20.1 Introduction

Human beings, along with other mammals, consciously interact with the surrounding world by means of seven sensing mechanisms. In addition to the five senses, the abilities to detect temperature and variations in elevation are almost as important. One cannot help but be awed by the evolutionary process that brought about the development of such senses and by their integration into a brain capable of information processing and storage.

If a better awareness of our surroundings, food supplies, and predators was the main driver for this evolutionary process, a parallel might be drawn with our own human enterprise of creating sensors that help us understand the world with which we interact. A sensor can be viewed as the “primary element of a measurement chain, which converts the input variable into a signal suitable for measurement.” A sensing scheme is usually based on a transduction principle or mechanism; an input variable is transformed into an output variable through a transduction mechanism. Transduction principles are known physical or chemical effects that correlate observations in different domains. For example, the photoelectric effect is used to correlate number of photons with electric current. The piezoelectric effect does the same for stress and electricity, and Biot-Savart’s law correlates magnetic field and electric current. In other words, the operating principle of a sensor involves transforming signals between different domains, from a domain we cannot directly access to one we can measure.
A biosensor is a special type of sensor often used in bioanalysis. Humankind has been performing bioanalysis since the dawn of time, using the sensory nerve cells of the nose to detect scents and those of the tongue to taste dissolved substances. As time has progressed, so has our level of understanding about the function of living organisms in detecting trace amounts of biochemicals in complex systems. The abilities of biological organisms to recognize foreign substances are unparalleled and have to some extent been mimicked by researchers in the development of biosensors. Using bioreceptors from biological organisms or receptors patterned after biological systems, scientists have developed a new means of chemical analysis that often has the high selectivity of biological recognition systems. These biorecognition elements, in combination with various transduction methods, have helped to create the rapidly expanding fields of bioanalysis and related technologies known as biosensors and biochemical sensors.

### 20.2 Biosensors: Definition and Classification

Two fundamental operating principles of a biosensor are “biological recognition” and “sensing.” Therefore, a biosensor can be generally defined as a device that consists of two basic components connected in series: (1) a biological recognition system, often called a bioreceptor, and (2) a transducer. The basic principle of a biosensor is to detect this molecular recognition and to transform it into another type of signal using a transducer. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The interaction of the analyte with the bioreceptor is designed to produce an effect measured by the transducer, which converts the information into a measurable effect such as an electrical signal. Figure 20.1 illustrates the conceptual principle of the biosensing process.

Biosensors can be classified by bioreceptor or transducer type (Figure 20.2). A bioreceptor is a biological molecular species (e.g., an antibody, an enzyme, a protein, or a nucleic acid) or a living biological system (e.g., cells, tissue, or whole organisms) that utilizes a biochemical mechanism for recognition. The sampling component of a biosensor contains a biosensitive layer that can contain...
bioreceptors or be made of bioreceptors covalently attached to the transducer. The most common forms of bioreceptors used in biosensing are based on (1) antibody/antigen interactions, (2) nucleic acid interactions, (3) enzymatic interactions, (4) cellular interactions (i.e., microorganisms, proteins), and (5) interactions using biomimetic materials (i.e., synthetic bioreceptors). For transducer classification, the previously mentioned techniques (optical, electrochemical, and mass-sensitive) are used.

Bioreceptors are the key to specificity for biosensor technologies. They are responsible for binding the analyte of interest to the sensor for the measurement. These bioreceptors can take many forms; the different bioreceptors that have been used are as numerous as the different analytes that have been monitored using biosensors. However, bioreceptors can generally be classified into five different major categories: (1) antibody/antigen, (2) enzymes, (3) nucleic acids/DNA, (4) cellular structures/cells, and (5) biomimetic. Figure 20.3 shows a schematic diagram of two types of bioreceptors: the structure of an immunoglobulin G (IgG) antibody molecule (Figure 20.3A), and DNA and the principle of base pairing in hybridization (Figure 20.3B).

Since the first biosensors were reported in the early 1960s, growth of research activities in this area has accelerated. Biosensors have seen a wide variety of applications, primarily in three major areas: biological monitoring, biomedical diagnostics and environmental sensing applications.

### 20.3 Transduction Systems

Biosensors can be classified based on the transduction methods they employ. Transduction can be accomplished through a large variety of methods. Most forms can be categorized in one of three main classes: (1) optical detection methods, (2) electrochemical detection methods, and (3) mass-based detection methods. Other detection methods include voltaic and magnetic methods, and new types of transducers are constantly being developed for use in biosensors. Each of these three main classes contains many different subclasses, creating a large number of possible transduction methods or combination of methods. This section provides a brief overview of the various detection methods used in biosensors. Special emphasis will be placed on the description of optical transducing principles, which is the focus of this chapter.

#### 20.3.1 Optical Detection

Optical detection offers the largest number of possible subcategories of all three of the transducer classes. This is because optical biosensors can be used with many different types of spectroscopies (e.g., absorption, fluorescence, phosphorescence, Raman, surface-enhanced Raman scattering
FIGURE 20.3  Schematic diagrams of two types of bioreceptors: (A) IgG antibody; (B) DNA and the hybridization principle. (From Vo-Dinh, T. et al., Fresenius J. Anal. Chem., 366, 540, 2000. With permission.)
(SERS), refraction, dispersion spectrometry) to measure different spectrochemical properties of target species. These properties include amplitude, energy, polarization, decay time, and phase. Amplitude is the most commonly measured parameter of the electromagnetic spectrum because it can generally be correlated with the concentration of the analyte of interest.

The energy of the electromagnetic radiation measured can often provide information about changes in the local environment surrounding the analyte, its molecular vibrations (i.e., Raman or infrared absorption spectroscopies), or the formation of new energy levels. Measurement of the interaction of a free molecule with a fixed surface can often be investigated with polarization measurements. Polarization of emitted light is usually random when emitted from a free molecule in solution; however, when a molecule becomes bound to a fixed surface, the emitted light often remains polarized. The decay time of a specific emission signal (i.e., fluorescence or phosphorescence) can also be used to gain information about molecular interactions because these decay times are highly dependent upon the excited state of the molecules and their local molecular environment. Another property that can be measured is the phase of the emitted radiation. When electromagnetic radiation interacts with a surface, the speed or phase of that radiation is altered, based on the refractive index of the medium (analyte). When the medium changes via binding of an analyte, the refractive index may change, thus changing the phase of the impinging radiation. This property of electromagnetic radiation has been successfully exploited in commercial applications using surface plasmon resonance sensors.

20.3.1.1 Fluorescence

Fluorescence is one of the most sensitive spectroscopic techniques, and its sensitivity makes it uniquely suited for the detection of very low concentrations of bioanalytes. Background information on the photophysical principles of the fluorescence emission process can be found in Chapter 28 of this handbook. When coupled with a high-power light source such as a laser, it can yield very high signal-to-noise (S/N) values. Single-molecule detection using laser-induced fluorescence has been reported in many studies. Because of its inherently high sensitivity, fluorescence has traditionally been the technique of choice for optical detection of trace-level analytes (at the femtomole level or lower). For high-quantum-yield fluorophores, the effective fluorescence cross sections can be as high as \(10^{-16} \text{ cm}^2/\text{molecule}\).

A typical optical setup for a fluorescence biosensor using a laser as the light source is shown in Figure 20.4.29 The instrument consists of an optical fiber with antibodies immobilized at the sensor tip. Excitation light from a laser is sent through a beam splitter onto the incidence end of the optical fiber. The laser beam is transmitted inside the fiber onto the sensor tip, where it excites the analyte molecules bound to the antibodies. The excited antigen fluorescence is collected and retransmitted to

![FIGURE 20.4 Schematic diagram of an optical system for an antibody-based biosensor. (From Vo-Dinh, T. et al., Appl. Spectrosc., 41, 735, 1987. With permission.)](image-url)
the incidence end of the fiber, directed by the beam splitter onto the entrance slit of a monochromator, and recorded by a photomultiplier. This fluoroimmunosensor (FIS) was used to detect the carcinogen benzo[a]pyrene (BaP). 29

Fluorescence detection is also suitable for time- or phase-resolved measurements, yielding additional information from the system of interest. Vo-Dinh and co-workers reported the development of a phase-resolved fiberoptic fluoroimmunosensor (PR-FIS) that can differentiate BaP and its metabolite benzopyrene tetrox (BPT) based on the difference in their fluorescence lifetimes.

A diagram for a phase-resolved optical setup is shown in Figure 20.5. 30 The excitation laser beam is modulated with an acousto-optic modulation system. A function generator provides the waveforms to drive the modulator. Laser light is delivered to the sample by an optical fiber and the fluorescence is collected by the same fiber. The fluorescence from the sensing probe is collimated by appropriate optics and focused onto the entrance slit of a monochromator equipped with a photomultiplier. A lock-in amplifier synchronized with the function generator is used to measure phase-resolved signals. With this setup, BaP and BPT could be detected simultaneously using phase-resolved fluorescence. Their phase-dependent spectrum is shown in Figure 20.6. 30

Figure 20.6A shows the fluorescence of BPT with the use of the optimal phase shift for maximum BPT signal. On the other hand, Figure 20.6B shows the fluorescence of BaP with the use of the optimal phase shift for maximum BaP signal. The results illustrate the capability of the PR-FIS device to reveal the spectrum of 30 femtomoles of BPT in the presence of much higher amounts of interfering BaP.

Femtomolar sensitivities for fluorescently labeled proteins were reported by Herron and co-workers using a channel-etched thin film waveguide fluoroimmunosensor. 31 A silicon-oxynitride thin optical waveguide film was etched to create a channel for small volumes of analyte. Two different types of assays were performed and compared using this biosensor. The first was a direct assay of a fluorescently tagged protein ligand to a protein receptor that had been immobilized onto the waveguide. The second assay was an indirect sandwich-type assay of a nonfluorescent protein ligand, where the analyte (the protein ligand) binds to a protein bioreceptor immobilized on the waveguide; then a fluorescently tagged secondary receptor was used for measurement purposes. The fluorescent dye used to tag the proteins was Cy-5, a red emitting cyanine dye that reduced the chance of excitation of possible interferents.
An interesting application of fluorescence spectroscopy involves the detection of lipopolysaccharide endotoxin (LPS), which is the most powerful immune stimulant known and a causative agent in the clinical syndrome known as sepsis. Sepsis is responsible for more than 100,000 deaths annually, in large part due to the lack of a rapid, reliable, and sensitive diagnostic technique. LPS has been detected in Escherichia coli at concentrations as low as 10 ng/ml in 30 s, using an evanescent wave fiber-optic biosensor. Polymyxin B covalently immobilized onto the surface of the fiber-optic probe selectively bound fluorescently labeled LPS. The competitive assay format worked in buffer and in plasma with similar sensitivities. This method can be used with other LPS capture molecules such as antibodies, lectins, or antibiotics to detect LPS and determine the LPS serotype simultaneously. This LPS assay using the fiber-optic biosensor can be applied in clinical and environmental testing.

20.3.1.2 Surface Plasmon Resonance

Since the first application of the surface plasmon resonance (SPR) phenomenon for sensing almost two decades ago, this method has made great strides in terms of instrumentation development and in applications. SPR sensor technology has been commercially available and SPR biosensors have become a useful tool for characterizing and quantifying biomolecular interactions.

SPR makes it possible to monitor the binding process as a function of time by following the increase in refractive index that occurs when one of the interacting partners binds to its ligand immobilized on the surface of a SPR sensor substrate. A technique that does not require the reactants to be labeled is a major advantage, simplifying the data collection process. Biosensor binding data are also useful for selecting peptides to be used in diagnostic solid-phase immunoassays. Very small changes in binding affinity can be measured with good precision, which is a prerequisite for analyzing the functional effect and thermodynamic implications of limited structural changes in interacting molecules. For example, the on-rate ($k_o$) and off-rate ($k_d$) kinetic constants of the interaction between a protein and an antibody.

![Phase-resolved fluorescence spectra of BaP and BPT](image)
can be readily measured and the equilibrium affinity constant, \( K \), can be calculated from the ratio \( \frac{k_a}{k_d} = K \).\(^{34}\)

The transduction principle involved in surface plasmon resonance sensors is based on the arrangement of a dielectric/metal/dielectric sandwich so that, when light impinges on a metal surface, a wave is excited within the plasma formed by the conduction electrons of the metal.\(^{35,36}\) A surface plasmon is a surface charge density wave occurring at a metal surface. When a plasmon resonance is induced in the surface of a metal conductor by the impact of light of a critical wavelength and angle, the effect is observed as a minimum in the intensity of the light reflected off the metal surface. The critical angle is naturally very sensitive to the dielectric constant of the medium immediately adjacent to the metal and therefore lends itself to exploitation for bioassay. For example, the metal can be deposited as, or on, a grating; upon illumination with a wide band of frequencies, the absence of reflected light at the frequencies at which the resonance matching conditions are met can be observed.

Because of the intrinsic dependence with the index of refraction at the surface, surface plasmon resonance can be used as a sensor transducer to indicate when alterations at the surface happen. The binding event involving antibody–antigen recognition or DNA hybridization at the SPR sensor surface is the most common SPR application. SPR is able to detect small variations of the index of refraction at the metal-coated interface caused by changes in a few monolayers above the surface.

In biosensor devices, the surface plasmon resonance is detected as a very sharp decrease of the light reflectance when the angle of incidence is varied. The resonance angle is very sensitive to variations in the refractive index of the medium just outside the metal film. Because the electric field probes the medium within only a few hundreds of nanometers from the metal surface, the condition for resonance is very sensitive to variations in thin films on this surface. Changes in the refractive index of about \( 10^{-5} \) are easily detected.

The surface plasmon wave penetrates in both directions normal to the interface; consequently, the incident angle or frequency at which resonance is observed is dependent on the refractive index of dielectric at the interface. Liedberg and co-workers\(^{35}\) have shown that surface plasmon resonance can be used as the basis of a genuine reagentless immunoassay if large analytes are to be monitored. The antibody is immobilized on the metal. When a large antibody binds, it displaces solution (having a refractive index of approximately 1.34) with, for example, protein (having a refractive index of 1.5). The effective refractive index of the dielectric adjacent to the metal is thus changed in proportion to the amount of analyte bound, and the surface plasmon resonance (incident angle or resonance frequency) is shifted accordingly. Flanagan and Pantell\(^{36}\) have shown that the amount of analyte bound can be directly related to the resonance shift even when the resonance curve is distorted by scattering caused by surface roughness, thus relieving one of the constraints of precise control of metalization, which would be unattractive in the mass production of inexpensive sensors.

SPR biosensors can provide qualitative information on macromolecular assembly processes under a variety of conditions. Quantitative information, in the form of affinity constants for complex formation, can be obtained in a manner similar to conventional solid phase assays. The major advantage of SPR biosensors is that the formation and breakdown of complexes can be monitored in real time. This offers the possibility of determining the mechanism and kinetic rate constants associated with a binding event. This information is essential for understanding how biological systems function at the molecular level.

However, accurate interpretation of biosensor data is not always straightforward.\(^{37}\) A few software programs can interpolate SPR data and provide an estimate binding constants. The program CLAMP is software developed to interpret complex interactions recorded on biosensors.\(^{37}\) It combines numerical integration and nonlinear global curve-fitting routines. The BIACore\(^{TM}\) system is one of the most used among the several commercially available optical biosensors.

For example, the interactions between adenylate kinase (AK) and a monoclonal antibody against AK (McAb3D3) were examined with an optical biosensor, and the sensograms were fitted to four models using numerical integration algorithms.\(^{38}\) The interaction of AK in solution with immobilized McAb3D3 followed a single exponential function and the data fitted well to a pseudo first-order reaction model.
The application of surface plasmon resonance biosensors in life sciences and pharmaceutical research continues to increase. Several reviews providing a comprehensive analysis of the commercial SPR biosensor literature and highlights of emerging applications are available. Some general guidelines to help increase confidence in the results reported from biosensor analyses (because of the variability in the quality of published biosensor data) have been compiled as well.

20.3.1.3 Near-Infrared Absorption

Near-infrared (NIR) spectroscopy utilizes wavelengths above 800 nm to excite vibrational overtones and low-energy electronic levels of chemical species. Use of NIR usually profits from lower fluorescence background and higher specificity for appropriate dyes. Longer wavelengths also offer better penetration of translucent tissue, another major advantage of NIR for biomedical diagnostics.

A biomolecular probe utilizing NIR for the detection of biological molecules (immunochemical samples) with a semiconductor laser diode (780 nm) has been reported. This probe consists of a modified fiber tip binding site, an NIR dye, and a photodiode detector. Preparation of the NIR biosensor involved the immobilization of anti-IgG antibody to the activated binding site of the fiber, followed by coating with IgG for a sandwich-type probe. The antibody was labeled with the commercially available NIR dye IR-144. The low background signal of the detector allowed the detection of 2.72 ng/ml of IgG in a probe coated with 10 ng/ml antibody at 820 nm.

20.3.1.4 Reflectometric Interference

Gauglitz and co-workers have developed a unique technique based on reflectometric interference spectroscopy (RiFS) for detection in biosensors. RiFS was used for the detection of biomolecular interactions and applied for small-molecule detection by chemical sensor surfaces. The principle of RiFS, which does not require the use of labels, is illustrated in Figure 20.7. A thin silica layer on a glass substrate is illuminated from the back side using white light. Light beams are reflected at the different layers and superimposed to form a characteristic interference pattern. Changes in the thickness of the transducer surface caused by biomolecular interactions lead to a shift of the interference pattern, which can be analyzed in real time.

20.3.1.5 Raman

The possibility of using Raman or surface-enhanced Raman scattering (SERS) labels as gene probes has been reported. The SERS technique has been recently applied to the detection of DNA fragments of the human immunodeficiency virus (HIV) and of the breast cancer gene. Raman spectroscopy
has also proved to be a very useful tool for chemical analysis because it can identify chemical groups. This technique, however, suffers from poor sensitivity, often requiring powerful and expensive laser excitation sources. However, the discovery of the SERS effect, which results in increased sensitivities of up to $10^8$-fold for some compounds, has renewed interest in this technology for analytical purposes. The feasibility of using surface-enhanced Raman gene (SERGen) probes, which exhibit an extremely narrow small spectral bandwidth, has been demonstrated. The ability to use labeled primers extends the utility of the SERGen probes for medical diagnostic purposes.

Because SERGen probes rely on chemical identification rather than emission of radioactivity, they have a significant advantage over radioactive probes. SERGen probes are formed with stable chemicals that do not emit potentially dangerous ionizing radiation. Furthermore, the probes offer the excellent specificity inherent in Raman spectroscopy. While isotope labels are few, many chemicals can be used to label DNA for SERS detection. Potentially, dozens to hundreds of different SERGen probes could be constructed and used to probe several DNA sequences of interest (label multiplexing) simultaneously, thus decreasing the time and cost for gene diagnostics and DNA mapping. The multispectral imaging (MSI) system developed in this work, with its rapid wavelength switching of the acoustooptic tunable filter (AOTF) system could allow very rapid scanning and high-throughput data collection.

For biomedical diagnostics, the SERGen probe could have a wide variety of applications in areas where nucleic acid identification is involved. The SERGen probes may also be used in polymerase chain reaction (PCR) applications for medical diagnostic applications, e.g., for HIV detection. In genomics applications as well as in high-throughput analysis, the SERS gene probe technology could lead to the development of detection methods that minimize the time, expense, and variability of preparing samples by combining the BAC mapping approach with SERS "label multiplex" detection. Large numbers of DNA samples can be simultaneously prepared by automated devices. With the SERGen technique, multiple samples can be separated and directly analyzed using multiple SERGen labels simultaneously (label multiplex scheme). The use of the SERS technique for biomedical application is further described in Chapter 64 of this handbook.

### 20.3.2 Electrochemical Detection

Electrochemical detection is another possible means of transduction that has been extensively used in biosensors. This technique is complementary to optical detection methods such as fluorescence, the most sensitive of the optical techniques. Since many analytes of interest are not strongly fluorescent and tagging a molecule with a fluorescent label is often labor-intensive, electrochemical transduction can be very useful. By combining the sensitivity of electrochemical measurements with the selectivity provided by bioreception, detection limits comparable to those of fluorescence biosensors are often achievable.

Electrochemical detection is usually based on the chemical potential of a particular species in solution (the analyte), as measured by comparison to a reference electrode. Therefore, the electrochemical response is dependent on the activity of the analyte species, not their concentration. However, for dilute solutions of low ionic strength, the thermodynamic parameter activity approaches the physical parameter concentration (in molar terms). In comparison, the signal intensity associated with optical detection is usually directly proportional to the number of a specific chromophore within a certain pathlength and, therefore, directly dependent on the concentration of the chromophore. The linear relationship between signal intensity and concentration of a species is known as Beer–Lambert's law. The cases in which discrepancies from this linear relationship occur are usually caused by secondary effects such as self-absorbance, and equilibrium conditions.

Multiple examples of electrochemical sensors applied to biological systems are known. For example, electrochemical flow-through enzyme-based biosensors for the detection of glucose and lactate have been developed by Cammann and co-workers. Glucose oxidase and lactate oxidase were immobilized in conducting polymers generated from pyrrole, N-methylpyrrole, aniline, and o-phenylenediamine on platinum surfaces. These various sensor matrices were compared on basis of amperometric measurements of glucose and lactate, and the o-phenylenediamine polymer was found to be the most sensitive.
This polymer matrix was also deposited on a piece of graphite felt and used as an enzyme reactor as well as a working electrode in an electrochemical detection system. Using this system, a linear dynamic range of 500 μM to 10 mM glucose was determined with a detection limit of <500 μM. For lactate, the linear dynamic range covered concentrations from 50 μM to 1 mM with a detection limit of <50 μM.

A biosensor for protein and amino acid estimation has been reported by Sarkar and Turner. A screen-printed biosensor based on a rhodinized carbon-paste working electrode was used in the three-electrode configuration for a two-step detection method. Electrolysis of an acidic potassium bromide electrolyte at the working electrode produced bromine, which was consumed by the proteins and amino acids. The bromine production occurred at one potential while monitoring of the bromine consumption was performed using a lower potential. The method proved very sensitive to almost all of the amino acids, as well as some common proteins, and was even capable of measuring L- and D-proline, which gave no response to enzyme-based biosensors. This sensor has been tested by measuring proteins and amino acids in fruit juice, milk, and urine.

Scheller and co-workers have developed an electrochemical biosensor for the indirect detection of L-phenylalanine via NADH. This sensor is based on a three-step multi-enzymatic/electrochemical reaction. Three enzymes — L-phenylalanine dehydrogenase, salicylate hydroxylase, and tyrosinase — are immobilized in a carbon paste electrode. The principle behind this reaction/detection scheme is as follows. First, the L-phenylalanine dehydrogenase, upon binding and reacting with L-phenylalanine, produces NADH. The second enzyme, salicylate hydroxylase, then converts salicylate to catechol in the presence of oxygen and NADH. The tyrosinase then oxidizes the catechol to o-quinone, which is detected electrochemically, and reduced back to catechol with an electrode potential of ~50 mV vs. an Ag/AgCl reference electrode.

This reduction step results in an amplification of signal due to the recycling of catechol from o-quinone. Prior to the addition of the L-phenylalanine dehydrogenase to the electrode, it was tested for its sensitivity to NADH, its pH dependence, and its response to possible interferents, urea, and ascorbic acid. From these measurements, it was found that the sensor sensitivity for NADH increased 33-fold by introducing the recycling step over the salicylate hydroxylase system alone. When this sensor was tested for the detection of L-phenylalanine in human serum, the linear dynamic range was found to cover concentrations ranging from 20 to 150 μM with a detection limit of 5 μM, which is well within the clinical range of 78 to 206 μM.

### 20.3.3 Mass-Sensitive Detection

Another form of transduction has also been used in biosensors to measure small changes in mass. Mass-based detection is the newest of the three classes of transducers and has already been shown to be capable of sensitive measurements. Mass analysis relies on the use of piezoelectric crystals that can be made to vibrate at a specific frequency with the application of an electrical signal of a specific frequency. The frequency of oscillation is dependent on the electrical frequency applied to the crystal as well as the crystal’s mass. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency of the crystal changes and the resulting change can be measured electrically and used to determine the mass added to the crystal. In most cases the added mass consists of antibodies or DNA fragments bound to their biospecific counterparts that have been immobilized on the sensor surface.

Guilbault and co-workers developed a quartz crystal microbalance biosensor for the detection of *Listeria monocytogenes*. Several approaches were tested for the immobilization of *Listeria* onto the quartz crystal through a gold film on the surface. Once bound, the microbalance was then placed in a liquid flow-cell, where the antibody and antigen were allowed to form a complex, and measurements were obtained. Calibration of the sensor was accomplished using a displacement assay and was found to have a response range from $2.5 \times 10^4$ to $2.5 \times 10^5$ cells/crystal. More recently, Guilbault and co-workers have also developed a method for covalently binding antibodies to the surface of piezoelectric crystals via sulfur-based self-assembled monolayers. Prior to antibody binding, the monolayers are activated with 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride
and N-hydroxysulfosuccinimide. Using this binding technique, a real-time capture assay based on mouse IgG was performed and results were reported.

The first use of a horizontally polarized surface acoustic wave biosensor has been reported by Hunklinger and co-workers. This sensor has a dual path configuration, with one path acting as an analyte-sensitive path and the other acting as a reference path. A theoretical detection limit of 33 pg was calculated based on these experiments, and a sensitivity of 100 kHz/(ng/mm²) is reported. In addition, a means of inductively coupling a surface acoustic wave biosensor to its radio-frequency generating circuitry has been reported recently. This technique could greatly reduce problems associated with wire bonding for measurements made in liquids because the electrodes are coated with a layer of SiO₂.

A relatively new type of mass-based detection system uses microcantilevers. Constructed of silicon, these devices are generally shaped like a microsize diving board. Their advantages include their miniature size, high degree of sensitivity, simplicity, low power consumption, low manufacturing cost, and compatibility with array designs. The extremely low mass of the device allows it to sense perturbing forces because of the adsorbed masses at the picogram level, the viscosity of a gas or liquid over several orders of magnitude, and the acoustic and seismic vibrations. Special coatings on the silicon will adapt the cantilever to sense relative humidity, temperature, mercury, lead, ultraviolet radiation, and infrared (IR) radiation. By using current micromachining technology, multiple arrays could be used to make multielement or multitarget sensor arrays involving hundreds of cantilevers without significantly increasing the size, complexity, or overall package costs.

20.4 Bioreceptors and Biosensor Systems

20.4.1 Antibody

20.4.1.1 Antibody Bioreceptors

The basis for the specificity of immunoassays is the antigen-antibody (Ag-Ab) binding reaction, which is a key mechanism by which the immune system detects and eliminates foreign matter. The enormous range of potential applications of immunosensors is due, at least in part, to the astonishing diversity possible in one of their key components, antibody molecules. Antibodies are complex biomolecules made up of hundreds of individual amino acids arranged in a highly ordered sequence. The structure of an IgG antibody molecule is schematically illustrated in Figure 20.3A. The antibodies are actually produced by immune system cells (B cells) when such cells are exposed to substances or molecules called antigens. The antibodies called forth following antigen exposure have recognition/binding sites for specific molecular structures (or substructures) of the antigen.

The way in which an antigen and an antigen-specific antibody interact may perhaps be understood as analogous to a lock-and-key fit, in which the specific configurations of a unique key enable it to open a lock. In the same way, an antigen-specific antibody fits its unique antigen in a highly specific manner, so that hollows, protrusions, planes, and ridges on the antigen and the antibody molecules. (in a word, the total three-dimensional structure) are complementary. Further details of how such complementarity is achieved will be discussed later in this chapter. It is sufficient at this point simply to indicate that, due to this three-dimensional shape fitting, and the diversity inherent in individual antibody makeup, it is possible to find an antibody that can recognize and bind to any one of a huge variety of molecular shapes.

This unique property of antibodies is the key to their usefulness in immunosensors; the ability to recognize molecular structures allows one to develop antibodies that bind specifically to chemicals, biomolecules, microorganism components, etc. One can then use such antibodies as specific detectors to identify and find an analyte of interest that is present, even in extremely small amounts, in a myriad of other chemical substances.

The other antibody property of paramount importance to their analytical role in immunosensors is the strength or avidity/affinity of the antigen–antibody interaction. Because of the variety of interactions that can take place as the Ag-Ab surfaces lie in close proximity to each other, the overall strength of the interaction can be considerable, with correspondingly favorable association and equilibrium constants. What this means
in practical terms is that the Ag–Ab interactions can take place very rapidly (for small antigen molecules, almost as rapidly as diffusion processes can bring antigen and antibody together) and that, once formed, the Ag–Ab complex has a reasonable lifetime. Figure 20.10 shows a schematic of how biosensor probes can be prepared from antibody production, isolation, and binding to the sensing surface.

For an immune response to be produced against a particular molecule, a certain molecular size and complexity are necessary: proteins with molecular weights greater than 5000 Da are generally immunogenic. Radioimmunoassay (RIA), which utilizes radioactive labels, has been one of the most widely used immunoassay methods. RIA has been applied to a number of fields, including pharmacology, clinical chemistry, forensic science, environmental monitoring, molecular epidemiology and agricultural science. The usefulness of RIA, however, is limited by several shortcomings, including the cost of instrumentation, the limited shelf life of radioisotopes, and the potential deleterious biological effects inherent to radioactive materials. For these reasons, extensive research efforts are aimed at developing simpler, more practical immunochemical techniques and instrumentation that offer comparable sensitivity and selectivity to RIA. In the 1980s, advances in spectrochemical instrumentation, laser miniaturization, biotechnology, and fiber-optic research provided opportunities for novel approaches to the development of sensors for the detection of chemicals and biological materials of environmental and biomedical interest.

Since the development of a remote fiber-optic immunosensor for in situ detection of the chemical carcinogen benzo[a]pyrene, antibodies have become common bioreceptors used in biosensors today. The schematics for the optical detection of a bioanalyte are shown in Figure 20.11. In the arrangement shown, a single optical fiber carries the excitation light source to the sample and the fluorescence signal back to a spectrometer.

Due to fiber-to-fiber differences in fiber-optic biosensors, there is often a great difficulty in normalizing the spectral signal obtained with one fiber to another fiber. Ligler and co-workers reported on a method for calibrating antibody-based biosensors using two different fluorescent dyes. To accomplish this, they labeled the capture antibodies bound to the fiber with one fluorescent dye and the antigen with a different dye. Both dyes were excited at the same wavelength and their fluorescence was monitored. The resultant emission spectrum of the fluorescence signal from the capture antibodies was used to normalize the signal from the tagged antigen.

Another example of antibody-based biosensors for bioanalysis is the development of an electrochemical immunoassay for whole blood. This work involved the development of a sandwich type of separationless amperometric immunoassay without any washing steps. The assay is performed on a conducting redox hydrogel on a carbon electrode on which avidin and choline oxidase have been co-immobilized. Biotinylated antibody is then bound to the gel. When the antigen binds to the sensor, another solution of complementary horseradish peroxidase-labeled antibody is bound to the antigen, thus creating an electrical contact between the redox hydrogel and the peroxidase. The hydrogel then acts as an electrocatalyst for the reduction of hydrogen peroxide water.

An important aspect of biosensor fabrication is the binding of the bioreceptor to the sensor solid support or to the transducer. Vogel and co-workers report on a method for the immobilization of histidine-tagged antibodies onto a gold surface for surface plasmon resonance measurements. A synthetic thio-alkane chelator is self-assembled on a gold surface. Reversible binding of an anti-lysozyme Fab fragment with a hexahistidine-modified extension on the C terminal end is then performed. Infrared spectroscopy was used to determine that the secondary structure of the protein was unaffected by the immobilization process. Retention of antibody functionality upon immobilization was also demonstrated.

Due to the reversible binding of such a technique, this could prove a valuable method for regeneration of biosensors for various applications. Enzyme immunoassays can further increase the sensitivity of detection of antigen–antibody interactions by the chemical amplification process, whereby one measures the accumulated products after the enzyme has been allowed to react with excess substrate for a period of time.

With the use of nanotechnology, submicron fiber-optic antibody-based biosensors have been developed by Vo-Dinh and co-workers for the measurements of biochemicals inside a single cell. Nanometer-scale
fiberoptic biosensors were used for monitoring biomarkers related to human health effects associated with exposure to polycyclic aromatic hydrocarbons (PAHs). These sensors use a monoclonal antibody for benzo[a]pyrene tetrode (BPT), a metabolite of the carcinogen benzo[a]pyrene, as the bioreceptor. Excitation light is launched into the fiber and the resulting evanescent field at the tip of the fiber is used to excite any of the BPT molecules that have bound to the antibody. The fluorescent light is then collected via a microscope. Using these antibody-based nanosensors, absolute detection limits for BPT of approximately 300 zeptomol (10⁻²¹ mol) have been reported. These nanosensors allow probing of cellular and subcellular environments in single cells. The development and applications of optical nanosensors are described in further detail in Chapter 60 of this handbook.

20.4.1.2 Immunoassay Formats

Biomolecular interactions can be classified into two categories according to the test format performed (direct or indirect). In a direct format the immobilized target molecule interacts with a ligand molecule or the immobilized ligand interacts with a target molecule directly. For immunosensors, the simplest situation involves in situ incubation followed by direct measurement of a naturally fluorescent analyte. For nonfluorescent analyte systems, in situ incubation is followed by development of a fluorophor-labeled second antibody. The resulting antibody sandwich produces a fluorescence signal directly proportional to the amount of bound antigen. The sensitivity obtained when using these techniques increases with increasing amounts of immobilized receptor. The indirect format involves competition between fluorophor-labeled and unlabeled antigens. In this case, the unlabeled analyte competes with the labeled analyte for a limited number of receptor binding sites. Assay sensitivity therefore increases with decreasing amounts of immobilized reagent. Figure 20.8 illustrates the principles of (1) a competitive assay, (2) a direct assay, and (3) a sandwich assay.

A sandwich immunoassay using fluorescently labeled tracer antibodies has been developed to detect cholera toxin (CT). Using this fluorescence-based biosensor, researchers analyzed six samples simultaneously in 20 min. The biochemical assays utilized a ganglioside-capture format: ganglioside GM1, utilized for capture of the analyte, was immobilized in discrete locations on the surface of the optical waveguide. Binding of CT to the immobilized GM1 was demonstrated with direct assays (using fluorescently labeled CT). The limits of detection for CT were 200 ng/ml in direct assays and 40 ng/ml and 1 ng/ml in sandwich-type assays performed using rabbit and goat tracer antibodies. Binding of CT to other glycolipid capture reagents was also observed. While significant CT binding to loci patterned with GD1b, Gb3, and Gb4 was observed, CT did not bind significantly to immobilized GT1b at the concentrations tested.

A similar planar array, equipped with a charge-coupled device (CCD) as a detector, was used to detect three toxic analytes simultaneously. Wells approximately 2 mm in diameter were formed on glass slides using a photoactivated optical adhesive. Antibodies against staphylococcal enterotoxin B (SEB), ricin, and Yersinia pestis were covalently attached to the bottoms of the circular wells to form the sensing surface. Rectangular wells containing chicken immunoglobulin were used as alignment markers and to generate control signals. After the optical adhesive was removed, the slides were mounted over a CCD operating at ambient temperature in inverted (multipin phasing) mode. Cy5-labeled antibodies were used to determine the identity and amount of toxin bound at each location, using quantitative image analysis. Concentrations as low as 25 ng/ml of ricin, 15 ng/ml of pestis F1 antigen, and 5 ng/ml of SEB could be routinely measured.

A similar assay for ricin, using an immobilized anti-ricin IgG on an optical fiber surface, was reported. Two immobilization methods were tried: in the first, the antibody was directly coated onto the silanized fiber using a cross linker; in the second, avidin-coated fibers were incubated with biotinylated antiricin IgG to immobilize the antibody using an avidin–biotin bridge. The assay using the avidin–biotin-linked antibody demonstrated higher sensitivity and wider linear dynamic range than the assay using antibody directly conjugated to the surface. The limits of detection for ricin in buffer solution and river water are 100 pg/ml and 1 ng/ml, respectively.

The use of protein A, an immunoglobulin-binding protein, for antibody immobilization on the surface of these fiber probes has been investigated as an alternative immobilization method to the
classical avidin–biotin and IgG–anti-IgG interactions. No difference was observed in the binding of fluorescently labeled goat IgG by rabbit anti-goat IgG, regardless of whether the capture antibody was bound to the probe surface via protein A or covalently attached. However, in a sandwich immunoassay for the F1 antigen of *Yersinia pestis*, probes with rabbit anti-plague IgG bound to the surface via protein A generated twice the signal generated by the probes with the antibody covalently attached. Assay regeneration was also examined with protein A probes because Ab–Ag complexes have been successfully eluted from protein A under low pH conditions.

The regeneration of antibodies covalently immobilized to an optical fiber surface is also an important parameter that classifies the usefulness of a biosensor. Ideally, the antibody–antigen complex can be disso- ciated under mild conditions to regenerate the sensor. In a study by Liegler and co-workers, three different restoring solutions were tested and compared: (1) 0.1 M glycine hydrochloride in 50% (v/v) ethylene glycol, pH = 1.75; (2) a basic solution 0.05 M tetraethylamine in 50% (v/v) ethylene glycol, pH = 11.0; and (3) 50% (v/v) ethanol in PBS. In this study, optical fibers coated with polyclonal rabbit anti-goat antibody against a large protein retained 70 and 65% of the original signal after five consecutive regenerations with acidic and basic solvent systems, respectively. The fibers coated with monoclonal mouse anti-trinitrobenzene antibody specific for a small organic molecule retained over 90% of the original signal when regenerated with basic and ethanol solutions.

Using a 635-nm laser diode light source, a biosensor based on labeled Cy5 antibodies was used to detect the F1 antigen of *Y. pestis* and the protective antigen of *Bacillus anthracis*. In a blind test

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**FIGURE 20.8** Immunoassay formats: (A) competitive assay; (B) direct assay; (C) sandwich assay.
containing F1 antigen spiked into 30 of 173 serum samples, this immunosensor was able to achieve 100% detection success for samples with 100 ng/ml or more of F1 antigen, with a specificity of 88%.

### 20.4.1.3 Antibody Probe Regeneration

Removal of antigens bound to antibodies covalently attached to optical fiber surfaces is one of the limiting factors in the development of reusable, inexpensive, and reliable optical fiber immunosensors for environmental and clinical analysis. Chemical reagents were supposed to cleave the binding between antibody and antigen, thus regenerating the biosensor — a chemical procedure that is simple but ineffective after multiple regeneration operations (less than five cycles), due to possible denaturation of the antibody. Another approach in the development of regenerable biosensors involves the design of microcapillary systems capable of delivering and removing reagents and antibody-coated microbeads into the sensing chamber without removing the sensor from the sample. Several investigators have searched for fast dissociation protocols able to regenerate immobilized antibodies while maintaining their stability for use in routine analysis, commercial immunosorbents, or optical fiber sensors.

Antigen–antibody interactions can be classified in three different groups: hydrophobic interactions, electrostatic (or Coulombic) interactions, and interactions due to a combination of both forces. Hydrophobic interactions are due to the propensity of nonpolar groups and chains to aggregate when immersed in water. This type of interaction is maximized between the hydrophobic complementary determining regions (CDR) of the antibody paratopes and the predominantly hydrophilic groups found in the antigen epitope. Electrostatic interactions between antigen and antibody are caused by one or more ionized sites of the epitope and ions of opposite charge on the paratope. After primary binding has occurred through hydrophobic and electrostatic interactions, the epitope and the paratope will be close enough to allow van der Waals and hydrogen bonds to become operative. In order to dissociate the antigen–antibody complexes, the strength of these forces may be reduced by changing the pH, ionic strength, and temperature through the addition of dehydrating agents and/or organics. In this sense, strong acids such as HCl or H₂SO₄, mixtures such as glycine-HCl, or basic solutions of tetraethylamine, for example, have been used when the primary attractive forces in the bond can be considered electrostatic interactions.

Lu et al. pointed out that the use of organic solutions such as ethylene glycol could improve the washing efficiency by reducing the van der Waals and hydrogen bonds to become operative. In order to dissociate the antigen–antibody complexes, the strength of these forces may be reduced by changing the pH, ionic strength, and temperature through the addition of dehydrating agents and/or organics. In this sense, strong acids such as HCl or H₂SO₄, mixtures such as glycine-HCl, or basic solutions of tetraethylamine, for example, have been used when the primary attractive forces in the bond can be considered electrostatic interactions.

Higher-frequency ultrasound (high-kHz to low-MHz range) is used to remove small (micrometer to submicrometer) particles from the surfaces of silicon wafers. This so-called megasonic cleaning process does not damage the surfaces, suggesting that its mode of action does not depend on the strong effects of inertial cavitation that occur with lower frequency (e.g., 20 kHz) ultrasonic horns and baths. The mechanisms of megasonic cleaning likely involve nonlinear effects such as acoustic streaming and radiation pressure as well as the oscillatory linear forces. Stable cavitation (a less intense process than inertial cavitation) may also play an important role, particularly in enhancing streaming effects. The success of the gentle but effective megasonic cleaning process suggests that MHz-range ultrasound may have some utility in regeneration of biosensor surfaces.

Vo-Dinh and co-workers described a novel procedure for regenerating antibodies immobilized on a fiber optic surface with ultrasonic irradiation using a broadband imaging transducer operating near 5 MHz. This type of ultrasound device is commonly used for the detection of flaws in the nondestructive evaluation of engineering materials and measurement of the mechanical properties of various media, including biological tissues. The use of ultrasound for the regeneration of optical fiber immunosensors could be an important advance in the application of these devices for in vivo
and in situ measurements because it would no longer be necessary to supply a regeneration solution to the sensor system that could lead to the denaturation of the immobilized antibody.

20.4.2 Enzyme

Enzymes are often used as bioreceptors because of their specific binding capabilities as well as their catalytic activity. In biocatalytic recognition mechanisms, the detection is amplified by a catalytic reaction. This is the basis for the now commonplace enzyme-linked immunosorbent assay (ELISA) technique.

With the exception of a small group of catalytic ribonucleic acid molecules, all enzymes are proteins. Some enzymes require no chemical groups other than their amino acid residues for activity. Others require an additional component called a cofactor, which may be either one or more inorganic ions, such as Fe$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$, or a more complex organic or organometallic molecule called a coenzyme. The catalytic activity provided by enzymes allows for much lower limits of detection than would be obtained with common binding techniques. As expected, the catalytic activity of enzymes depends upon the integrity of their native protein conformation. If an enzyme is denatured, dissociated into its subunits, or broken down into its component amino acids, its catalytic activity is destroyed. Enzyme-coupled receptors can also be used to modify the recognition mechanisms. For instance, the activity of an enzyme can be modulated when a ligand binds at the receptor. This enzymatic activity is often greatly enhanced by an enzyme cascade, which leads to complex reactions in the cell.

Gauglitz and co-workers have immobilized enzymes onto an array of optical fibers for use in the simultaneous detection of penicillin and ampicillin. These biosensors provide an interferometric technique for measuring penicillin and ampicillin based on pH changes during their hydrolysis by penicillinase. Immobilized onto the fibers with the penicillinase is a pH indicator, phenol red. As the enzyme hydrolyzes the two substrates, shifts in the reflectance spectrum of the pH indicator are measured. Various types of data analysis of the spectral information were evaluated using a multivariate calibration method for the sensor array, which consisted of different biosensors.

Rosenzweig and Kopelman described the development and use of a micrometer-sized fiber-optic biosensor for the detection of glucose. These biosensors are 100 times smaller than existing glucose optodes and represent the beginning of a new trend in nanosensor technology. They are based on the enzymatic reaction of glucose oxidase, which catalyses the oxidation of glucose and oxygen into gluconic acid and hydrogen peroxide. To monitor the reaction, an oxygen indicator, tris(1,10-phenanthroline)ruthenium chloride, is immobilized into an acrylamide polymer with the glucose oxidase, and this polymer is attached to the optical fiber via photopolymerization. A comparison of the response of glucose sensors created on different sizes of fibers found that the micrometer-size sensors have response times at least 25 times faster (2 s) than the larger fibers. In addition, these sensors are reported to have absolute detection limits of approximately 10 to 15 mol and an absolute sensitivity five to six orders of magnitude greater than current glucose optodes.

A fiber-optic evanescent wave immunosensor for the detection of lactate dehydrogenase has been developed. Two different assay methods, a one-step and a two-step process, using the sensor based on polyclonal antibody recognition were described. The response of this evanescent wave immunosensor was then compared to a commercially available SPR-based biosensor for lactate dehydrogenase detection using similar assay techniques, and similar results were obtained. It was also demonstrated that, although the same polyclonal antibody can be used for the one- and the two-step assay techniques, the two-step technique is significantly better when the antigen is large.

20.4.3 Nucleic Acid

20.4.3.1 Nucleic Acid Bioreceptors

Another biorecognition mechanism involves hybridization of DNA or RNA. In the last decade, interest in nucleic acids as bioreceptors for biosensor and biochip technologies has increased. The complementarity of the pairing of the nucleotides adenine:thymine (A:T) and cytosine:guanine
(C,G) in a DNA ladder (Figure 20.3B) forms the basis for the specificity of biorecognition in DNA biosensors, often referred to as genosensors. If the sequence of bases composing a certain part of the DNA molecule is known, then the complementary sequence, often called a probe, can be synthesized and labeled with an optically detectable compound (e.g., a fluorescent label). By unwinding the double-stranded DNA into single strands, adding the probe, and then annealing the strands, the labeled probe can be made to hybridize to its complementary sequence on the target molecule.

Grabley and co-workers have reported the use of DNA biosensors for monitoring DNA–ligand interactions. Surface plasmon resonance was the analytical method used to monitor real-time binding of low-molecular-weight ligands to DNA fragments that were irreversibly bound to the sensor surface via Coulombic interactions. The sensor was capable of detecting binding effects between 10 and 400 pg/mm². Binding rates and equilibrium coverages were determined for various ligands by changing the ligand concentration. In addition, affinity constants, association rates, and dissociation rates were also determined for these various ligands.

Sandwich-type biosensors based on liquid-crystalline dispersions formed from DNA-polycation complexes have been described by Yevdokimov and co-workers. These sandwich biosensors have been shown to be useful for detection of compounds and physical factors that affect the ability of specific DNA cross linkers — polycationic molecules — to bind between adjacent DNA molecules. The specific case of dispersions from DNA/protamine complexes was investigated and it was demonstrated that, by using this type of sensor with this complex, the hydrolytic enzyme trypsin could be measured down to concentrations of approximately 10⁻¹⁴ M.

Karube and co-workers demonstrated another type of biosensor that uses a peptide nucleic acid as the biorecognition element. The peptide nucleic acid is an artificial oligo amide capable of binding very strongly to complementary oligonucleotide sequences. By use of a surface plasmon resonance sensor, the direct detection of double stranded DNA that had been amplified by a polymerase chain reaction (PCR) has been demonstrated. This technique was capable of monitoring the target DNA over a concentration range of 40 to 160 nM, corresponding to an absolute detection limit of 7.5 pmol.

Using a unique analytical technique, Vo-Dinh and co-workers have developed a new type of DNA gene probe based on SERS detection. The SERS probes do not require the use of radioactive labels and have great potential to provide sensitivity and selectivity via label multiplexing due to the intrinsically narrow bandwidths of Raman peaks. The effectiveness of the new detection scheme is demonstrated using the gag gene sequence of the human immunodeficiency (HIV) virus. A SERS-based DNA assay for the breast cancer susceptibility gene (BRCA1) has also been developed. The assay is based on the immobilization of oligonucleotides on a thin silver surface and hybridization with Rhodamine-labeled probes. The silver surface serves as the hybridization support and the means for Raman signal enhancement. The development of a biosensor for DNA diagnostics using visible and NIR dyes has been reported. The system employed a two-dimensional charge-coupled device and was used to detect the cancer suppressor p53 gene.

### 20.4.3.2 DNA Biosensors

DNA sensors are usually based on hybridization assays and may incorporate simultaneous analytical capability to detect a large number of oligonucleotide fragments. A fiber-optic DNA biosensor microarray for the analysis of gene expression has been reported for the simultaneous analysis of multiple DNA sequences using fluorescent probes.

Fluorescent intercalating and groove-binding dyes that can associate with double-stranded DNA (dsDNA) are used for detection of hybridization in some sensor and biochip designs. It is possible that dye–dye interactions at concentrations relevant to biosensor use can lead to unexpected and undesired emission wavelength shifts and fluorescence quenching interactions. To maximize signal to noise, many biosensors utilize dye concentrations in large excess in comparison to the quantity of immobilized DNA. The linearity of fluorescence intensity response of dyes intercalated to dsDNA may vary with different dye:base pair ratios.
A very common alternative to intercalating fluorescent dyes for the detection of dsDNA are covalently bound dyes. Dyes attached to the terminus of a strand of DNA through a short hydrocarbon chain (also known as a tether) are continuously available for hybridization and allow the biosensor to be fully reversible.25

Another important characteristic of the biosensor assay is the choice of the fluorescent dye that acts as the signal transducer. Traditionally, ethidium bromide (EB) has been used extensively to detect hybridization of DNA in applications such as electrophoresis, gene chips, and biosensors. A number of dyes with greater quantum efficiency than EB for detection of hybridization have been reported. Furthermore, other practical spectroscopic advantages can be gained in terms of improved signal-to-noise ratio (SNR) by use of dyes with excitation that is red-shifted relative to EB. Pyrilium iodide has been shown to be an intercalator of high quantum efficiency and long excitation wavelength.25

One type of DNA sensor utilized the direct synthesis of an ssDNA sequence directly onto optical fibers, using the well established solid-phase phosphoramidite methodology. The covalently immobilized oligomers were able to hybridize with available complementary ssDNA, which was introduced into the local environment to form dsDNA. This event was detected by the use of the fluorescent DNA stain ethidium bromide (EB). The sampling configuration utilized total internal reflection of optical radiation within the fiber, resulting in an intrinsic-mode optical sensor. The nonoptimized procedure used standard hybridization assay techniques to provide a detection limit of 86 ng ml–1 cDNA, a sensitivity of 83% fluorescence intensity increase per 100 ng ml –1 of cDNA initially present, with a hybridization analysis time of 46 min. The sensor has been observed to sustain activity after prolonged storage times (3 months) and harsh washing conditions (sonication).26 A similar sensor fabricated using quartz optical fibers as the support provided very similar results.26

A very interesting biosensor capable of detecting triple-helical DNA formation was also based on the direct synthesis of oligonucleotides on the surface of fused silica optical fibers, using a DNA synthesizer. Two sets of oligonucleotides on different fibers were grown in the 3’ to 5’ and 5’ to 3’ directions, respectively. Fluorescence studies of hybridization showed unequivocal hybridization between oligomers immobilized on the fibers and complementary oligonucleotides from the solution phase, as detected by fluorescence from intercalated EB. The complementary oligonucleotide, dT(10), which was expected to hybridize when the system was cooled below the duplex melting temperature, provided a fluorescence intensity with a negative temperature coefficient. Upon further cooling, to the point where the pyrimidine motif T*AT triple-helix formation occurred, a fluorescence intensity change with a positive temperature coefficient was observed.27

In another study, the same type of sensor, with directly immobilized ssDNA on optical fibers, was used to monitor variation in the melt temperature of dsDNA. Because of microenvironment conditions, the local ionic strength, the pH, and the dielectric constant at the surface can be substantially different from those in bulk electrolyte solution. The local conditions influence the thermodynamics of hybridization and can be studied by the melt temperature of dsDNA. Fiber-optic biosensors with dT(20) oligonucleotides attached to their surfaces were used to determine the Tm from the dissociation of duplexes of fluorescein-labeled and unlabeled dA(20) and d(A(9)GA(10)). Each thermal denaturation of dsDNA at the surface of the optical fibers was accompanied by a two- to threefold reduction in standard enthalpy change relative to values determined for denaturation in bulk solution. The experimental results suggested that the thermodynamic stability of duplexes immobilized on a surface is dependent on the density of immobilized DNA.

Additionally, the deviation in melt temperature, arising as a result of the presence of a centrally located single base-pair mismatch, was significantly larger for thermal denaturation occurring at the surface of the optical fibers (ΔTm = 6 to 10°C) relative to that observed in bulk solution (ΔTm = 3.8 to 6.1°C). These results suggest that hybridization at an interface occurs in a significantly different physical environment from hybridization in bulk solution, and that surface density can be tuned to design analytical figures of merit.109 Increased immobilization density resulted in significantly higher sensitivity but reduced dynamic range in all hybridization assays conducted. Sensitivity and selectivity
were functions of temperature; however, the selectivity of hybridization assays done using the sensors could not be predicted by consideration of thermal denaturation temperatures alone.  

20.4.4 Cell-Based Systems

Cellular structures and cells comprise a broad category of bioreceptors that have been used in the development of biosensors and biochips. These bioreceptors are based on biorecognition by an entire cell or microorganism or by a specific cellular component capable of specific binding to certain species. There are presently three major subclasses of this category: (1) cellular systems, (2) enzymes, and (3) nonenzymatic proteins. Due to the importance and large number of biosensors based on enzymes, these have been given their own classification and were previously discussed in Section 20.4.2. This section deals with cellular systems and nonenzymatic proteins.

20.4.4.1 Cellular Bioreceptors

Microorganisms offer a form of bioreceptor that often allows a whole class of compounds to be monitored. Generally these microorganism biosensors rely on the uptake of certain chemicals into the microorganism for digestion. Often, a class of chemicals is ingested by a microorganism, therefore allowing a class-specific bioreceptor to be created. Microorganisms such as bacteria and fungi have been used as indicators of toxicity or for the measurement of specific substances. For example, cell metabolism (e.g., growth inhibition, cell viability, substrate uptake), cell respiration, and bacterial bioluminescence have been used to evaluate the effects of toxic heavy metals. Many cell organelles can be isolated and used as bioreceptors. Cell organelles are essentially closed systems, so they can be used over long periods of time. Whole mammalian tissue slices or in vitro cultured mammalian cells are used as biosensing elements in bioreceptors. Plant tissues are also used in plant-based biosensors; they are effective catalysts because of the enzymatic pathways they possess.

Bilitewski and co-workers have developed a microbial biosensor for monitoring short-chain fatty acids in milk. Arthrobacter nicotianae microorganisms were immobilized in a calcium-alginate gel on an electrode surface. To this gel was added 0.5 mM CaCl₂ to help stabilize it. Monitoring the oxygen consumption of the A. nicotianae electrochemically allowed its respiratory activity to be monitored, thereby providing an indirect means of monitoring fatty acid consumption. Detection of short-chain fatty acids in milk, ranging from 4 to 12 carbons in length, was accomplished with butyric acid as the major substrate. A linear dynamic range from 9.5 to 165.5 µM was reported, with a response time of 3 min.

20.4.4.2 Nonenzymatic Proteins

Many proteins found within cells often serve the purpose of bioreception for intracellular reactions that will take place later or in another part of the cell. These proteins could simply be used for transport of a chemical from one place to another, e.g., a carrier protein or channel protein on a cellular surface. In any case, these proteins provide a means of molecular recognition through one or another type of mechanism (i.e., active site or potential sensitive site). By attaching these proteins to various types of transducers, many researchers have constructed biosensors based on nonenzymatic protein biorecognition.

In one recent application, Cusanovich and co-workers developed micro- and nanobiosensors for nitric oxide that are free from most potential interferents. These sensors are based on bioreception of nitric oxide by cytochrome c. Two different techniques of immobilization of the cytochrome c to fibers were tested: polymerization in an acrylamide gel and reversible binding using a gold colloid-based attachment. The cytochrome used in this work was labeled with a fluorescent dye that is excited via an energy transfer from the hemoprotein. Response times of faster than 1 s were reported along with a detection limit of 20 µM. Cytochrome c samples from three different species of bacteria were evaluated.

Vogel and co-workers have reported on the use of lipopeptides as bioreceptors for biosensors. A lipopeptide containing an antigenic peptide segment of VP1, a capsid protein of the picornavirus that causes foot-and-mouth diseases in cattle, was evaluated as a technique for monitoring antigen–antibody interactions. The protein was characterized via circular dichroism and infrared spectroscopy to verify that upon self-assembly onto a solid surface it retained the same structure as in
its free form. Based on surface plasmon resonance measurements, it was found that the protein was still fully accessible for antibody binding. This technique could provide an effective means of developing biomimetic ligands for binding to cell surfaces.

### 20.4.5 Biomimetic Receptors

A receptor fabricated and designed to mimic a bioreceptor is often termed a “biomimetic receptor.” Several different methods have been developed over the years for the construction of biomimetic receptors.123–128 These methods include genetically engineered molecules, artificial membrane fabrication, and molecular imprinting. The molecular imprinting technique, which has recently received great interest, consists of mixing analyte molecules with monomers and a large number of cross-linkers. Following polymerization, the hard polymer is ground into a powder, and the analyte molecules are extracted with organic solvents to remove them from the polymer network. As a result, the polymer has molecular holes or binding sites that are complementary to the selected analyte.

Recombinant techniques, which allow for the synthesis or modification of a wide variety of binding sites using chemical means, have provided powerful tools for designing synthetic bioreceptors with desired properties. Hellinga and co-workers reported the development of a genetically engineered single-chain antibody fragment for monitoring phosphorylcholine.129 In this work, protein engineering techniques are used to fuse a peptide sequence that mimics the binding properties of biotin to the carboxyterminus of the phosphorylcholine-binding fragment of IgA. This genetically engineered molecule can be attached to a streptavidin monolayer, and total internal reflection fluorescence was used to monitor the binding of a fluorescently labeled phosphorylcholine analog.

Artificial membranes have been developed for many different bioreception applications. Stevens and co-workers developed one by incorporating gangliosides into a matrix of diacetylenic lipids (5 to 10% of which were derivatized with sialic acid).130 The lipids were allowed to self-assemble into Langmuir-Blodgett layers and were then photopolymerized via ultraviolet irradiation into polydiacetylene membranes. When cholera toxins bind to the membrane, its natural blue color changes to red; absorption measurements were used to monitor the toxin concentration. Using these polydiacetylenic lipid membranes coupled with absorption measurements, concentrations of cholera toxin as low as 20 μg/ml could be monitored.

Molecular imprinting has been used for the construction of a biosensor based on electrochemical detection of morphine.131 A molecularly imprinted polymer for the detection of morphine was fabricated on a platinum wire using agarose and a cross-linking process. The resulting imprinted polymer was used to specifically bind morphine to the electrode. Following morphine binding, an electroinactive competitor, codeine, was used to wash the electrode and thus release some of the bound morphine. The freed morphine was then measured by oxidation at the electrode; concentrations ranging from 0.1 to 10 μg/ml were analyzed, with a reported detection limit of 0.05 μg/ml. One of the major advantages of the molecular imprinting technique is the rugged nature of a polymer relative to a biological sample. The molecularly imprinted polymer can withstand harsh environments such as those experienced in an autoclave or chemicals that would denature a protein.

### 20.5 Probe Development: Immobilization of Biomolecules

Many of the methods used in biosensor fabrication involve binding the recognition probe (oligonucleotide strand, antibody, etc.) to a sensor-sensitive surface or to an optically active tag (fluorescent dye, etc.). For enzyme-based sensors, immobilization of an enzyme is a critical step and can be accomplished via simple physical adsorption or through more elaborate covalent binding schemes.

Molecules may be physically immobilized in a solid support through hydrophobic or ionic interactions or covalently immobilized by attachment to activated surface groups.132 Noncovalent immobilization is effective for many applications and usually requires easier and faster preparation steps.133–135 In addition, the adsorbed molecules usually preserve their original properties (e.g., wavelength of absorption, excitation
or emission, enzymatic activity) because they do not require the structural modification inherent in covalent immobilization to a solid support. However, continuous leaching of the adsorbate from the solid support may reduce the sensor’s durability and even render it useless in the worst cases.

Covalent immobilization is often necessary for binding molecules that do not adsorb, adsorb very weakly, or adsorb with improper orientation and conformation to noncovalent surfaces. Covalent immobilization may provide greater stability, reduced nonspecific adsorption, and greater durability.136,137

Several synthetic techniques are available for the covalent immobilization of biomolecules or labeling of a sensor probe with a fluorescent dye.138 Most of these techniques use free amine groups in a polypeptide (enzymes, antibodies, antigens, etc.) or in an amino-labeled DNA strand to react with a carboxylic acid moiety to form amide bonds. As a general rule, a more active intermediate (labile ester) is first formed with the carboxylic acid moiety and in a later stage reacted with the free amine, increasing the coupling yield. A few coupling procedures are described below.

**Carbodiimide coupling.** Surfaces modified with mercaptoalkyldiols can be activated with 1,1,1,1-tetramethyl-3-azabicyclo[3.2.2]nonane-3-carboximidoyl chloride (CDI) to form a carbonylimidazole intermediate. A biomolecule with an available amine group displaces the imidazole to form a carbamate linkage to the alkylthiol tethered to the surface.139

**N-hydroxysuccinimide (NHS) and its derivatives.** Using a succinimide ester intermediate in acylation reactions of 5'-amino-labeled DNA is also a very efficient protocol. The NHS-activated carboxyl group has a much longer lifetime than the reaction intermediates produced by carbodiimide coupling.140 NHS can also be used to facilitate amide formation between a carboxylic acid moiety and free amine groups in a polypeptide (enzymes, antibodies, antigens, etc.). NHS reacts almost exclusively with primary amine groups, with the exception of mercaptans. This nucleophilic substitution reaction covalently immobilizes biomolecules via available amine moieties by forming stable amide bonds. Covalent immobilization can be achieved in as little as 30 min. This surface has been shown to immobilize 5' amine-modified oligonucleotides, providing an ideal template for hybridization and amplification. Because the DNA is bound at one end rather than at numerous sites along the molecule, the result is high specificity and low background. Because H₂O competes with –NH₂ in reactions involving these very labile esters, it is important to consider the hydrolysis kinetics of the esters used in this type of coupling. A derivative of NHS, O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate, increases the coupling yield by utilizing a leaving group that is converted to urea during the carboxylic acid activation, hence favorably increasing the negative enthalpy of the reaction. The schematic in Figure 20.9 illustrates this approach.

![Binding scheme for biomolecule immobilization.](image)
**Maleimide.** Maleimide can be used to immobilize biomolecules through available –SH moieties. Coupling schemes with maleimide have been proven useful for the site-specific immobilization of antibodies, Fab fragments, peptides, and SH-modified DNA strands. Sample preparation for the maleimide coupling of a protein involves the simple reduction of disulfide bonds between two cysteine residues with a mild reducing agent, such as dithiothreitol, 2-mercaptoethanol, or tris(2-carboxyethyl)phosphine hydrochloride. However, disulfide reduction will usually lead to the protein losing its natural conformation and might impair enzymatic activity or antibody recognition. The modification of primary amine groups with 2-iminothiolane hydrochloride (Traut’s reagent) to introduce sulfhydryl groups is an alternative for biomolecules lacking them. Free sulfhydryls are immobilized to the maleimide surface by an additional reaction to unsaturated carbon–carbon bonds.

**Hydrazide.** Hydrazide is used for the covalent coupling of periodate-activated carbohydrates or glycosylated biomolecules. It can be used for the site-specific immobilization of antibodies, carbohydrates, glycolipids, glycoproteins, and many enzymes. Antibodies are immobilized to the hydrazide surface through the carbohydrate moieties on the Fc region, which allows the Fab regions to be properly oriented.

Non-covalent immobilization of biomolecules includes sol-gel or polymer entrapment of enzymes, and adsorption of oligonucleotides onto poly-ionic membranes (poly-lysine, polymers with quaternium ammonium groups, etc). For sol-gel or polymeric immobilization, solubility of the biomolecules in the polymerization medium is a major constraint for the use of this technique. In the case of surface adsorption, conformation changes of proteins that might diminish antibody recognition or enzyme activity need to be considered.

A sensor for the nitrate ion based on the encapsulation of an enzyme in a sol-gel structure is reported to be effective even after a storage period of up to 6 months. The enzyme of choice was the periplasmic nitrate reductase (Nap), extracted from the denitrifying bacterium *Thiosphaera pantotropha*, which reacts specifically with the nitrate (NO₃⁻) anion.

Edmiston and co-workers found evidence that the immobilization of proteins by entrapment in a porous silica matrix prepared by sol-gel techniques may significantly change the conformation of the proteins. These researchers examined two model proteins, bovine serum albumin (BSA) and horse heart myoglobin (Mb), entrapped in wet sol-gel glass bulbs. They investigated the fluorescence behavior of dissolved and entrapped BSA in the presence of acid, a chemical denaturant, and a collisional quencher. The results show that a large fraction of the BSA added to the sol is entrapped within the gelled glass in a native conformation. However, the reversible conformational transitions that BSA undergoes in solution are sterically restricted in the gel. In contrast, the native properties of Mb are largely lost upon entrapment, as judged by the changes in the visible absorbance spectra of dissolved and entrapped Mb in acidic solutions. Fluorescence studies of dissolved and entrapped apomyoglobin supported this conclusion.

### 20.6 Biomedical Applications

This section presents an overview of various medical applications of biosensors, especially in the diagnosis of diseases. A large body of work was accomplished using SPR commercial instruments and involves the study of protein or DNA interactions relevant for medical applications. Other widely used methods include fluorescence spectroscopy, NIR, and circular dichroism.

#### 2.6.1 Cellular Processes

Staining cellular organelles is a classic laboratory method that utilizes visible or fluorescent dyes that have high affinity for specific organelles. After staining, visible or UV illumination is used for microscopy identification. Newer techniques explore the same principles to obtain more information on cellular processes as well.

For example, G protein-coupled receptors (GPCRs) represent one of the most important drug targets for medical therapy, and information from genome sequencing and genomic databases has substantially accelerated their discovery. The lack of a systematic approach to identify the function
of a new GPCR or to associate it with a cognate ligand has added to the growing number of orphan receptors. A novel approach to this problem using optical detection of a beta-arrestin2/green fluorescent protein conjugate (beta arr2-GFP) has been reported. Confocal microscopy demonstrates the translocation of beta arr2-GFP to more than 15 different ligand-activated GPCRs, providing a real-time and single-cell-based assay to monitor GPCR activation and GPCR-Gr protein-coupled receptor kinase or GPCR-arrestin interactions. The use of beta-arr2-GFP as a biosensor to recognize the activation of pharmacologically distinct GPCRs should accelerate the identification of orphan receptors and permit the optical study of their signal transduction biology, which is intractable to ordinary biochemical methods.150

Abscisic acid (ABA) is a plant hormone involved in many developmental and physiological processes, but no ABA receptor has been identified yet.151 In an attempt to demonstrate that the monoclonal antibody JIM19 recognizes carbohydrate epitopes of cell surface glycoproteins, researchers have used flow cytometry of rice protoplasts and immunoblotting of purified plasma membranes (PMs).151 Through use of SPR technology, specific binding of PMs to JIM19 was observed. The interaction was antagonized significantly by ABA but not by the biologically inactive ABA catabolite, phaseic acid. Pretreatment with JIM19 resulted in significant inhibition of ABA-inducible gene expression. Taken together, these data suggest that JIM19 interacts with a functional PM complex involved in ABA signaling.151

Another receptor binding study done with optical biosensor technology investigated the affinity and specificity of the putative proximal tubular scavenging receptor for protein reabsorption and the specificity of AGE-modified protein interactions with primary human mesangial cells.152 An SPR biosensor with a carboxy-methyl dextran surface was used for binding competition analysis of five different proteins of the LLCPK cell line (ranging in size and charge). The biosensor data show evidence to support the existence of a single scavenging receptor for all the proteins tested. The proteins competed with each other, differing only in their relative binding affinity for the common receptor. This study has also showed that human mesangial cells can bind to AGE-modified human serum albumin (AGE-HSA) immobilized onto the carboxylate surface and that binding can be inhibited by using increasing concentrations of soluble AGE-HSA. However, increasing concentrations of soluble non-AGE-modified HSA can also inhibit binding to a similar extent, which implies relatively little AGE-receptor expression on cultured primary human mesangial cells. The SPR biosensor is a potential tool to explore cellular interactions with renal cells.152

### 20.6.2 Viral Agents

The use of biosensors to detect specific viruses in biological samples offers a great diagnostic tool for medical applications. To date, studies have targeted several viruses, including HIV (discussed separately), measles virus, herpes simplex virus, rhinoviruses, and foot-and-mouth disease prions. Some of the techniques used in their detection are outlined below.

Identifying viruses in clinical materials during the acute phase of infections could give necessary information for the treatment of infections by human immunoglobulin (hIg) or interferon (IF). However, because of lack of information, most virus infections are not treated. A real-time detection system for viruses in general has been developed using an optical biosensor and a model virus: herpes simplex virus Type 1 (HSV-1). The HSV-1 virus was found to propagate in Vero cells and, when diluted in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), could be detected with an SPR sensor with high sensitivity and a detection limit of 10 infectious units (50% tissue culture infective dose [TCID50] units). When a crude homemade rabbit antiserum was used against measles virus with host cell debris as a ligand, the SPR sensor performed with lower sensitivity, detecting less than 500 infectious (TCID50) units of virus in a 100-μl solution. This real-time viral detection and titration system has sensitivity high enough for clinical purposes.153

The herpes virus was also the object of a study of epitope mapping using an SPR optical biosensor. The human herpes virus entry mediator C (HveC), also known as the poliovirus receptor-related protein 1 (PRR1) and as nectin-1, allows the entry of HSV-1 and HSV-2 into mammalian cells. The interaction of the virus envelope glycoprotein D (gD) with such a receptor is an essential step in
the process leading to membrane fusion.154 HveC is a member of the immunoglobulin (Ig) super-family and contains three Ig-like domains in its extracellular portion. The gD binding site is located within the first Ig-like domain (V domain) of HveC. In a careful study using SPR, 11 monoclonal antibodies (MAbs) against the ectodomain of HveC were chosen to detect linear or conformational epitopes within the V domain. Besides the biosensor analysis, the HveC was detected by enzyme-linked immunosorbent assay, Western blotting, and directly on the surface of HeLa cells and human neuroblastoma cell lines, as well as simian Vero cells. A few of the 11 monoclonal antibodies blocked HSV entry. Competition assays on an optical biosensor showed that CK6 and CK8 (linear epitopes) inhibited the binding of CK41 and R1.302 (conformational epitopes) to HveC and vice versa.154

One of the reasons for the traditionally low success for the direct identification of viruses by simple immunological assays is the large variability of their surface epitopes. For example, more than 100 immunologically distinct serotypes of human rhinoviruses (HRV) have been discovered, making detection of surface-exposed capsid antigens impractical. However, the nonstructural protein 3C protease (3Cpro) is essential for viral replication and is relatively highly conserved among serotypes, making it a potential target for diagnostic testing of HRVs. An SPR biosensor with a modified silicon surface with broadly reactive serotype antibodies to 3Cpro has been developed.155

The in vitro sensitivity, specificity and multiserotype cross-reactivity of the 3Cpro assay were tested using the SPR sensor in a 28-min, noninstrumented room-temperature test with a visual detection limit of 12 pM of 3Cpro (1000 TCID50 equivalents). Nasal washes from naturally infected individuals were used as test samples. The assay detected 87% (45 of 52) of the HRV serotypes tested but showed no cross reactivity to common respiratory viruses or bacteria. The SPR assay detected 3Cpro in expelled nasal secretions from a symptomatic individual on the first day of illness. In addition, 82% (9 of 11) concentrated nasal wash specimens from HRV-infected children were positive in the 3Cpro test. Thus the assay is suitable as a diagnostic test for a point-of-care setting, where rapid HRV diagnostic test results could contribute to clinical decisions regarding appropriate antibiotic or antiviral therapy.155

Another SPR system was applied to the quantitative analysis of the binding of HSV-1 to Vero cells. A commercially available sulfonated human immunoglobulin preparation was used as the neutralization antibody titer against this virus.156

Virus-like particles (VLPs) are multimeric proteins expressed by Saccharomyces cerevisiae. These particles are approximately 80 nm in diameter and are used as a framework for a range of biological products, including carriers of viral antigens. An SPR biosensor was developed for rapid monitoring of purified VLPs; this device can be used for real-time bioprocess monitoring of VLPs. Problems of mass transfer of the analyte were overcome through selection of a planar biosensor surface instead of the traditional polymer-coated surface. To prolong the surface activity for interaction analysis, a sandwich assay was developed that involved the use of a secondary capture species. It was shown that VLP concentration in pure solution could be determined within 10 min.157

An SPR biosensor has been used for screening synthetic peptides mimicking the immunodominant region of C-S8c1 foot-and-mouth disease virus. The main antigenic site (site A) of the foot-and-mouth disease virus (FMDV, strain C-S8c1) may be adequately reproduced by a 15-peptide with the amino acid sequence H-YTASARGDLAHL TT-NH2 (A15), corresponding to the residues 136 to 150 of the viral protein VP1.158 The SPR sensor surface was modified with monoclonal antibodies raised against antigenic site A. Although these antigenicities have previously been determined from ELISA methods, the SPR-based technique is superior in that it allows a fast and straightforward screening of antigens while simultaneously providing kinetic data for the Ag–Ab interaction.158

### 20.6.3 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) has been the target of intense research in the past two decades. Some of the research efforts involving optical biosensors are outlined below.

The two main proteins on the HIV envelope are glycoproteins gp120 and gp41 (named for their approximate size in kilodaltons). Glycoproteins gp120 and gp41 are associated together noncovalently. Binding of
HIV-1 gp120 to T-cell receptor CD4 initiates conformational changes in the viral envelope that trigger viral entry into the host cells. SPR has been used in a number of HIV studies. This technique was applied to observe the conformational changes in gp120 upon binding to certain ligands, to compare the gp120-activation effects of CD4 mimetics, and to examine for CD4 competition and gp120 activation. SPR optical biosensors provide a means of looking at the interaction between macromolecules as it occurs in real time, providing information about the kinetics of the interaction, in addition to estimating affinity constants.

SPR optical biosensor assays for the screening of low-molecular-weight compounds, using an immobilized protein target, have been developed. HIV-1 protease was immobilized on the sensor surface by direct amine coupling. A large number of inhibitors and noninteracting reference drugs were applied to the sensor surface in a continuous flow of buffer to estimate binding constants. The optimized assay could correctly distinguish HIV-1 inhibitors from other compounds in a randomized series, indicate differences in their interaction kinetics, and reveal artifacts due to nonspecific signals, incomplete regeneration, or carryover.

SPR biosensors have also been used to study the interaction between HIV-1 protease and reversible inhibitors. The steady-state binding level and the time course of association and dissociation could be observed by measuring the binding of inhibitors injected in a continuous flow of buffer to the enzyme immobilized on the biosensor surface. Fourteen low-molecular-weight inhibitors (500 to 700 Da), including four clinically used HIV-1 protease inhibitors (indinavir, nelfinavir, ritonavir, and saquinavir) were analyzed. Inhibition constants \( K_i \) were determined by a separate enzyme inhibition assay. Indinavir had the highest affinity \( (B_{50} = 11 \text{ nM}) \) and the fastest dissociation \( (t_{1/2} = 500 \text{ s}) \) among the clinically used inhibitors, while saquinavir had a lower affinity \( (B_{50} = 25 \text{ nM}) \) and the slowest dissociation rate \( (t_{1/2} = 6500 \text{ s}) \). Because these two inhibitors have similar affinities, the differences in dissociation rates reveal important characteristics in the interaction that cannot be obtained by the inhibition studies alone.

Characterization of another set of HIV-1 protease inhibitors using binding kinetics data from an SPR biosensor-based screen has also been reported.

Fluorescence polarization, circular dichroism, and SPR optical biosensor binding studies were used to investigate the novel virucidal protein cyanovirin-n (cv-n). Cv-n binds with equally high affinity to soluble forms of either h9 cell-produced or recombinant glycosylated HIV-1 gp120 (sgp120) or gp160 (sgp160). Studies showed that cv-n is also capable of binding to the glycosylated ectodomain of the HIV-envelope protein gp41 (sgp41), albeit with considerably lower affinity than the sgp120/cv-n interaction. These optical techniques shed light on the binding of cv-n with sgp120 and sgp41, providing direct evidence that conformational changes are a consequence of cv-n interactions with both HIV-1 envelope glycoproteins.

### 20.6.4 Bacterial Pathogens

Several physicochemical instrumental techniques for direct and indirect identification of bacteria such as IR and fluorescence spectroscopy, flow cytometry, chromatography, and chemiluminescence have been reviewed as feasible biosensor technologies.

*Staphylococcus aureus* is a pathogen that commonly causes human infections and intoxication. An evanescent-wave optical sensor was developed for the detection of protein A, a product secreted only by *S. aureus*. A 488-nm laser was used in conjunction with a plastic optical fiber with adsorbed antibodies for protein. A sandwich immunoassay with fluorescein isothiocyanate conjugated with anti-(protein A) IgG was used to monitor the Ag–Ab reaction. The detection limit was 1 ng/ml of protein A.

In a different approach, an optical biosensor based on resonant mirrors was used in the detection of whole cells of *S. aureus* (Cowan-1). The bacterium cells, which express protein-A at their surface, were detected through their binding to human IgG immobilized on an aminosilane-derivatized sensor surface at concentrations in the range \( 8 \times 10^5 \text{ to } 8 \times 10^7 \text{ cells/ml} \). A control *S. aureus* strain (Wood-46) that does not express protein-A produced no significant response. The sensitivity of the technique was increased by three orders of magnitude when a human IgG-colloidal gold conjugate

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(30 nm) was used in a sandwich assay format. *S. aureus* (Cowan-1) cells were detected in spiked milk samples at cell concentrations from $4 \times 10^3$ to $1.6 \times 10^6$ cells/ml using the sandwich assay.

The same resonant mirror optical biosensor technology was used to characterize *Helicobacter pylori* strains according to their sialic acid binding. In that work, intact bacteria were used in real-time measurements of competition and displacement assays using different glycoconjugates. The authors found that that several, but not all, *H. pylori* strains express sialic acid-binding adhesin specific for alpha-2,3-sialylactose. The adhesin, removable from the bacterial surface by water extraction, is not related to other reported *H. pylori* cell surface proteins with binding ability to sialylated compounds such as sialylglycoceramides.

A fiber-optic evanescent-wave sensing system that features all-fiber optical design and red semiconductor-laser excitation has been developed and tested. A 650-nm laser was used because biological matrices demonstrate minimal fluorescent background in the red; this helps reduce the background signal of nonessential biomolecules. The fiber directs the fluorescent signal of a sandwich immunoassay to detect *Salmonella* back to a charge-coupled device (CCD) fiber spectrophotometer. The system could detect *Salmonella* with a concentration as low as $10^5$ colony-forming units (CFU) per milliliter.

With a similar biodetection approach utilizing laser-induced fluorescence, an optic biosensor was used to detect the fraction 1 (F1) antigen from *Y. pestis*, the etiologic agent of plague. An argon ion laser (514 nm) was used to launch light into a long-clad fiber, and the fluorescence produced by an immunofluorescent complex formed in the evanescent wave region was measured with a photodiode. Capture antibodies, which bind to F1 antigen, were immobilized on the core surface to form the basis of the sandwich fluoroimmunoassay. The evanescent wave has a limited penetration depth ($<1$ lambda), which restricts detection of the fluorescent complexes bound to the fiber’s surface. The direct correlation between the concentration of the F1 antigen and the signal provided an effective method for sample quantitation; the method was able to detect F1 antigen concentrations from 50 to 400 ng/ml in phosphate-buffered saline, serum, plasma, and whole blood, with a 5-ng/ml detection limit.

A very different detection approach for *Salmonella typhimurium* involved immunomagnetic separation and a subsequent enzyme-linked assay with alkaline phosphatase. The magnetic microbeads coated with anti-*Salmonella* were used to separate *Salmonella* from sample solutions at room temperature for 30 min. A sandwich complex with alkaline phosphatase and the *Salmonella* immobilized on the magnetic beads was formed, separated from the solution by a magnetic filtration, and incubated with a p-nitrophenyl phosphate substrate at 37°C for 30 min to produce p-nitrophenol by the enzymatic hydrolysis. *Salmonella* was detected by measuring the absorbance of p-nitrophenol at 404 nm, with a linear response between $2.2 \times 10^4$ and $2.2 \times 10^6$ CFU/ml.

### 20.6.5 Cancer

Optical biosensors have been utilized as tools to aid in the direct diagnosis of carcinogenesis, the identification of genetic markers associated with it, and the quantification of known carcinogens.

Fluorescent detection has become a technique of choice for oligonucleotide hybridization detection. Pairs of high density oligonucleotide arrays (DNA chips) consisting of more than 96,000 oligonucleotides were designed to screen the entire 5.53-kb coding region of the hereditary breast and ovarian cancer BRCA1 gene for all possible sequence changes in the homozygous and heterozygous states. Fluorescent hybridization signals from targets, containing the 4 natural bases to more than 5592 different, fully complementary 25mer oligonucleotide probes on the chip, varied over two orders of magnitude. To examine the thermodynamic contribution of rU.dA and rA.dT target probe base pairs to this variability, modified uridine [5-methyluridine and 5-(1-propynyl)-uridine] and modified adenosine (2,6-diaminopurine riboside) 5'-triphosphates were incorporated into BRCA1 targets. Hybridization specificity was assessed based upon hybridization signals from >33,200 probes containing centrally localized single base pair mismatches relative to target sequence. Targets containing 5-methyluridine displayed promising localized enhancements in the hybridization signal, especially in pyrimidine-rich target tracts.
while maintaining single nucleotide mismatch hybridization specificities comparable with those of unmodified targets.\textsuperscript{175}

In another study, the breast cancer susceptibility gene \textit{BRCA1} was also detected by a relatively new technique based on SERS. A single 24-mer sequence was used as the capture probe and was immobilized on a silver-coated microarray platform for hybridization.\textsuperscript{21} Breast cancer was also the target of a biosensor design to measure the interaction of S100A4 and potential binding partners.\textsuperscript{176} Elevated levels of S100A4 induce a metastatic phenotype in benign mammary tumor cells \textit{in vivo}. In humans, the presence of S100A4 in breast cancer cells correlates strongly with reduced patient survival. There was significant interaction of S100A4 with nonmuscle myosin and p53 but not with actin, tropomyosin, or tubulin.\textsuperscript{176}

A regenerable immunosensor utilizing an antibody against breast cancer antigen has been described. A 65% removal of the antigens bound to the Mab immobilized on the fiber surface is attained after ultrasound regeneration.\textsuperscript{22} A multiarray biosensor utilizing DNA probes labeled with visible and NIR dyes has also been developed. The detection system uses a two-dimensional CCD to detect the \textit{p53} cancer gene.\textsuperscript{23}

Prostate cancer is the cause of death of many thousands of men worldwide. Screening men for elevated prostate-specific antigen (PSA) levels is believed to be an important tool for the diagnosis and management of the disease. An assay for measuring PSA in whole blood using the fluorescence capillary fill device has been developed for use in prostate cancer screening programs.\textsuperscript{177}

A unique example of the technology being developed is optical nano-biosensors capable of interrogating the contents of a single isolated cell.\textsuperscript{178} These submicrometer fiber-optic biosensors have been used to measure carcinogens within single cells. Optical fibers were pulled to a distal-end diameter of 40 nm and coated with antibodies to selectively bind benzo[a]pyrene tetrol (BPT), a metabolite of benzo[a]pyrene, an extremely potent carcinogen. Two different cell lines have been investigated: human mammary carcinoma cells and rat liver epithelial cells. The detection limit of these nanosensors has been determined to be \((0.64 \pm 0.17) \times 10^{-11} \) M for BPT.\textsuperscript{75} The development and application of nanosensors are further described in the chapter on nanosensors for single-cell analysis in this handbook.

The carcinogen benzo[a]pyrene (BAP) was the target of an antibody-based fiberoptics biosensor.\textsuperscript{7} In that biosensor, BAP was the analyte and fluorophore because it has a large fluorescence cross section. An antibody with high specificity for BAP was immobilized on the tip of an optical fiber. Upon exposure to contaminated samples, the optical fiber was irradiated with a laser and the resulting fluorescence correlated with the BAP concentration.

### 20.6.6 Parasites

Detection of antibodies specific for the parasite \textit{L. donovani} in human serum samples has been reported. The method is based on an evanescent wave fluorescence collected by optical fibers that have the purified cell surface protein of \textit{L. donovani} immobilized on their surface. The sensing fibers are incubated with the patient serum for 10 min and then incubated with goat anti-human IgG. Fluorescence was proportional to \textit{L. donovani}–specific antibodies present in the test sera.\textsuperscript{179}

### 20.6.7 Toxins

Ricin, a potently toxic protein, has been detected with an evanescent-wave fiber-optic biosensor with a detection limit of 100 pg/ml and 1 ng/ml for buffer solutions and river water, respectively. Athis detection was based on a sandwich immunoassay scheme, using an immobilized anti-ricin IgG on the surface of the optical fiber. Two coupling methods were used. In the first, the antibody was directly coated to the silanized fiber using a cross linker; the second method utilized avidin-coated fibers incubated with biotinylated antiricin IgG to immobilize the antibody using an avidin-biotin bridge. The assay using the avidin–biotin-linked antibody demonstrated higher sensitivity and a wider linear dynamic range than the assay using the antibody directly conjugated to the surface. The linear dynamic range of detection for ricin in buffer using the avidin–biotin chemistry is 100 pg/ml to 250 ng/ml.\textsuperscript{78}
The lipopolysaccharide (LPS) endotoxin is the most powerful immune stimulant known and a causative agent in the clinical syndrome known as sepsis. Sepsis is responsible for more than 100,000 deaths annually, in large part due to the lack of a rapid, reliable, and sensitive diagnostic technique. An evanescent wave fiberoptic biosensor was developed for the detection of LPS from *E. coli* at concentrations as low as 10 ng/ml in 30 s. Polymyxin B covalently immobilized onto the surface of the fiber-optic probe was able to bind fluorescently labeled LPS selectively. Unlabeled LPS present in the biological samples was detected in a competitive assay format, by displacing the labeled LPS. The competitive assay format worked in buffer and in plasma with similar sensitivities. This method might also be used with other LPS capture molecules, such as antibodies, lectins, or antibiotics, to simultaneously detect LPS and determine the LPS serotype.

An immunoaffinity fluorometric biosensor was developed for the detection of aflatoxins, a family of potent fungi-produced carcinogens commonly found in a variety of agriculture products. Developed into a fully automated instrument based on immunoassays with fluorescent tags, the detection system was able to detect aflatoxins from 0.1 to 50 ppb in 2 min with a 1-ml sample volume. Parathion was detected with a biosensor based on total internal reflection using a competitive-displacement immunoassay. This biosensor utilizes casein–parathion conjugates, immobilized by adsorption on quartz fibers, and selectively adsorbed antiparathion rabbit antibodies raised against bovine serum albumin (BSA)–parathion conjugates from polyclonal immune sera. The presence of free parathion inhibited the binding of the rabbit’s anti-(BSA)-parathion. Fluorescein isothiocyanate (FITC) goat antirabbit IgG was used to generate the optical signal. It could detect 0.3 ppb of parathion and had a detection limit 100-fold higher for the detection of its oxygen analog, paraoxon. Other biosensor approaches for the detection of parathion include another competition assay that inhibits the alkaline phosphatase generation of a chemiluminescent substance and a direct detection method based on another enzymatic sensor.

A very interesting approach for biosensing toxins involves the integration of multiple transducers with different affinities for a large range of biotoxins. The different transducers are based on membranes made of mixtures of biologically occurring lipids deposited on the sensing surface of an SPR optical biosensor. Eight surfaces were prepared, some of which contained various glycolipids as minor components, and one was supplemented with membrane proteins. The researchers analyzed the binding of six protein toxins (cholera toxin, cholera toxin B subunit, diphtheria toxin, ricin, ricin B subunit, and staphylococcal enterotoxin B) and of bovine serum albumin at pH 7.4 and pH 5.2 to each of the sensor surfaces. Each of the seven proteins produced a distinct binding pattern to the multitransducer sensor. The same concept had been used earlier for the development of “artificial noses,” which are sensor arrays able to detect a small collection of distinct analytes.

Cholera toxin (CT) has been also detected with a fluorescence-based biosensor using a waveguide platform. The biochemical assay utilized a ganglioside-capture format, where the ganglioside (GM1) that captures the analyte was immobilized in discrete locations on the surface of an optical waveguide. Binding of CT to immobilized GM1 was demonstrated with direct assays (using fluorescently labeled CT) and with “sandwich” immunoassays (using fluorescently labeled tracer antibodies). The detection limits for CT were 200 ng/mL in direct assays and 40 ng/mL and 1 μg/mL in sandwich-type assays performed using rabbit and goat tracer antibodies. A slightly different biosensing approach was also used to detect cholera toxin. Instead of direct fluorescence detection, fluorescence quenching was used. The ganglioside GM1 was again used as the recognition unit for CT, and was covalently labeled with fluorophores and then incorporated into a biomimetic membrane surface. In a very nice application of surface plasmon resonance, SPR biosensors have been used to estimate the immunoreactivity of tetanus toxin and *Vipera aspis* venom against new pasteurized preparations of their horse F(ab’2) preparations, in order to investigate immunoreactivity–immunoprotection efficacy relationships. The immunoreactivity data were compared with seroneutralization titres. The association–dissociation rate and affinity constants of the current and the new tetanus toxin-specific F(ab’2) preparations were similar, at about 10^4 M^-1 s^-1, 10^4 s^-1 and 10^4 M^-1, respectively.
20.6.8 Blood Factors

SPR was used to determine absolute heparin concentration in human blood plasma. Protamine and poly-ethylene-imine (PEI) were used to modify the sensor surface and were evaluated for their affinity to heparin. Heparin adsorption onto protamine in blood plasma was specific with a lowest detection limit of 0.2 U/ml and a linear detection range of 0.2 to 2 U/ml. Although heparin adsorption onto PEI in buffer solution had indicated superior sensitivity to that on protamine, in blood plasma it was not specific for heparin and adsorbed plasma species to a steady-state equilibrium. By reducing the incubation time and diluting the plasma samples with buffer to 50%, the nonspecific adsorption of plasma could be controlled and a PEI pretreated with blood plasma could be used successfully for heparin determination. Heparin adsorption in 50% plasma was linear between 0.05 and 1 U/ml so that heparin plasma levels of 0.1 to 2 U/ml could be determined with a relative error of 11% and an accuracy of 0.05 U/ml.\textsuperscript{188}

20.6.9 Congenital Diseases

SPR and biospecific interaction analysis (BIA) have been used to detect the Delta F508 mutation (F508del) of the cystic fibrosis transmembrane regulator (CFTR) gene in homozygous as well as heterozygous human subjects.\textsuperscript{189} The detection method involved the immobilization on an SA5 sensor chip of two biotinylated oligonucleotide probes (one normal, N-508, and the other mutant, Delta F508) that are able to hybridize to the CFTR gene region involved in F508del mutation. A hybridization step between the oligonucleotide probes immobilized on the sensor chips and (1) wild-type or mutant oligonucleotides, as well as (2) ssDNA. These nucleic acid samples were obtained using asymmetric polymerase chain reaction (PCR), performed using genomic DNA from normal individuals and from F508del heterozygous and Delta 508del homozygous patients. The different stabilities of DNA/DNA molecular complexes generated after hybridization of normal and Delta F508 probes immobilized on the sensor chips were then evaluated. The results strongly suggest that the SPR technology enables a one-step, nonradioactive protocol for the molecular diagnosis of F508del mutation of the CFTR gene. This approach could be of interest in clinical genetics, because the hybridization step is often required to detect microdeletions present within PCR products.\textsuperscript{189}

20.7 Conclusions

The past decade has witnessed the rapid development of a wide variety of biosensors for many different analyses and has even seen them begin to advance to clinical and, in some cases, commercially available technologies, such as SPR biosensors. The increasing interest in the field of optical biosensors has provided a great deal of information about the biochemistry of clinically important ailments and has provided drug discovery research with faster analytical tools to investigate drug–receptor interactions. Optical methods have also been extensively applied to DNA fingerprinting and genotyping, techniques that might find enormous applications in clinical diagnosis in the near future.

As a very positive sign, optical biosensors are coming of age as a bioanalytical tool. A larger number of researchers in different areas now have access to a more user-friendly technology, and are able to develop custom applications specific to their research needs, expanding the range of applications for the technology.

For practical medical diagnostic applications, there is a strong need for a truly integrated biosensor system that can be easily operated by relatively unskilled personnel. Some of the current commercially available technologies have dramatically simplified the data collection operation. Although these systems have demonstrated their usefulness in genomic detection, protein interaction analysis, carcinogen monitoring, etc., they are laboratory oriented and involve relatively expensive equipment and trained, supervised operation.

The outlook is promising for optical biosensor systems; the near future should bring a larger number of multichannel applications for the simultaneous detection of multiple biotargets; improvements in size and performance; and lower production costs due to a more integrated package. Highly integrated systems lead
to reduction in noise and increase in signal due to the improved efficiency of sample collection and the
reduction of interfaces. The capability of large-scale production using low-cost integrated circuit (IC)
technology is an important advantage that cannot be overlooked. For medical applications, the development
of low-cost, disposable biosensor surfaces that can be used for clinical diagnostics at the point of care can
be a major driving force for the expansion of optical biosensor technologies.

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