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REVIEW

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Biosensors and biochips: advances in biological and medical diagnostics

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Abstract In the past two decades, the biological and medical fields have seen great advances in the development of biosensors and biochips capable of characterizing and quantifying biomolecules. This review is meant to provide an overview of the various types of biosensors and biochips that have been developed for biological and medical applications, along with significant advances over the last several years in these technologies. It also attempts to describe various classification schemes that can be used for categorizing the different biosensors and provide relevant examples of these classification schemes from recent literature.

1 Introduction

Humankind has been performing bioanalysis since the dawn of time, using the sensory nerve cells of the nose to detect scents or the enzymatic reactions in the tongue to taste food. 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. Because biological organisms are some of the most efficient machines ever created, scientists have sought to apply and copy their efficiency for use in man made creations. In particular, the recognition abilities of biological organisms for foreign substances is unparalleled. Using bioreceptors from biological organisms or receptors that have been 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 biochips.

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A biosensor can be generally defined as a device that consists of a biological recognition system, often called a bioreceptor, and a transducer. 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 1 illustrates the conceptual principle of the biosensing process. Biosensors that include transducers based on integrated circuit microchips are often referred to as biochips. In general, a biochip consists of an array of individual biosensors that can be individually monitored and generally are used for the analysis of multiple analytes.

Biosensors and biochips can be classified either by their bioreceptor or their transducer type (see Fig. 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

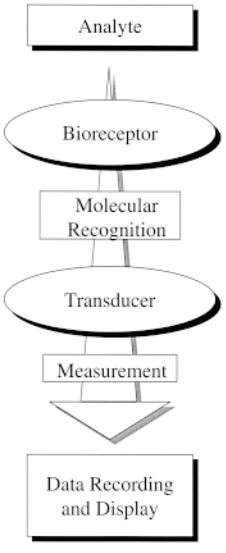

Fig. 1 Conceptual diagram of the biosensing principle

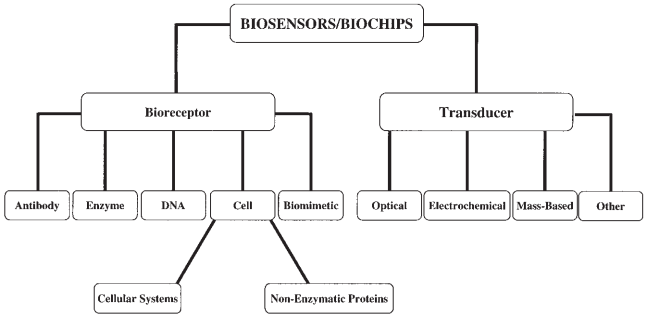

Fig. 2 Schematic of biosensor/biochip classification schemes

sampling component of a biosensor contains a bio-sensitive layer. The layer can either 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, conventional techniques include: 1) optical measurements (i.e. luminescence, absorption, surface plasmon resonance, etc.) 2) electrochemical and 3) mass-sensitive measurements (i.e. surface acoustic wave, microbalance, etc.).

Since the first biosensors were reported in the early 1960s [1], there has been an explosive growth of research activities in this area [2–5]. Biosensors have seen a wide variety of applications primarily in two major areas, biological monitoring and environmental sensing. This review covers the recent and significant advances in biosensor and biochip technologies for the analysis of samples of biological and biomedical interest. Since over 1500 articles have been published in the area of biosensors and biochip technology from January 1998 to August 1999, this paper is not meant to be a comprehensive review, but rather a critical review, presenting a selection of the significant advances in the field of biosensors and biochips. A computer search of the Science Citation Index provided most of the references for this review, and was in general limited to journal articles and generally did not include patents, conference proceedings, reports or dissertations.

2 Biosensors

2.1 Bioreceptors

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 and 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. These categories include: 1) antibody/antigen, 2) enzymes, 3) nucleic acids/DNA, 4) cellular structures/cells and 5) biomimetic. Figure 3 shows a schematic diagram of two types of bioreceptors: the struc-

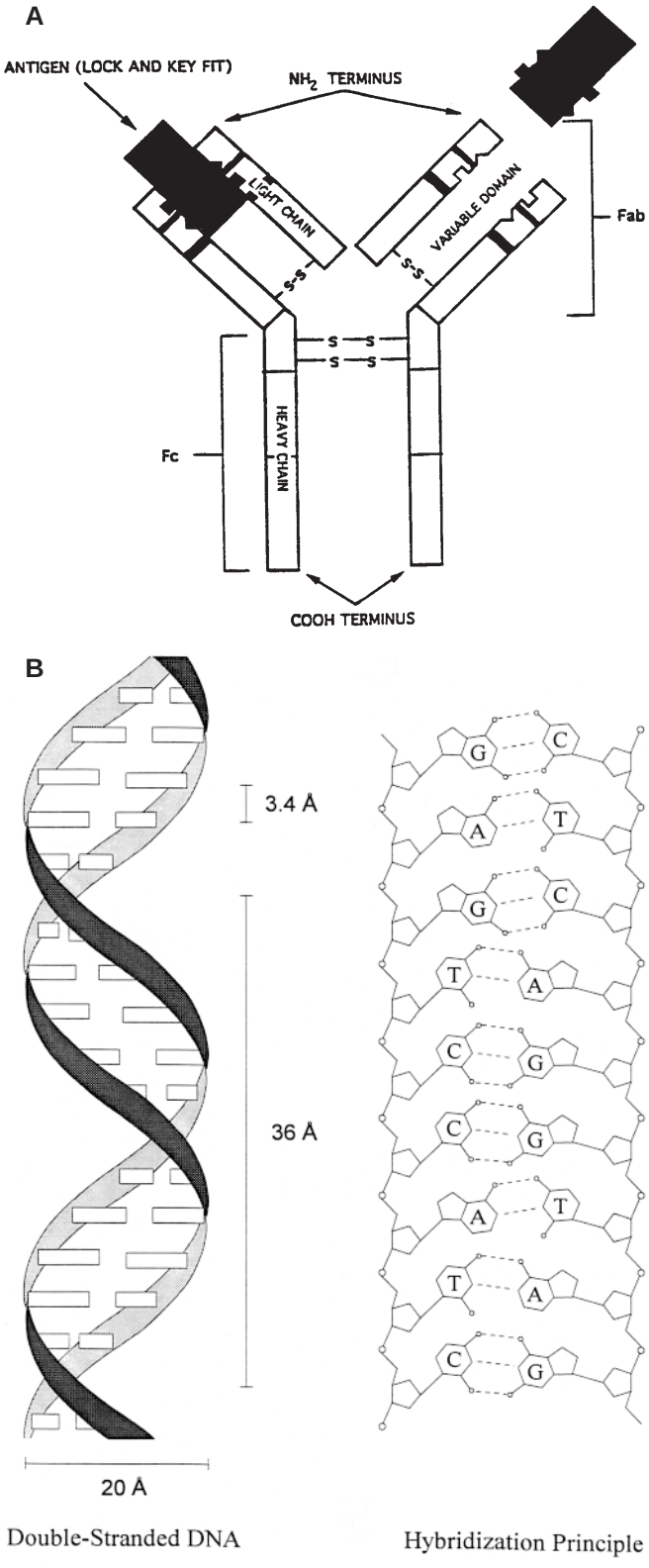

Fig. 3 Schematic diagrams of two types of bioreceptors: **A** IgG antibody, **B** DNA and the hybridization principle

ture of an immunoglobulin G (IgG) antibody molecule (Fig. 3A), and DNA and the principle of base pairing in hybridization (Fig. 3B).

2.1.1. Antibody/antigen

Antibodies are biological molecules that exhibit very specific binding capabilities for specific structures. This is very important due to the complex nature of most biological systems. An antibody is a complex biomolecule, made up of hundreds of individual amino acids arranged in a highly ordered sequence. 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. The way in which an antigen and its antigen-specific antibody interact may be understood as analogous to a lock and key fit, by which specific geometrical configurations of a unique key enables it to open a lock. In the same way, an antigen-specific antibody "fits" its unique antigen in a highly specific manner. This unique property of antibodies is the key to their usefulness in immunosensors where only the specific analyte of interest, the antigen, fits into the antibody binding site.

Radioimmunoassay (RIA) utilizing radioactive labels has been the most widely used immunoassay method. Radioimmunoassays have 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, there are extensive research efforts aimed at developing simpler, more practical immunochemical techniques and instrumentation, which offer comparable sensitivity and selectivity to RIA. In the 1980s, advances in spectrochemical instrumentation, laser miniaturization, biotechnology and fiberoptic research have 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 first development of a remote fiberoptic immunosensor for *in situ* detection of the chemical carcinogen benzo[a]pyrene [6], antibodies have become common bioreceptors used in biosensors today [7–14]. Biomolecular interactions can be classified in two categories, according to the test format performed (i.e., direct and 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 [6]. For non fluorescent analyte systems, *in situ* incubation is followed by development of a fluorophor-labeled second antibody. The resulting antibody sandwich produces a fluorescence signal that is 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 [14]. 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.

Due to the fiber-to-fiber differences in fiber optic biosensors, there is often a great difficulty in normalizing the signal from one fiber to the signal from another fiber. Ligler and coworkers reported on a method for calibrating antibody-based biosensors using two different fluorescent dyes [15]. To accomplish this, they labeled the capture antibodies, bound to the fiber, with one fluorescence dye and the antigen with a different dye. Both dyes were excited at the same wavelength and their fluorescence 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 progress by Heller and coworkers towards the development of an electrochemical immunoassay for whole blood [16]. They describe the development of a sandwich-type 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 was 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 to water.

An important aspect of biosensor fabrication is the binding of the bioreceptor to the measurement support or the transducer. Vogel and coworkers report on a method for the immobilization of histidine-tagged antibodies onto a gold surface for surface plasmon resonance measurements [17]. A synthetic thioalkane 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 [17]. 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 [18].

With the use of nanotechnology, submicron fiberoptic antibody-based biosensors have been developed by Vo-Dinh and coworkers for the measurements of biochemicals inside a single cell [19–21]. Nanometer scale fiberop-

tic biosensors were used for monitoring biomarkers related to human health effects that are associated with exposure to polycyclic aromatic hydrocarbons (PAHs). These sensors use a monoclonal antibody for benzo[a]pyrene tetrol (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 ca. 300 zeptomol (10^{-21} moles) have been reported [19]. These nanosensors allow the probing of cellular and subcellular environments [20, 21].

2.1.2 Enzymes

Enzymes are often chosen as bioreceptors based on their specific binding capabilities as well as their catalytic activity. In biocatalytic recognition mechanisms, the detection is amplified by a reaction catalyzed by macromolecules called biocatalysts. 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 chemical 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 metalloorganic 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. 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 [22].

Gauglitz and coworkers have immobilized enzymes onto an array of optical fibers for use in the simultaneous detection of penicillin and ampicillin [23]. These biosensors provide an indirect 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 containing biosensors of different compositions.

Kopelman and coworkers described the development and use of a micrometer-sized fiber-optic biosensor for the detection of glucose [24]. These biosensors are 100 times smaller than existing glucose optodes and represent the beginning of a new trend in nanosensor technology [25]. These sensors are based on the enzymatic reaction of glucose oxidase that catalyzes 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 fiber-optic via photopolymerization. A comparison of the response of glucose sensors created on different size fibers was made, and it was found that the micrometer size sensors have response times at least 25 times faster (only 2 s) than the larger fibers. In addition, these sensors are reported to have absolute detection limits of ca. 10^{-15} mol and an absolute sensitivity 5–6 orders of magnitude greater than current glucose optodes [24].

2.1.3 Nucleic acids

Another biorecognition mechanism involves hybridization of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), which are the building blocks of genetics. In the last decade, nucleic acids have received increasing interest as bioreceptors for biosensor and biochip technologies [26–31]. The complementarity of adenine : thymine (A : T) and cytosine : guanosine (C : G) pairing in DNA (Fig. 3 B) 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 will hybridize to its complementary sequence on the target molecule.

Grabley and coworkers have reported on the use of DNA biosensors for the monitoring of DNA-ligand interactions [32]. Surface plasmon resonance was 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 DNA layer remained stable over a period of several days and was confirmed using ellipsometry. The sensor was capable of detecting binding effects between 10 and 400 pg/mm^2. 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 coworkers [33]. These sandwich biosensors have been shown to be useful for detection of compounds and physical factors that affect the ability of specific DNA crosslinkers, 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 that the hydrolytic en-

zyme trypsin could be measured down to concentrations of approximately 10^{-14} M.

Karube and coworkers demonstrate another type of biosensor that uses a peptide nucleic acid as the biorecognition element [34]. The peptide nucleic acid is an artificial oligo amide that is capable of binding very strongly to complimentary oligonucleotide sequences. Using 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 40–160 nM, corresponding to an absolute detection limit of 7.5 picomol.

Vo-Dinh and coworkers have developed a new type of DNA gene probe based on surface-enhanced Raman scattering (SERS) detection [35, 36]. These SERS probes have great potential to provide both 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 immunodefficiency (HIV) virus [36]. The development of a biosensor for DNA diagnostics using visible and near infrared (NIR) dyes has also been reported [37]. This system employed a two-dimensional charge-coupled device and was used to detect the cancer suppressor *p53* gene.

2.1.4 Cellular structures/cells

Cellular structures and cells comprise a broad category of bioreceptors that have been used in the development of biosensors and biochips [38–72]. These bioreceptors are either based on biorecognition by an entire cell/microorganism or a specific cellular component that is capable of specific binding to certain species. There are presently three major subclasses of this category: 1) cellular systems, 2) enzymes and 3) non-enzymatic proteins. Due to the importance and large number of biosensors based on enzymes, these have been given their own classification and were previously discussed. One of the major benefits associated with using this class of bioreceptors is that often the detection limits can be very low because of signal amplification. Many biosensors developed with these types of bioreceptors rely on their catalytic or pseudocatalytic properties.

Cellular systems. 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 are ingested by a microorganism, therefore allowing a class-specific biosensor 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. Since cell organelles are essentially closed systems, 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 because they are effective catalysts as a result of the enzymatic pathways they possess [22].

Bilitewski and coworkers have developed a microbial biosensor for the monitoring of short-chain fatty acids in milk [69]. Arthrobacter nicotianae microorganisms were immobilized in a calcium-alginate gel on an electrode surface. To this gel was added 0.5 mM $CaCl_2$ to help stabilize it. By monitoring the oxygen consumption of the Arthrobacter nicotianae electrochemically, its respiratory activity could be monitored, thereby providing an indirect means of monitoring fatty acid consumption. Detection of short-chain fatty acids, ranging from 4 to 12 carbons in length, in milk was accomplished with butyric acid being the major substrate. A linear dynamic range from 9.5–165.5 μM is reported with a response time of 3 min. Methods for shortening the response time and recovery time of microbial sensors are also discussed.

Non-enzymatic proteins. Many proteins that are 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, such as 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 non-enzymatic protein biorecognition. In one recent application, Cusanovich and coworkers have developed micro- and nano-biosensors for nitric oxide that are free from most potential interferents [25]. 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 are reported along with a detection limit of 20 μM. Cytochrome c' samples from three different species of bacteria were evaluated.

Detection of endotoxin using a protein bioreceptor based biosensor has been reported by James et al. [73]. The liposaccharide endotoxin is a causative agent in the clinical syndrome known as sepsis, which causes more than 100 000 deaths annually. This work describes an evanescent wave fiber optic biosensor that makes use of a covalently immobilized protein, polymyxin B, as the biorecognition element. The sensor is based on a competitive assay with fluorescently tagged lipopolysaccharide. When this sensor was applied to the detection of lipopolysaccharides

in *E. coli*, detection of concentrations of 10 ng/mL in 30 s was reported.

Vogel and coworkers have reported on the use of lipopeptides as bioreceptors for biosensors [74]. 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 bioreceptor 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.

2.1.5 Biomimetic receptors

A receptor that is 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 [75–87]. 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 amount of crosslinkers. 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 also provided powerful tools for designing synthetic bioreceptors with desired properties. Development of a genetically engineered single-chain antibody fragment for the monitoring of phosphorylcholine has been reported by Hellinga and coworkers [88]. 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 was capable of being attached to a streptavidin monolayer and total internal reflection fluorescence was used to monitor the binding of a fluorescently labeled phosphorylcholine analog.

Artificial membrane fabrication for bioreception has been performed for many different applications. Stevens and coworkers have developed an artificial membrane by incorporating gangliosides into a matrix of diacetylenic lipids (5–10% of which were derivatized with sialic acid) [89]. 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 and 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 were capable of being monitored.

Molecular imprinting has been used for the construction of a biosensor based on electrochemical detection of morphine [90]. A molecularly imprinted polymer for the detection of morphine was fabricated on a platinum wire using agarose and a crosslinking 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. This freed morphine was then measured by oxidation at the electrode and concentrations ranging from 0.1–10 μg/mL were analyzed, with a reported limit of detection 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.

2.2 Transducers

Biosensors can also be classified based upon the transduction methods they employ. Transduction can be accomplished via a great variety of methods. Most forms of transduction can be categorized in one of three main classes. These classes are: 1) optical detection methods, 2) electrochemical detection methods and 3) mass detection methods. However, new types of transducers are constantly being developed for use in biosensors.

Each of these three main classes contain many different subclasses, creating a nearly infinite number of possible transduction methods or combination of methods.

2.2.1 Optical techniques

Optical transduction offers the largest number of possible subcategories of all three of the transducer classes [6, 14, 18–25, 35–37, 91–122]. This is due to the fact that optical biosensors can be used for many different types of spectroscopy (e.g., absorption, fluorescence, phosphorescence, Raman, SERS, refraction, dispersion spectrometry, etc.) with different spectrochemical properties recorded. These properties include: amplitude, energy, polarization, decay time and/or phase. Amplitude is the most commonly measured parameter of the electromagnetic spectrum, as 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 intramolecular atomic 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 based on polar-

ization measurements. Polarization of emitted light is often 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 since these decay times are very dependent upon the excited state of the molecules and their local molecular environment. Vo-Dinh and coworkers reported the development of a phase-resolved fiberoptic fluoroimmunosensor (PR-FIS), which can differentiate the carcinogen benzo[a]pyrene and its metabolite benzopyrene tetrol based on the difference of their fluorescence lifetimes [120]. 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 (i.e. analyte). When the medium changes. via binding of an analyte, the refractive index may change. thus changing the phase of the impinging radiation.

Wolfbeis and coworkers provide a classical example of an optical biosensor in their development of an optode for the detection of urea [123]. Absorption measurements of a pH sensitive dye are used to quantify the amount of urea present. A lipophilic carboxylated polyvinyl chloride membrane containing a pH sensitive dye was used as the sensor transducer. Urease was covalently bound to this membrane, forming a very thin layer. As various concentrations of urea were tested using the sensor, the effective pH change caused a shift in the absorbance profile of the dye that was measured. This sensor allowed for the rapid determination of urea over the concentration range 0.3–100 mM. In addition, the reproducibility and stability of the biosensor is also discussed.

A fiber-optic evanescent wave immunosensor for the detection of lactate dehydrogenase has been developed [124]. Two different assay methods, a one-step and a two-step assay process, using the sensor based an polyclonal antibody recognition were described. The response of this evanescent wave immunosensor was then compared to a commercially available surface plasmon resonance 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 both the one- and two-step assay techniques, the two-step technique is significantly better when the antigen is large.

Femtomolar sensitivities for fluorescently labeled proteins are reported by Herron and coworkers using a channel-etched thin film waveguide fluorimmunosensor [125]. A siliconoxynitride thin film optical waveguide 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 is an indirect sandwich type assay of a non-fluorescent protein ligand, where the analyte (the protein ligand) binds to a protein bioreceptor that had been 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 absorbing cyanine dye to prevent excitation of possible interferences.

2.2.2 Electrochemical techniques

Electrochemical detection is another possible means of transduction that has been used in biosensors [126–147]. This technique is very 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 fluorescence biosensors are often achievable.

Electrochemical flow-through enzyme-based biosensors for the detection of glucose and lactate have been developed by Cammann and coworkers [148]. 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 based on amperometric measurements of glucose and lactate and it was found that the o-phenylenediamine polymer was 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–10 mM glucose was determined with a limit of detection of < 500 μM. For lactate, the linear dynamic range covered concentrations from 50 μM–1 mM with a detection limit of < 50 μM.

A biosensor for protein and amino acid estimation is reported by Turner and Sarkar [148]. 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 give no response to enzyme based biosensors. This sensor has been tested by measuring proteins and amino acids in fruit juice, milk and urine and consumes approximately 10 μL of sample for direct detection.

Scheller and coworkers have developed an electrochemical biosensor for the indirect detection of L-phenylalanine via NADH [150]. 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 fol-

lows. 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 electrochemically detected and reduced back to catechol with an electrode potential of –50 mV vs. a 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 just 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 20–150 μM with a detection limit of 5 μM, which is well within the clinical range of 78–206 μM.

2.2.3 Mass-sensitive techniques

Another form of transduction that has been used for biosensors is the measurement of small changes in mass [151–159]. This is the newest of the three classes of measurements, however, it has already been shown to capable of very sensitive measurements. The principle means of mass analysis relies on the use of piezoelectric crystals. These crystals 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 therefore 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 be used to determine the additional mass of the crystal.

Guilbault and coworkers developed a quartz crystal microbalance biosensor for the detection of Listeria monocytogenes [160]. Several different approaches were tested for 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 complex, and measurements were obtained. Calibration of the sensor was accomplished using a displacement assay and was found to have a response range from 2.5×10^5–2.5×10^7 cells/crystal. More recently, Guilbault and coworkers have developed a method for covalently binding antibodies to the surface of piezoelectric crystals via sulfur based self-assembled monolayers [161]. 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 usage of a horizontally polarized surface acoustic wave biosensor has been reported by Hunklinger and coworkers [162]. This sensor has a dual path configuration, with one path acting as an analyte sensitive path and the other path acting as a reference path. Antibodies were immobilized onto the sensor via protein A, with a mass density of 0.4 ng/mm^2. A theoretical detection limit of 33 pg was calculatcd based on these experiments, and a sensitivity of 100 kHz/(ng/mm^2) is reported. In addition, a means of inductively coupling a surface acoustic wave biosensor to its RF generating circuitry has been reported recently [163]. This technique could greatly reduce wire bonding associated problems for measurements made in liquids, since the electrodes are coated with a layer of SiO_2.

3 Biochips

Within the last couple of decades, the development of integrated biosensors for the detection of multiple biologically relevant species has begun to take place [164–168]. These integrated biosensor arrays that use the same excitation source for all of the elements and the same measurement process have been termed many things: gene chips, DNA-chips, etc. Most of the different array chips have been based on the use of nucleic acids (i.e. DNA) as the bioreceptors. Other types of bioreceptors such as antibodies, enzymes and cellular components can also be used. It is noteworthy that substrates having microarrays of bioreceptors are often referred to as biochips although most of these systems do not have integrated microsensor detection systems. A few of the more recent applications and advances in biochip technology will be discussed in this review.

In 1997, Dempsey et al. described the development of a microarray of electrochemical biosensors for the detection of glucose and lactate on line [169]. This array of electrochemical biosensors was prepared by photolithographic techniques, using glucose oxidase and lactate oxidase as the bioreceptors. The glucose oxidase or lactate oxidase at each of the different sites in the array produces hydrogen peroxide when its appropriate substrate, glucose or lactate, is present. The hydrogen peroxide produced was measured at each element amperometrically. The entire electrode/bioreceptor array is capable of very sensitive measurements, 1–5 nA/mM, for glucose and lactate with dynamic ranges of 0.1–35 and 0.05–15 mM, respectively.

An optical microarray system using a charge-coupled device (CCD) detector and DNA probes has been developed by Vo-Dinh and coworkers [37]. The evaluation of various system components developed for the DNA multiarray biosensor were discussed. DNA probes labeled with visible and near infrared (NIR) dyes are evaluated. Examples of applications of gene probes in DNA hybridization experiments and in biomedical diagnosis (detection of the *p53* cancer suppressor gene) illustrated the usefulness and potential of the DNA multiarray device. An optical microarray for the detection of toxic agents using

a planar array of antibody probes was described by Ligler and coworkers [170]. Their system was composed of a CCD for detection, an excitation source and a microscope slide with a photoactivated optical adhesive. Antibodies against three different toxins, staphylococcal enterotoxin B (SEB), ricin and Yersinia pestis, were covalently attached to small wells in the slide formed by the optical adhesive. The microscope slide was then mounted over the CCD with a gradient refractive index (GRIN) lens array used to focus the wells onto the CCD. Toxins were then introduced to the slide followed by Cy5-labeled antibodies. The bound antibodies were then excited and the resulting fluorescence from all of the sensor locations were monitored simultaneously. Concentrations ranging from 5–25 ng/mL were capable of being measured for the different toxins.

High-density oligonucleotide arrays, consisting of greater than 96 000 oligonucleotides have been designed by Hacia et al. for the screening of the entire 5.53 kb coding region of the hereditary breast and ovarian cancer *BRCAI* gene for all possible variations in the homozygous and heterozygous states [171]. Single-stranded RNA targets were created by PCR amplification followed by *in vitro* transcription and partial fragmentation. These targets were then tested and fluorescence responses from targets containing the four natural bases to greater than 5 592 different fully complimentary 25mer oligonucleotide probes were found. To examine the effect of uridine and adenosine on the hybridization specificity, 33 200 probes containing centrally localized base pair mismatches were constructed and tested. Targets that contained modified 5-methyluridine showed a localized enhancement in fluorescence hybridization signals. In general, oligonucleotide microarrays, often referred to as "DNA chips", are generally made by a light-directed chemical reaction that uses photographic masks for each chip [172]. A maskless fabrication method of light-directed oligonucleotide microarrays using a digital microarray has been reported [173]. In this method, a maskless array synthesizer replaces the chrome mask with virtual masks generated on a computer, which are relayed to a digital microarray.

The development of a truly integrated biochip having a phototransistor integrated circuit (IC) microchip has been reported by Vo-Dinh and coworkers [173, 174]. This work involves the integration of a 4×4 and 10×10 optical biosensor array onto an integrated circuit (Fig. 4). Most optical biochip technologies are very large when the excitation source and detector are considered, making them impractical for anything but laboratory usage. In this biochip the sensors, amplifiers, discriminators and logic circuitry are all built onto the chip. In one biochip system, each of the sensing elements is composed of 220 individual phototransistor cells connected in parallel to improve the sensitivity of the instrument. The ability to integrate light emitting diodes (LEDs) as the excitation sources into the system is also discussed. An important element in the development of the multifunctional biochip (MFB) involves the design and development of an IC electro-optic system for the microchip detection elements using the complementary metal oxide silicon (CMOS) technology. With this technology, highly integrated biochips are made possible partly through the capability of fabricating multiple optical sensing elements and microelectronics on a single system. Applications of the biochip are illustrated by measurements of the HIV1 sequence-specific probes using the DNA biochip device for the detection of a gene segment of the AIDS virus [174]. Recently, a MFB which allows simultaneous detection of several disease end-points using different bioreceptors, such as DNA, antibodies, enzymes, and cellular probes, on a single biochip system was developed [175]. The MFB device was a self-contained system based on an integrated circuit including photodiode sensor arrays, electronics, amplifiers, discriminators and logic circuitry. The multi-functional capability of the MFB biochip device is illustrated by measurements of different types of bioreceptors using DNA probes specific to gene fragments of the *Mycobacterium Tuberculosis* (TB) system, and antibody probes targeted to the cancer related tumor suppressor gene *p53*.

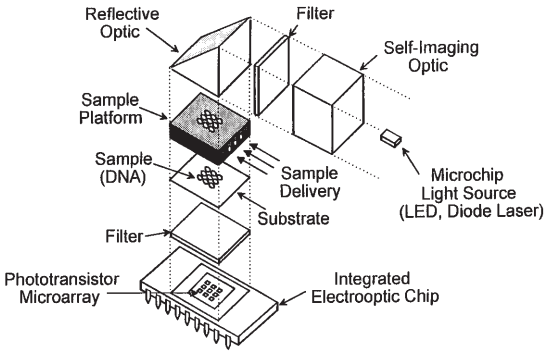

Fig. 4 Schematic diagram of an integrated DNA biochip system (Source: Ref [173])

4 Conclusion ond outlook

The past decade has seen great advancements in the field of bioanalysis along many fronts. Among the most rapidly advancing of these fronts is the area of biosensing, whether it is single analyte detection methods or multiarray-based biochip technology. The 1990s have seen the development of biosensors for many different analyses, and even seen them begin to advance to clinical and in some cases commercially available technologies [176, 177]. In 1998 and 1999, over 500 papers per year in the area of biosensor technology were published, and this rate is increasing. This great interest in the field of biosensors and biochips has revealed a great deal of information about the biology of all living things and may eventually provide an easy method for people to one day test themselves for certain illnesses at home or aid in the understanding of genetically transmitted illnesses.

For practical medical diagnostic applications, there is currently a strong need for a truly integrated biochip system that comprises probes, samplers, detector as well as amplifier and logic circuitry. Such a system will be useful in

physician's offices and could be used by relatively unskilled personnel. Most DNA biosensors previously reported are based on fiberoptic probes or glass and silica plates used as the probe substrates which are externally connected to a photosensing system generally consisting of a conventional detection device, such as a photomultiplier, or a charge-coupled device (CCD). Although the probes on the sampling platform are small (often referred to as a "DNA chip" or "gene chip"), the entire device containing excitation laser sources and detection systems (often a confocal microscope system) is relatively large, e.g., table-top size systems. While these systems have demonstrated their usefulness in gene discovery and genomics research, they are laboratory-oriented and involve relatively expensive equipment.

Biochip technologies could offer a unique combination of performance capabilities and analytical features of merit not available in any other bioanalytical system currently available. With its multichannel capability, biochip technology allows simultaneous detection of multiple biotargets. Biochip systems have great promise to offer several advantages in size, performance, fabrication, analysis and production cost due to their integrated optical sensing microchip. The small sizes of the probes (microliter to nanoliter) minimize sample requirement and reduce reagent and waste requirement. Highly integrated systems lead to a reduction in noise and an 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. The assembly process of various components is made simple by integration of several elements on a single chip. For medical applications, this cost advantage will allow the development of extremely low cost, disposable biochips that can be used for in-home medical diagnostics of diseases without the need of sending samples to a laboratory for analysis.

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