelectric crystals is at a higher frequency (30–200 MHz), and an acoustic wave is generated by application of an alternating voltage across a pattern of interlaced metal electrodes (e.g., titanium or gold), known as an interdigital transducer. The acoustic signal produced is detected by a second interdigital transducer situated a few millimeters away. The adsorption of sample to the crystals slows the acoustic wave, and the recorded change in velocity is proportional to the analyte concentration. Besides mass, however, factors such as temperature, pressure, and surface conductivity may also alter the properties of the acoustic wave; this has inhibited the successful design of such devices. Acoustic wave devices have been used in specific areas such as environmental gas monitoring [73] and chemical detection [74, 75]; more recently, their incorporation in immunosensor design has been described, including rapid assays of antigen present in foodstuffs and human IgG measurements [76]. Given the similarities between surface acoustic waves and piezoelectricity, the problem of nonspecificity may also plague the development of acoustic wave immunosensors.

Heat-Detecting Immunosensors

The calorimetric biosensor, first reported in 1974 [77], was designed by attaching the biological component to a heat-sensing transducer, the thermistor; alternatively, the biological component was immobilized on a column in which the thermistor was embedded [78]. These biosensors have a large number of applications, given that most enzyme-catalyzed reactions are accompanied by heat production of 25–100 kJ/mol [79]. A wide range of clinical analytes can be measured this way, e.g., cholesterol, glucose, ATP, urea, triglycerides, and ascorbic acid [80, 81]. However, one of the most promising fields for calorimetric biosensor development is that of biotechnology; in specific areas such as fermentation and environmental analysis, lactose, ethanol, cellobiose, penicillin, and sucrose have been measured [82]. The construction of microcalorimetric biosensors composed of miniaturized thin-film thermistors [83], and the incorporation of immobilized antibodies for determination of antigens, indicate the possibility for producing devices of reasonable size and simplicity. The latter technique, known as thermometric ELISA, has been successfully used in immunological analysis for determining concentrations of human pro-

Table 2. Optical Immunosensors.

Indirect		
Luminescence	Bioluminescence	
	Chemiluminescence	
Direct		
Fluorescence		
reflectance		
Ellipsometry		
SPR	BIAcore	
Waveguides	Fiber-optic waveguides	
	Interferometers	Mach-Zehnder
		Difference
	Grating couplers	Input
	IASys	Output

insulin (measured in media from genetically engineered *Escherichia coli* with a detection limit of 0.1 mg/L and a reaction time of 7 min) [84], albumin, and gentamicin [85].

Optical Immunosensors

The measurement of electromagnetic radiation absorbed or emitted by either reactants or products of a biological system has been extremely popular in immunosensor design, this being the largest and perhaps most promising group of transducers [86]. Optical transducers can be designed to respond to ultraviolet or visible radiation or to the production of bio- and chemiluminescence and can be adapted for fiber-optic-containing devices (Table 2). Early optical systems were based on spectrophotometry, enzymes immobilized on a column, and the absorbance of the product measured [87]. Later, the enzymes were immobilized on nylon coils and the system was linked to flow-injection or bubble analyzers [88]. In an effort to reduce the size of the device, fiber-optic technology was then introduced; the reagent phase was immobilized on a single optical fiber or a fiber bundle [89], so that changes in the optical properties of the reagent phase attributable to the analyte could be monitored. These devices, now known as optrodes [90], have been widely used and offer several advantages over electrodes, including the lack of a requirement for a reference electrode [91]. Furthermore, fiber-optics have increased versatility, being suitable for clinical applications, in vivo monitoring, and measurement of hazardous materials.

Optical transducers may be used for sensing with or without the use of a label; thus, some sensors make use of labeled reagents, e.g., an enzyme or fluorophore, to provide the detected signal. These require sophisticated instrumentation because of the low light levels detected. Direct optical sensors, which do not involve labeling, constitute a substantial proportion of the immunosensors currently available. These include attenuated total internal reflection [92, 93], ellipsometry [94], SPR [95], and monomode dielectric waveguides [96]. In some cases labels have been incorporated into these immunosensors to increase sensitivity [97, 98].

OPTICAL SENSING WITH A SIGNAL-GENERATING LABEL

An enzyme can be used as a label to generate a range of products that absorb light, fluoresce, or luminesce—the last offering partic-

ularly high sensitivities. Biosensors have been designed to monitor luminescence spectroscopy, either bioluminescence (light emitted from a biological reaction) or chemiluminescence (light emitted from a chemical reaction). Unlike other optical biosensors, no light source is required for these systems, which greatly simplifies instrument design. Bioluminescence-based systems such as that of the luciferin–luciferase reaction, in which luciferin is oxidized by firefly luciferase with the production of light, have been used in assays for oxygen, ATP, and NADH/NAD(P)H in dehydrogenase systems and in environmental monitoring [99]—all demonstrating a high sensitivity. Bioluminescent systems utilizing luminous bacteria have also been reported [100]. Fiber-optic bioluminescence sensors have been developed by immobilizing luciferase onto an optical fiber to detect ATP and NAD(P)H [101, 102]. Chemiluminescence systems include the luminol–peroxide system, which has been applied to many oxidase reactions involving hydrogen peroxide production [103]. Chemiluminescence adapted for fiber-optic immunosensors has facilitated the determination of various antigens, e.g., estradiol, α_2 -interferon, hCG, total IgG, and antibodies to influenza virus [104, 105]. Problems associated with luminescence sensors include reagent replenishment: For irreversible oxidation of substrate to take place, the luminescent compound must be available in excess. This can be arranged by immobilizing the enzyme onto an optical fiber, and loosely embedding coreactants for their slow release. As described above, fiber-optic sensors do not have to contain large volumes to sustain an adequate operation time, so their size can be minimized.

DIRECT OPTICAL SENSING

The development of optical techniques for direct monitoring of minuscule changes in adsorption, fluorescence, light scatter, or RI at a sensor surface is an extremely promising area in immunosensor technology. Optical transducers based on reflectance, ellipsometry, SPR, and waveguides have all been described for this purpose [106]. In these systems, light entering the device is directed towards the sensing surface and then reflected back out again. The light emerging from the device is then monitored, revealing information regarding the physical events occurring at the sensing surface.

The principles behind direct optical sensing lie in internal reflectance spectroscopy, which was reported in the late 1960s and early 1970s [107, 108]. This device consists of two materials of differing RI: a layer of high RI, often consisting of a glass prism, and a layer of lower RI that contains the sample (Fig. 5). A light beam is directed through the layer of higher RI to the interface between the two media. When the angle of the incident beam exceeds the critical angle, the light is totally internally reflected back out of the device. In the material of lower RI, a high-frequency electromagnetic field is generated, known as the evanescent wave [93]. This wave, a fraction of the wavelength of the incident light, advances along the x -axis in the plane of incidence, and penetrates into the sample medium (i.e., along the y -axis) for a short distance (approximately one wavelength) with exponentially decreasing amplitude. Biomolecules present in the sample that have been adsorbed at, or are in close contact with, the interface interact with the evanescent wave and cause a reduction in intensity of the reflected light beam. This

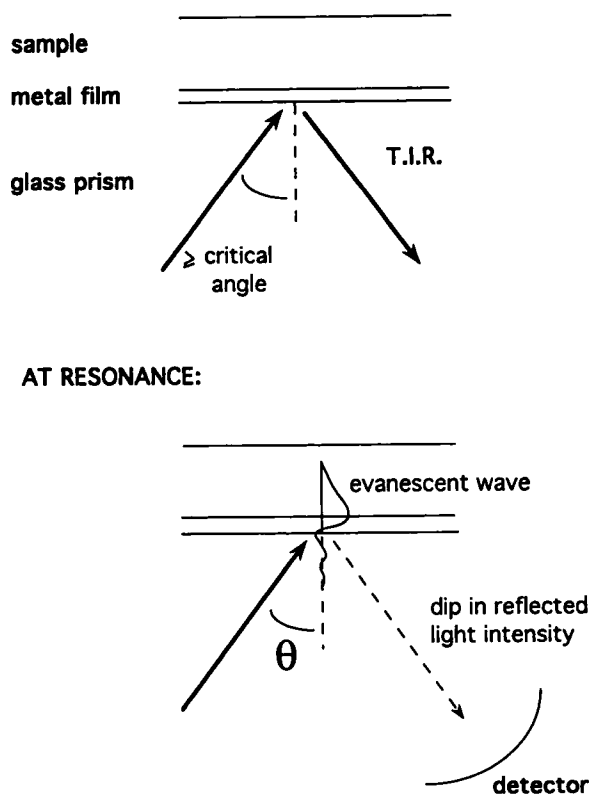


Fig. 5. Principles of SPR technology: Light is directed towards a layer of low RI from one of higher RI; at an angle equal to or greater than the critical angle, the light undergoes total internal reflection (TIR).

At the resonance angle (θ), surface plasmons in the metal film are excited by the light energy, causing them to oscillate and generate an evanescent wave. This condition of SPR results in a characteristic decrease in reflected light intensity.

distinctive decrease in light intensity therefore reflects any changes in RI occurring at the interface and is directly related to the mass (and concentration) of adsorbed biomolecules in the sample. As an alternative to light intensity, the optical interaction can also be monitored as a change in the phase of polarized light emerging from the sensing layer. This property has been successfully applied in several waveguide sensors.

Direct optical sensing has an advantage over indirect techniques, in that less-sophisticated instrumentation is required for detection of light, but problems have been encountered with nonspecific binding and poor sensitivity to small molecules. In some cases, therefore, the best features of both techniques have been combined to improve sensitivity, e.g., the use of SPR with fluorescent labels [109] and latex particles [110].

Fluorescence. The phenomenon of monitoring reactions occurring at the interface between the two layers of different RI has been adapted for the analysis of fluorescence. One example is the technique known as total internal reflection fluorescence, used to assess the fluorescence characteristics of a compound of interest [111]. In total internal reflection fluorescence, the incident light excites molecules near the sensor surface, which, in turn, creates a fluorescent evanescent wave. This couples back (i.e., reenters) into the waveguide and the emerging fluorescence is detected. Immu-

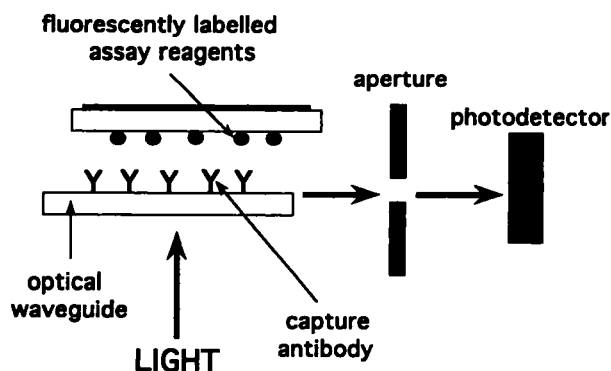


Fig. 6. The fluorescent capillary fill device (FCFD), in which the sample antigen competes with fluorescence-labeled antigen for binding to the immobilized capture antibody.

Light directed into the waveguide generates an evanescent wave at the sensing surface and causes direct excitation of the fluorophore. On its exit from the device, light passes through an aperture so that only the light arising from the bound fluorophore is detected.

nosensors that monitor fluorescence have been developed to detect light emitted from a fluorescence-labeled antibody bound to the antigen of interest at the surface of the device; in one demonstration, detection limits of 1.5 and 3.0 mg/L for IgG were achieved with fiber-optic and slide waveguides, respectively, and 2.7×10^{-7} mol/L for methotrexate with use of a slide waveguide [92]. More recently, a flow immunosensor has been designed to detect cocaine in aqueous samples; relying on the displacement of fluorescence-labeled antigen from immobilized antibody, this device produces results in <1 min with negligible cross-reactivity with other drugs [113].

Another example of a fluorescence-detecting immunosensor is the fluorescence capillary fill device (FCFD), a planar evanescent system (Fig. 6) [114]. In the FCFD, two parallel glass plates are held at 0.1 mm apart. All the reagents needed for the immunoassay are contained within the device—either covalently coupled to the lower plate, which functions as an optical waveguide, or dosed onto the upper plate in such a way as to dissolve in the presence of sample. Because samples enter the FCFD by capillarity, sample volume is controlled by the dimensions of the device. The light source is focused onto the lower waveguiding plate. Inside the FCFD, sample antigen competes with fluorescence-labeled antigen for binding to the immobilized capture antibody. Light directed into the waveguide generates an evanescent wave at the sensing surface, and the fluorophore becomes excited. On leaving the surface of the waveguide, light passes through an aperture and onto a photodetector, which monitors the intensity of light emitted by the fluorescence-labeled antigen. The aperture functions to reject the solution signal from the unbound fluorophore, which emerges at a different angle, and thus allows only the signal of the bound fluorophore into the path of the photodetector. The narrow gap separating the two plates of the FCFD also allows for fast assay times by requiring the reagents to diffuse over only a short distance. Thus, rates of reaction of assays performed by this device are limited by kinetics of antibody–antigen binding, not kinetics of diffusion. As an example, an assay for estrone-3-

Table 3. Examples of assays developed with an immunosensor for analytes in biological matrices.

Device	Analyte	Matrix	Detection limit, mol/L	Ref.
FCFD	E3G	Serum	1.1×10^{-9}	114
	hCG	Serum	4.5×10^{-11}	116
	PSA	Whole blood	3.3×10^{-12}	117
Difference interferometer	HBsA	Serum	2.0×10^{-13}	167
Fluoro-immunosensor	β_2 -M	Serum	1.0×10^{-10}	180
BIAcore	Theophylline	Serum	2.5×10^{-5}	138
	β_2 -M	Serum	1.6×10^{-9}	138
	IgE	Serum	4.0×10^{-10}	138

E3G, estrone-3-glucuronide; PSA, prostate-specific antigen; HBsA, hepatitis B surface antigen; β_2 -M, β_2 -microglobulin.

glucuronide in urine reached equilibrium 5 min after sample addition [114]. Encouraging results have been attained with the FCFD, including measurements of serum rubella antibody [115], hCG [116], and whole-blood prostate-specific antigen [117], demonstrating sensitivities of 4.5×10^{-11} and 3.3×10^{-12} mol/L, respectively, for the latter two antigens (Table 3).

The advantages of simple operation, with no need to measure the sample volume applied (this being controlled by capillarity), low-cost instrumentation, and a reagent shelf-life of 6 months even at 45 °C, ensure that the FCFD has much potential as a commercial immunosensor [106].

Reflectance. Attenuated total reflection is a form of internal reflection spectroscopy (IRS) in which light energy absorbed from the evanescent wave is monitored as an attenuation of the internally reflected light beam [107]. An optically absorbing film is present on the surface of an IRS sensor, resulting in a structure known as the Kretschmann configuration [118]. Incident light directed towards the upper surface is absorbed by this film, and the attenuated light intensity can be measured as a function of the incident wavelength. This technique has been applied to the monitoring of biological interactions in the infrared, visible, and ultraviolet regions of the spectrum. Attenuated total reflection has been applied to the study of antibody–antigen interactions [119] and a reflectance method has been reported for direct detection of immunological reactions at high-RI surfaces [120], but there have been few reports of the use of this technique in immunosensor design so far.

An example of a direct optical sensing technique exploiting the principles of optical diffraction is shown by the example of a commercial immunosensor (OBA™) [121]. Here, light is directed towards a silicon surface to which either antigen or antibody is immobilized. Binding of ligand to its immobilized partner creates a “grating” on this surface, and the light becomes diffracted from the surface. This technique has been demonstrated for a quantitative assay of hCG in serum at a working concentration range of 0–2500 IU/L.

Ellipsometry. In ellipsometry, the change in the state of polarization of light caused by reflection from a planar layered structure is monitored [94, 122, 123]. This method generated little interest for many years after the first observations, and miniaturization and low-cost fabrication were thought to be unfeasible for this technology [14]. However, ellipsometry has become more popular, particularly for studying the binding of proteins to surfaces [124]. Once reflected from a mirrored surface, the polarized light is interrogated so as to produce two readings; these are subsequently converted into changes in amplitude and in phase of light, respectively [125]. These properties are altered when a molecule is adsorbed to the surface, and the new readings can be interpreted as changes in RI and coating thickness, from which the concentration of bound analyte can be calculated. An example is the Isoscope™ ellipsometer (Sagax Instruments), which has been used to study receptor–ligand reactions [126]. As an immunosensor, the ellipsometer has been used successfully to determine gamma-interferon and human serum albumin [127] and to investigate immunological activity of immobilized immunoglobulins at solid surfaces [128].

Surface plasmon resonance. The phenomenon of SPR was first observed in 1902 [129] and is by now well documented [130, 131]. The optical arrangement for SPR is the Kretschmann configuration [118]; it differs from that of the previously described IRS set-up by the incorporation of an additional layer of a thin metal film between the prism and sample (see Fig. 5) [132]. The metal film characteristic of SPR usually consists of gold or silver. Specific ligands can be immobilized at the upper surface of the device (the sensor surface), to interact directly with biomolecules in the sample. SPR technology is based on the excitation of surface plasmons present within the metal film of the sensor. Surface plasmons are electrons at the surface of a metal (such as gold or silver) that behave differently from those in the bulk of the metal. This phenomenon is rather like surface tension, in which the bonding between molecules at the surface of a liquid is different from that of molecules in the bulk solution. Of the two types of plasmons—radiative and nonradiative—the latter is more commonly used for biosensor applications. Nonradiative plasmons are excited by light directed towards the metal film via a glass prism. When excited, the surface plasmons, which oscillate at a different frequency from that in the bulk of the metal film, absorb some of the light energy to generate an evanescent wave. This wave penetrates into the sample layer with exponentially decreasing amplitude, the condition known as SPR.

In the SPR system, polarized light is directed in one plane into the prism, which is of higher RI than the metal layer. When the angle of reflection of light is greater than or equal to the critical angle, light is totally internally reflected back out of the prism. At the resonance angle (θ), SPR is initiated; the absorption of light energy by the surface plasmons during resonance causes a sharp decrease in the intensity of the reflected light, which is monitored throughout the biomolecular interaction.

The evanescent wave does not propagate parallel to the prism interface in SPR, but is redirected upwards into the metal layer. This allows it to sense the metal–sample interface (the

sensor surface). The resonance angle is determined by the wavelength and polarization state of the incident light, as well as by the refractive indices of the prism, metal, and sample layers of the system. When all other factors are kept constant, the resonance angle measures the RI at the surface of the SPR system. Thus, changes in RI that take place when biomolecules become attached to their specific ligands at the sensor surface alter the angle at which the drop in light intensity occurs. Continuous monitoring of the reflected light intensity and the resonance angle therefore provides a direct profile of RI changes at the sensor surface and achieves real-time analysis of the binding events involved in the reaction.

Reports of the application of SPR to biosensing [133, 134] were followed by demonstrations that a wide range of molecules could be analyzed in this manner, from small peptides to larger analytes such as virus particles [135] and antibodies [136]. SPR has been commercially developed as a biosensor known as the BIAcore™ (Biosensor, Uppsala, Sweden), which is capable of label-free real-time analysis of biological interactions [137]; its use of integrated software to facilitate kinetic evaluation of binding reactions has made this technique useful in many areas [138, 139], e.g., biomolecular engineering, drug design, and monoclonal antibody characterization (i.e., epitope mapping and kinetic analyses of antibody binding) [140–143]. SPR immunosensors for sex-hormone-binding globulin [144] and syphilis screening have also been reported [145]. An earlier example of the technology had a detection limit for thyroxine of 5×10^{-9} mol/L [146].

A recent advance in SPR immunosensing is the incorporation of labels to increase sensitivity. The combination of fluorescent labeling with SPR to produce an SPR fluoroimmunoassay has successfully been used to assay hCG in serum [147]. Latex particles have been used to enhance the sensitivity of an assay for detection of antigen known as the Enhanced Surface Plasmon Resonance Inhibition Test (ESPRIT) [148]. This test consists of two steps, an inhibition step and an enhancement step. In the inhibition step, antibody is mixed with a known concentration of free antigen at the SPR sensor surface, to which antigen has already been immobilized. Free antigen inhibits antibody binding to the antigen-coated surface, and increasing concentrations of free antigen will therefore proportionally reduce the SPR signal. In the second step, latex particles coated with antigen are introduced; these bind to the antibody at the sensor surface and thus enhance the SPR signal. In this way the detection of antigens is independent of their molecular mass, which raises the possibility of sensing small-molecular-mass molecules (<5 kDa) in low concentrations.

Waveguides. There is a strong similarity between waveguide and SPR systems; in both, an evanescent wave is generated at the surface of the device. In SPR, surface plasmons in the metal layer become excited; in waveguides, the light wave itself is excited in a dielectric layer. The phenomenon of dielectrics, which has been widely exploited in immunosensor design, is described elsewhere [149]. Waveguides consist of a high-RI dielectric film sandwiched between two dielectric materials of lower RI [150]. The choice of materials to use when construct-

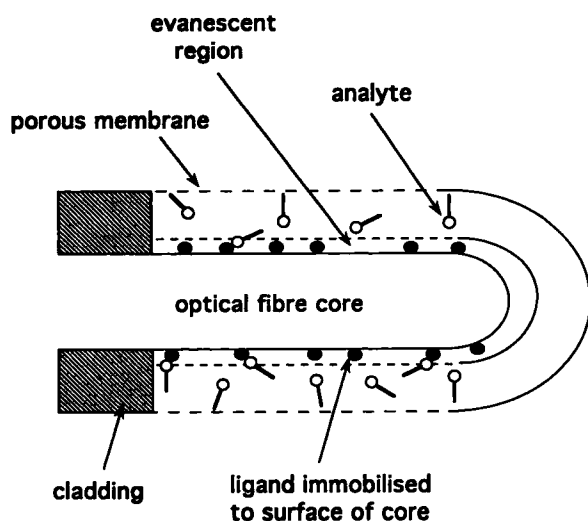


Fig. 7. The use of waveguides in fiber optics.

Fiber-optic waveguides are composed of a high-RI cylindrical core surrounded by a lower-RI cladding. Light directed towards the tip of the fiber undergoes total internal reflection at the interface between the core and the sample medium. An evanescent wave is generated here, which interacts with biomolecules adsorbed to the immobilized ligand. The reflected light signal is monitored once it has returned to the cladded region of the fiber.

ing a waveguide is important; commonly used are glass, silicon, polymers, III–V compounds, and lithium niobate, which have slightly different RIs and optical properties [151]. The miniaturization of waveguides to the size of optical chips and fibers has made them extremely popular for integration into biosensors, a phenomenon known as integrated optics (IO) [152]. Examples of waveguide systems currently adapted for IO biosensors are fiber-optics, interferometers, grating couplers, and more recently the IAsysTM device (Fisons Applied Sensor Technology, Cambridge, UK).

Fiber-optic waveguides. Waveguides may also be constructed in the form of fibers: high-RI cylindrical core made of glass, quartz, or polymer surrounded by lower-RI cladding (Fig. 7) [153]. Light is directed towards the tip of the fiber and undergoes total internal reflection at the interface between the core and sample medium, setting up an evanescent wave. Binding or absorption takes place between immobilized biomolecules and sample analyte within the evanescent field at the uncladded surface of the fiber. This reaction can be monitored by measuring the reflected light signal once it has returned from the uncladded region of the fiber into the cladded region. Fiber-optic waveguides have been successfully developed for immunosensors [154] and can be used to monitor the fluorescence signal generated in an immunoassay [155], as demonstrated recently by a cocaine-detecting fiber-optic immunosensor [156] and colorimetric measurements [157]. Optical fiber-based immunosensors have proved particularly useful for analyzing environmental and clinical samples that contain hazardous materials [158, 159]; the long fibers can be inserted into closed containers or biohazard hoods, thus preventing contamination of the operator and optical components.

An example of such a device is the fiber optic-based waveguide immunosensor for the detection of *Clostridium botulinum* toxin A [160]. Designed like a sandwich immunoassay, this labeled technique involves covalent coupling of antibodies to a tapered optical fiber; when this is brought into contact with a reaction mixture containing sample and rhodamine-labeled antibodies, the evanescent wave of the fiber-optic probe (connected to an argon laser) excites fluorophore molecules coupled to the immobilized antibodies by the presence of toxin but has a minimal influence on the fluorophore in the bulk of the reaction mixture. Another, similar device has been described for this purpose [161].

Interferometers. Interferometers may be used for measurements of RI, displacement, and surface flatness and have been successfully adapted for IO biosensors [162] to perform label-free molecular affinity analysis of biomolecular binding events. An example is the Fabry–Pérot interferometer, a single monomode channel waveguide [163]. In this system, light enters the device at a specific angle and is reflected backwards and forwards (by total internal reflection) several times between two mirrors situated at either end; each time light is reflected, some couples out of the interferometer. The light beams that emerge interfere with each other to form a characteristic wave pattern.

Some interferometers, such as the Mach–Zehnder interferometer [164], split the input light beam into two partial waves: the electric and magnetic fields (TE and TM, respectively) of a single polarized light wave (Fig. 8). In the Mach–Zehnder interferometer, the two components are reflected

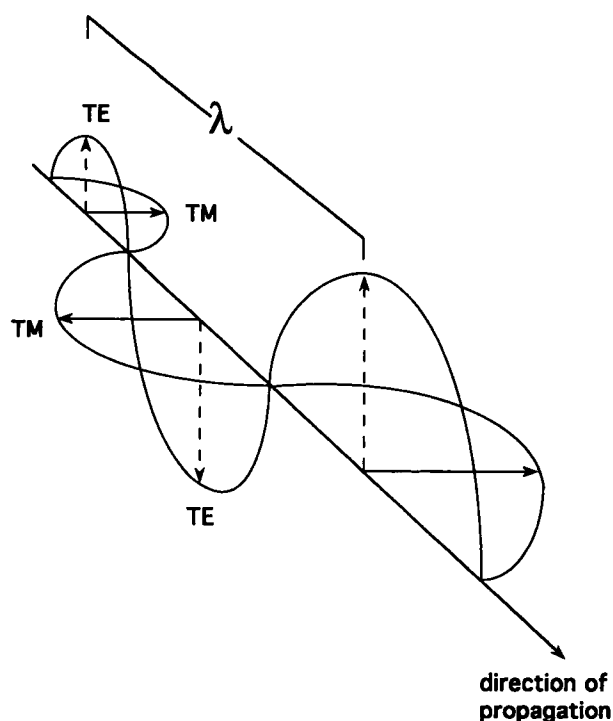


Fig. 8. Electric and magnetic components of polarized light: A single polarized light wave is composed of an electric field and a magnetic field (TE and TM, respectively), which are perpendicular to each other.

between different mirrors inside the device. One interacts with the sample and undergoes a change in phase so that, when the two polarizations are recombined at the output, the interference pattern alters.

The difference interferometer [165] is based on a two-mode interferometric thin-film waveguide [166] similar to the Mach-Zehnder set-up. One of its applications is as a direct immunosensor for label-free molecular-affinity analysis of biomolecular binding events such as antibody-antigen reactions [167], for which a detection limit of 2.0×10^{-13} mol/L was reported for hepatitis B surface antigen in serum. The molecular recognition component (ligand) is immobilized on the sensor surface, and a polarized laser beam is directed onto the butt-face of the waveguide, thus exciting the TE and TM polarizations. An evanescent field is set up via a thin film of waveguiding material (of high RI) at the sensor surface; as the field propagates across the sensor surface to the opposite side, it interacts with immobilized ligand on the sensor surface. A sample cell containing analyte molecules flows over the sensor surface, and any adsorption or binding to ligand within the penetration depth of the evanescent wave at the sensor surface will alter the effective refractive indices of TE and TM. When both polarizations couple out of the device, they interfere; continually monitoring changes in the relative phase between TE and TM gives a measure of the molecular surface concentration (i.e., that shown by changes in RI) at the sensor surface. The difference interferometer has been successfully used for the highly sensitive monitoring of a wide range of biomolecular interactions, and its ability to perform real-time monitoring makes it a valuable tool for characterizing specific processes such as antigen-antibody binding.

Planar waveguide interferometric immunosensors have been used to study immunoreactivity of antibodies immobilized to a surface, e.g., anti-hCG [168].

Grating couplers. The incorporation of a grating coupler—a series of fine corrugations etched into the waveguide surface—into an optical waveguide to produce an RI-detecting sensor was first proposed by Tiefenthaler and Lukosz (Fig. 9 [169]). This device monitors the coupling angle of either the input laser beam (the input grating coupler) [170, 171] or the output beam (the output grating coupler) [172, 173], either of which is proportional to the RI within the evanescent field at the surface of the device. The grating coupler is situated between a substrate layer of low RI (usually glass, RI = 1.64) and a waveguiding film of higher RI ($\text{SiO}_2\text{-TiO}_2$, RI = 1.75–1.82). Polarized light enters the device, and the grating causes light to couple in and out of the waveguide, thus setting up an evanescent wave at the waveguide-sample interface. The large difference in RI between the waveguiding layer and the substrate layer (>0.2) results in a large evanescent field, thereby increasing the activity towards RI changes at the surface. IO grating couplers are commercially available for chemical [174], biochemical [170], and gas sensing [175]. The use of grating couplers as immunosensors in pesticide detection has also been reported [176].

SPR and interferometry. The IAsys system [177], mentioned earlier, presents a novel form of optical biosensor, combining the technology of waveguides such as the difference interferom-

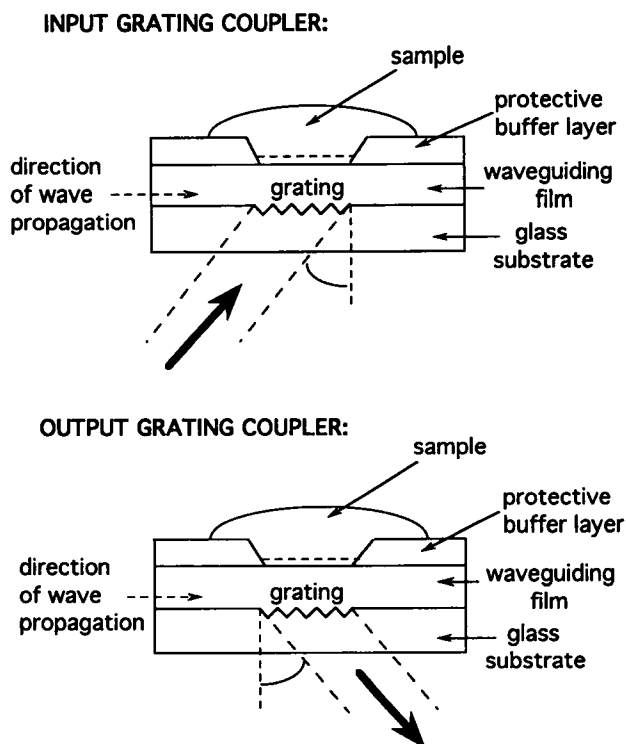


Fig. 9. Two types of grating couplers: the input grating coupler, which monitors the coupling angle of the input laser beam, and the output grating coupler, which monitors the angle of the output beam.

eter with that of SPR. It is similar to the SPR system in structure, but the metal layer is replaced by a dielectric resonant layer of high RI (Fig. 10). This layer, typically composed of titanium (RI = ~ 2), is separated from the glass prism (RI = 1.64) by a low-RI coupling layer of silica (RI = 1.46) thin enough to allow light to couple into the resonant layer via the evanescent field. These modifications produce a “sandwich” of three layers (high-low-high RI), on top of which is a layer of dextran for immobilization of the appropriate ligand.

At the interface between the prism and the coupling layer, an incident beam of laser light (directed at an angle $>\theta$) undergoes total internal reflection. When the resonance angle (θ) is reached, a fraction of this light couples into the coupling layer and is directed towards the resonant layer via the evanescent field. At the interface with the sample (of lower RI), total internal reflection takes place again and sets up another evanescent wave, which decays exponentially into the sample. This is the resonance condition. The wave propagates along this surface by ~ 1 mm before coupling back out of the device. Whereas in the SPR system resonance is observed as a decrease in the reflected light intensity, almost no change in the intensity of the reflected light occurs in the IAsys. However, as in all waveguide devices in which both TE and TM polarizations of light are monitored (e.g., the difference interferometer), there is a change in the phase of the reflected light at resonance, and this is the property utilized in this system [178].

In the IAsys, TE and TM polarizations are monitored separately. Before resonance is reached, these are both in phase

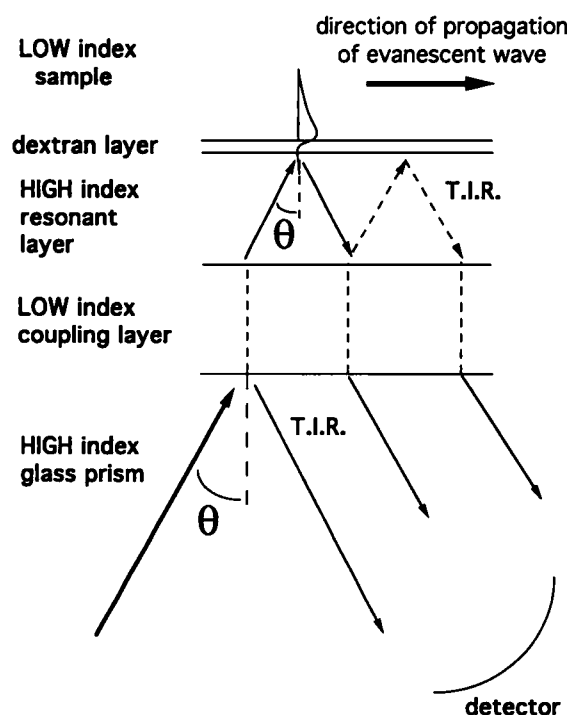


Fig. 10. Structure of the IAsys device: At the resonance angle (θ), light passes through a low-RI coupling layer, then couples into a resonant layer of higher RI, generating an evanescent wave at the interface with the sample layer that propagates some distance along the surface before coupling back out of the device.

Both TE and TM polarizations of light are detected, but unlike SPR there is no decrease in the intensity of the reflected light and the angle of incidence is monitored. TIR, total internal reflection.

and resemble sine waves (see Fig. 8). As the system approaches resonance, there is a brief delay/break in the reflected light, caused by light coupling into the resonant layer. This results in a change of 2π radians (360°) in the phase of light; the resulting light beam is still in phase, but the resonance angle (TE phase relative to TM) shifts by π radians (180°). When this occurs, the resulting polarization of the beam is rotated by 90° from that of the original beam and is now out of phase. This rotated beam can be monitored by a suitably orientated polarizer. Both incident polarizations (TE and TM) can undergo resonance and be detected simultaneously, because their respective resonance angles are widely separated. However, the TE polarization has a sharper resonance peak than does TM and is more sensitive to any changes in the resonance angle and therefore in RI. Accordingly, the system is optimized to track the TE resonance angle but uses the TM resonance angle as a reference.

Changes in RI occurring at or near the sensing surface because of the adsorption of biomolecules are within the region of the evanescent wave, and therefore alter the position of the resonance angle for TE. This angle shift is measured and recorded throughout the experiment, providing a direct interpretation of RI and therefore of mass changes occurring at the sample surface [178]. The IAsys, which provides an accurate and continuous representation of the RI changes taking place at the sensor surface, differs from SPR in two ways: First, there is no

pronounced variation in the intensity of the reflected light with angle; instead, some of the light undergoes a phase shift, which is observed as an intensity peak at the resonance angle. Second, the light propagating along the waveguide strikes the sample surface many times rather than once (as in SPR). Also, the dielectric materials comprising the resonant layer of the IAsys require lower absorption of light at the wavelengths used than does the metal film of the SPR system.

The IAsys has been used successfully to study reaction kinetics and to map epitopes, taking advantage of the opportunity for real-time monitoring. This facility has enabled the first clear demonstration of a matrix effect on the association constant, which is dependent on the viscosity of the matrix (Morgan CL, Newman DJ, Burrin JM, Price CP, ms. submitted for publication). The system has been used to quantify both haptens and proteins, although only limited published data for this are available.

Although optical immunosensors offer several advantages over other transducer types, they may still be subject to such problems as interferences from ambient light, long response times, and leakage of the reagent phase. Manufacturing/fabrication costs are also often high in comparison with some electrochemical devices. However, the extensive range of applications offered by optical immunosensing, particularly real-time monitoring of the antibody–antigen interaction, probably overcomes or outweighs these issues. Perhaps a combination of optical and electrochemical approaches, e.g., SPR at electrochemical interfaces or luminescence generated electrochemically, will be investigated in the future and developed with success [179].

Clinical Applications of Immunosensors

Not all of these sensing technologies developed for immunological quantification have achieved practical usage in biological fluids. Table 3 gives an overview of the sensing systems that have been demonstrated with a sample derived from a biological matrix, i.e., serum or whole blood. The most sensitive (i.e., lowest detection limit) is of the order of 2×10^{-13} mol/L for hepatitis B surface antigen in serum [167], which compares well with many conventional immunoassay techniques. Hapten assays operate in the low nanomolar region for digoxin and estradiol. There is even a highly sensitive assay for prostate-specific antigen in whole blood by FCFD (3.3×10^{-12} mol/L) technology [117], which is comparable with quoted detection limits for automated heterogeneous immunoassays.

Large sums of money have been invested by diagnostic companies to develop commercially viable immunosensors, as yet with little obvious success. The two widely available immunosensors are both direct optical systems—the BIAcore and the IAsys—and both have surfaces of carboxylated dextran. These have proved to have very low nonspecific binding in biological matrices and achieve good detection limits for a variety of molecules, but their major impact has been to revolutionize the kinetic rate analysis of biomolecular interactions. Surface-effect measurement of kinetic rate constants by real-time, stable automated immunosensors has received widespread usage in the diagnostic, biotechnology, and pharmaceutical industries as well as in basic clinical science.

The potential of immunosensing technologies has been around for quite a while and new opportunities, e.g., microspot technology, are continually developing. However, the main impact of these technologies has been (a) the innovative detection systems that have furthered conventional immunoassay systems and (b) the feasibility studies in both clinical and environmental testing areas (Table 4). Despite huge innovations and investments, however, immunosensors fill the pages of our literature rather than the benches of our laboratories, and

demonstration systems have failed to reach commercial-scale production—presumably because of fabrication difficulties or simply cost issues.

Conclusions

The diversity of the physical sciences that have been drawn into the realms of quantitative biochemical analysis is exciting and has proved to be challenging to both the physical and biological scientist. Several techniques clearly are worthy of development in the field of immunosensors, some of which are now beginning to emerge into the field of biochemical analysis. The requirement for highly reproducible fabrication of devices, particularly bioactive surfaces, appears to have limited the rapid exploitation of many of the systems described, as has the limited success in dealing with nonspecific binding effects and their impact on sensor performance.

There is no doubt that immunosensors are now established in the research laboratory and will increasingly become involved in bioprocess control systems. However, the place of immunosensors in the diagnostic field is not entirely clear. As disposable microfabricated immunoassay devices—e.g., immunochromatography [11], encapsulated liquid systems [184], and microengineered systems [185, 186]—continue to be developed, all offering the required sensitivity and speed of delivery of result, the role of true immunosensors has to be questioned. Perhaps their role will lie in continuous monitoring but, again, the limitations of the technology and a limited clinical need may stunt their growth.

Nonetheless, despite what might appear to be a rather slow development of the commercialization of sensor biotechnology in the face of a large investment, there is no doubt that the integration of the physical and biological sciences has been a profitable union in terms of knowledge gained.

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Table 4. Applications of Immunosensors for clinical and environmental or fermentational monitoring.

<i>Clinical</i>	
Potentiometric	Penicillin [31], insulin [33], urine hCG [183]
Amperometric	hCG [38], theophylline [39], Factor VIII-related antigen [40], α_1 -acid glycoprotein [41], apolipoprotein E [44], FSH and LH [45]
Piezoelectric	Drugs in urine [62], bovine hemoglobin [63], IgM [65], HIV-specific antibodies in serum [72]
SAW	Human IgG [76]
Calorimetric (TELISA)	Human proinsulin [84], albumin and gentamicin [85]
<i>Optical</i>	
Chemiluminescence	Estradiol, α_2 -interferon, hCG, total IgG, influenza antibodies [103, 104]
FCFD	Serum estrone-3-glucuronide [114], serum rubella antibodies [115], serum hCG [116], whole-blood prostate-specific antigen [117]
OBA	Serum hCG [121]
Ellipsometric	γ FN [127]
SPR	Serum hCG [147]
Optical fibers	Digoxin and ferritin [153], C. botulinum toxin A [160]
Difference interferometer	Serum hepatitis B surface antigen [167]
Fluoroimmunosensor	Serum β_2 -microglobulin [180]
BIACore	Serum theophylline [138], serum β_2 -microglobulin [138, 181, 182], serum IgE [138, 182]
<i>Environmental/fermentation</i>	
Amperometric	Herbicides [43]
Conductance	Pesticides [49]
Piezoelectric	Gaseous pollutants [56–58]; herbicides, including atrazine in drinking water [60, 61]
SAW	Gaseous pollutants [73], chemicals [74, 75]
Calorimetric	Sugars and sugar derivatives in fermentation [82]
<i>Optical</i>	
Bioluminescence	Environmental mercury [99]
Optical fibers	Pesticides [158], hazardous materials [159]
Grating couplers	Biochemicals [170], chemicals [174], gases [175], pesticides [176]
SAW, surface acoustic wave; FSH, follicle-stimulating hormone; LH, luteinizing hormone.	

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