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Micro and Nanoscale Biosensors and Materials

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Biosensors and Biochips

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This chapter provides an overview of the various types of biosensors and biochips that have been developed for biological and medical applications, along with significant advances and over the last several years in these technologies. Various classification schemes that can be used for categorizing the different biosensor and biochip systems are also discussed.

1.1. INTRODUCTION

A biosensor can be generally defined as a device that consists of a biological recognition system, often called a bioreceptor, and a transducer. 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. 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 1.1 illustrates the conceptual principle of the biosensing process. Biosensors that include transducers based on integrated circuit microchips are often referred to as biochips.

There are several classification schemes possible. Biosensors and biochips can be classified either by their bioreceptor or their transducer type (see Figure 1.2). 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 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,

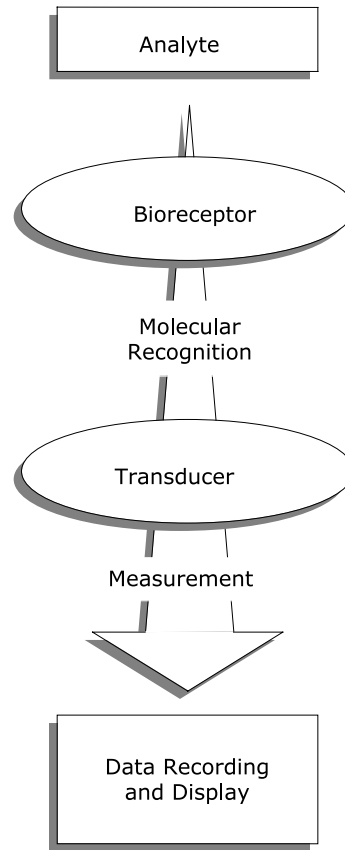


FIGURE 1.1. Conceptual diagram of the biosensing principle.

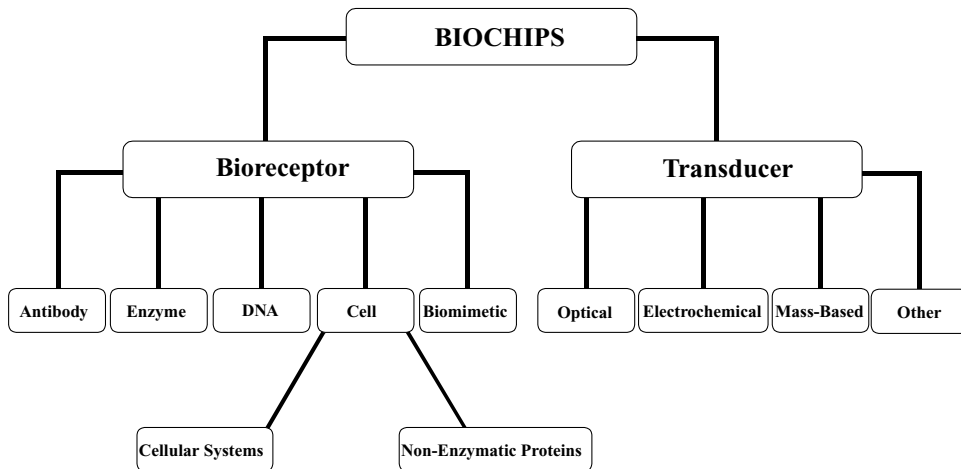


FIGURE 1.2. Schematic of biosensor/biochip classification schemes.

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.).

The development of biosensors was first reported in the early 1960s [6]. Biosensors have now seen an explosive growth and seen a wide variety of applications primarily in two major areas, biological monitoring and environmental sensing applications.

1.2. BIOSENSORS

1.2.1. Different Types of Bioreceptors

The key to specificity for biosensor technologies involves bioreceptors. 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 1.3 shows a schematic diagram of two types of bioreceptors: the structure of an immunoglobulin G (IgG) antibody molecule (Fig. 1.3A), and DNA and the principle of base pairing in hybridization (Fig. 1.3B).

1.2.1.1. Antibody Bioreceptors An antibody is a complex biomolecule, made up of hundreds of individual amino acids arranged in a highly ordered sequence. Antibodies are biological molecules that exhibit very specific binding capabilities for specific structures. 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 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

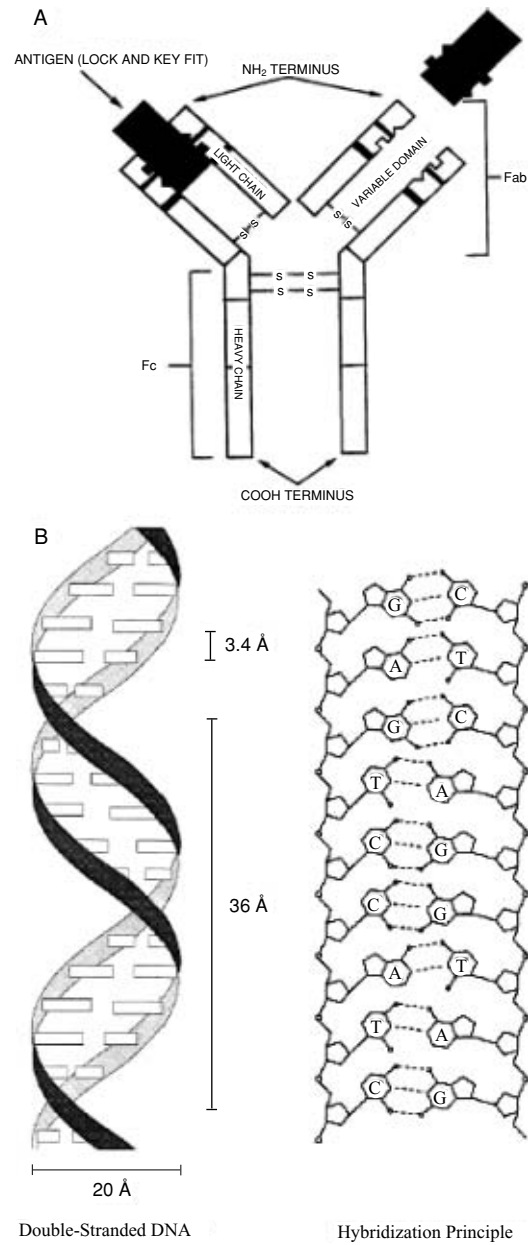


FIGURE 1.3. Schematic diagrams of two types of bioreceptors: **A)** IgG antibody. **B)** DNA and the hybridization principle.

for the detection of chemicals and biological materials of environmental and biomedical interest.

The first fiberoptic immunosensor was developed for *in situ* detection of the chemical carcinogen benzo[a]pyrene [52]. Nowadays, antibodies are often used in biosensors today. 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 [52]. 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 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 [42]. 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.

Antibody-based biosensors have been developed for use in an electrochemical immunoassay for whole blood [4]. 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 water.

Binding of the bioreceptor to the measurement support or the transducer is an important aspect of biosensor fabrication. A method for the immobilization of histidine-tagged antibodies onto a gold surface for surface plasmon resonance measurements was reported [15]. A synthetic thioalkane chelator is self-assembled on a gold surface. Reversible binding of an anti-lysozyme F-ab 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 [51].

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 [1, 8, 50]. Nanometer scale fiberoptic 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 [1]. These nanosensors allow the probing of cellular and subcellular environments in single cells [8, 50] as well as monitoring signaling processes in single cells [18, 46].

1.2.1.2. Enzyme Bioreceptors Another type of commonly used bioreceptors involves enzymes, which 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 [9].

Multiple enzymes have been immobilized onto an array of optical fibers for use in the simultaneous detection of penicillin and ampicillin [29]. 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.

The development and use of a micrometer-sized fiber-optic biosensor were reported for the detection of glucose [30]. These biosensors are 100 times smaller than existing glucose optodes and represent the beginning of a new trend in nanosensor technology [2]. These sensors are based on the enzymatic reaction of glucose oxidase that 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 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 [30].

1.2.1.3. Nucleic Acid Bioreceptors Nucleic acids have received increasing interest as bioreceptors for biosensor and biochip technologies. The complementarity of adenine:thymine (A:T) and cytosine:guanosine (C:G) pairing in DNA (Fig. 1.2b) 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.

DNA biosensors have been developed for the monitoring of DNA-ligand interactions [26]. 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/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.

Another type of biosensor uses a peptide nucleic acid as the biorecognition element [33]. 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.

Vo-Dinh and coworkers have developed a new type of DNA gene probe based on surface-enhanced Raman scattering (SERS) detection [16, 49]. The SERS probes do not require the use of radioactive labels and 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 immunodeficiency (HIV) virus [16]. The development of a biosensor for DNA diagnostics using visible and near infrared (NIR) dyes has been reported [48]. The system employed a two-dimensional charge-coupled device and was used to detect the cancer suppressor *p53* gene.

1.2.1.4. Cellular Bioreceptors Cellular structures and cells have been used in the development of biosensors and biochips [12]. 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.

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 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 or 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 [9].

A microbial biosensor has been developed for the monitoring of short-chain fatty acids in milk [34]. *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. 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.

Many proteins 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.

Detection of endotoxin using a protein bioreceptor based biosensor has been reported [17]. 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.

Lipopeptides have been used as bioreceptors for biosensors [3]. 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.

1.2.1.5. Biomimetic Receptors An artificial (man-made) 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. 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 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 [27]. 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.

Bioreceptor systems also used artificial membranes 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) [5]. 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 $\mu\text{g/mL}$ were capable of being monitored.

Bioreceptors based on molecular imprinting have been used for the construction of a biosensor based on electrochemical detection of morphine [20]. 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. 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. On the other hand, due to their rigid structures, molecular imprint probes do not have the same flexibility and selectivity as compared to actual bioreceptors.

1.2.2. Types of Transducers

Transduction can be accomplished via a great variety of methods. Biosensors can also be classified based upon the transduction methods they employ. 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 contains many different subclasses, creating a nearly infinite number of possible transduction methods or combination of methods.

1.2.2.1. Optical Techniques Optical biosensors can use many different types of spectroscopy (e.g., absorption, fluorescence, phosphorescence, Raman, SERS, refraction, dispersion spectrometry, etc.) with different spectrochemical properties recorded. For this reason, optical transduction, which offers the largest number of possible subcategories, have been developed in our laboratory over the last two decades [1, 2, 8, 9, 16, 29, 30, 42, 48–52]. 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 polarization 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 [19]. 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.

Absorption measurements of a pH sensitive dye are used to quantify the amount of urea present [23]. 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.

A fiber-optic evanescent wave immunosensor for the detection of lactate dehydrogenase has been developed [28]. Two different assay methods, a one-step and a two-step assay 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 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.

1.2.2.2. Electrochemical Techniques Electrochemical detection is another possible means of transduction that has been used in biosensors [11, 31, 41]. 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 [32]. 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 [14]. 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.

An electrochemical biosensor has been developed for the indirect detection of L-phenylalanine via NADH [25]. 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 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.

1.2.2.3. Mass-sensitive Techniques Measurement of small changes in mass is another form of transduction that has been used for biosensors [24, 40]. 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.

A quartz crystal microbalance biosensor has been developed for the detection of *Listeria monocytogenes* [43]. 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 $2.5 \times 10^5 - 2.5 \times 10^7$ 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 [53]. 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.

A horizontally polarized surface acoustic wave biosensor has been reported [10]. 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 [10]. 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 calculated based on these experiments, and a sensitivity of $100 \text{ kHz}/(\text{ng/mm}^2)$ is reported. In addition, a means of inductively coupling a surface acoustic wave biosensor to its RF generating circuitry has been reported recently [21]. 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 .

1.3. BIOCHIPS

1.3.1. *Microarray Systems*

Within the last couple of decades, the development of integrated biosensors for the detection of multiple biologically relevant species has begun to take place. 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. Figure 1.4 illustrates an example of DNA microarray system with its associated detection system. 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.

A microarray of electrochemical biosensors has been developed for the detection of glucose and lactate on line [54]. This array of electrochemical biosensors was prepared using 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.

An optical microarray system using a charge-coupled device (CCD) detector and DNA probes has been developed by Vo-Dinh and coworkers [48]. The evaluation of various system components developed for the DNA multi-array biosensor was discussed. The DNA probes labeled with visible and near infrared (NIR) dyes are evaluated. Examples of application 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

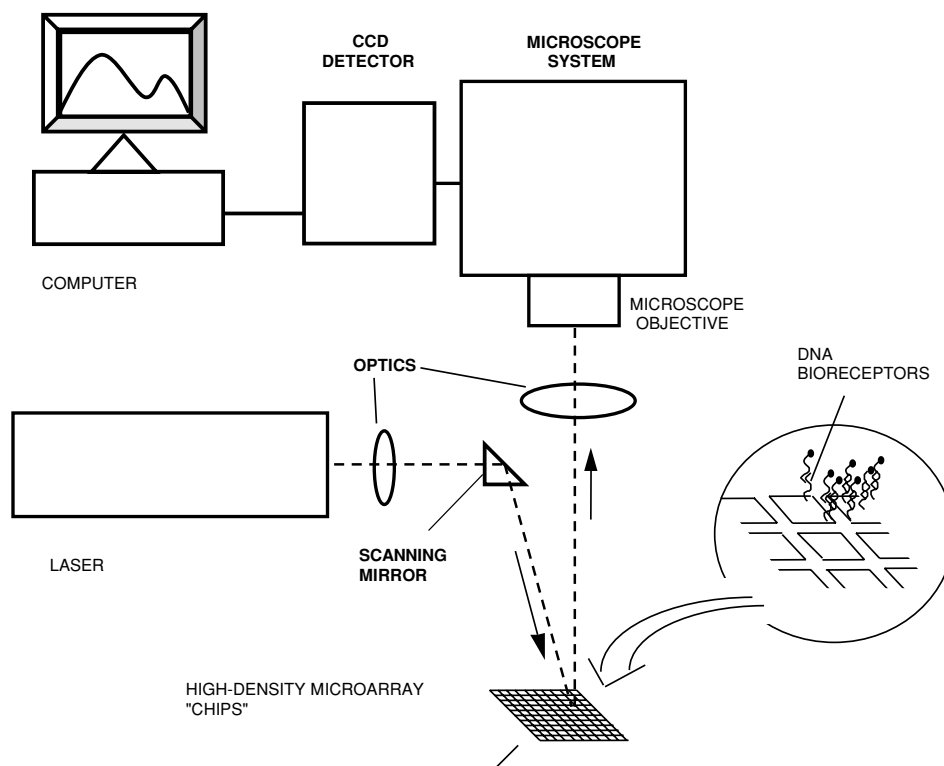


FIGURE 1.4. Schematic diagram of a DNA microarray with detection system.

multiarray device. An optical microarray for the detection of toxic agents using a planar array of antibody probes was described by Ligler and coworkers [13]. 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 *BRCA1* gene for all possible variations in the homozygous and heterozygous states [35]. 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 complementary 25 mer 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 [35]. A maskless fabrication method of light-directed oligonucleotide microarrays using a digital microarray has been reported [47]. 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.

1.3.2. Integrated Biochip Systems

The development of a truly integrated biochip having a phototransistor integrated circuit (IC) microchip has been reported by Vo-Dinh and coworkers [47, 48]. This work involves the integration of a 4×4 and 10×10 optical biosensor array onto an integrated circuit (Figure 1.5). 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

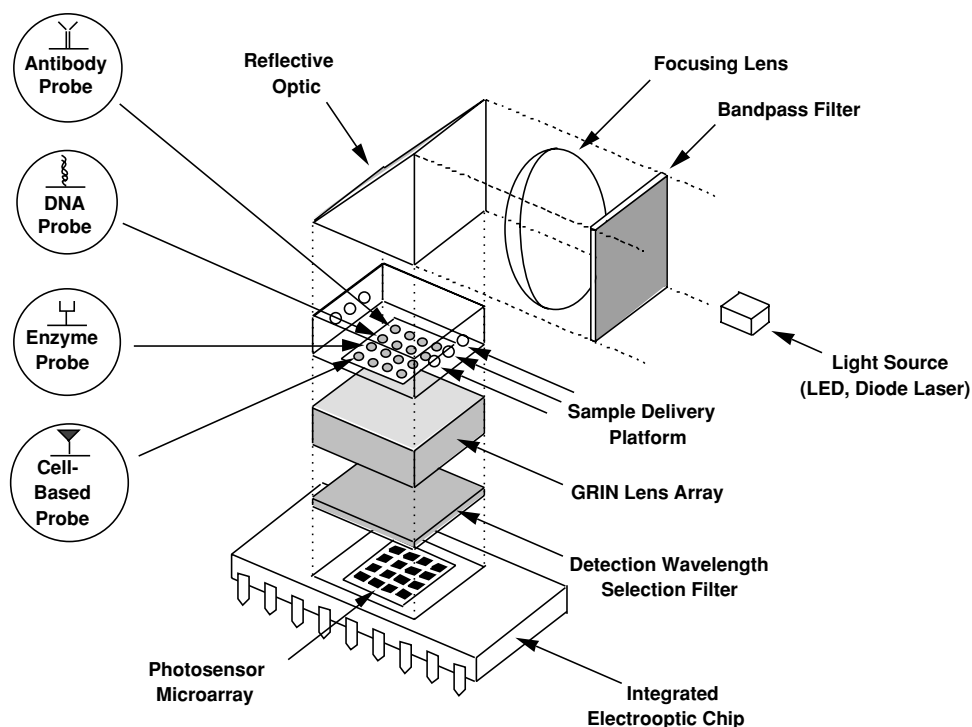


FIGURE 1.5. Schematic diagram of an integrated biochip system with microchip sensor.

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 [47]. Recently, a MFB which allows simultaneous detection of several disease endpoints using different bioreceptors, such as DNA, antibodies, enzymes, cellular probes, on a single biochip system was developed [22]. 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*.

A biochip equipped with a microfluidics sample/reagent delivery system for on-chip monitoring of bioassays has been developed for *E. coli* detection [39]. The microfluidics system includes a reaction chamber which houses a sampling platform that selectively captures detection probes from a sample through the use of immobilized bioreceptors. The independently operating photodiodes allow simultaneous monitoring of multiple samples. In this study the sampling platform is a cellulosic membrane that is exposed to *E. coli* organisms and subsequently analyzed using a sandwich immunoassay involving a Cy5-labeled antibody probe. Studies show that the biochip has a linear dynamic range of three orders of magnitude observed for conventional assays, and can detect 20 *E. coli* organisms. Selective detection of *E. coli* in a complex medium, milk diluent, is also reported for both off-chip and on-chip assays.

A CMOS biochip coupled to multiplex capillary electrophoresis (CE) system has been developed [36, 37]. This combination of multiplex capillary gel electrophoresis and the IC microchip technology represents a novel approach to DNA analysis on the microchip platform. Separation of DNA ladders using a multiplex CE microsystem of four capillaries was monitored simultaneously using the IC microchip system. The IC microchip-CE system has advantages such as low cost, rapid analysis, compactness, and multiplex capability, and has great potential as an alternative system to conventional capillary array gel electrophoresis systems based on charge-coupled device (CCD) detection.

Antibody-immobilized capillary reactors coupled to biochip detection have been developed for *E. coli* O157:H7 detection using enzyme-linked immunosorbent assay (ELISA), and a biochip system [38]. ISA is very sensitive and selective immunological method to detect pathogenic bacteria. ELISA is also directly adaptable to a miniature biochip system that utilizes conventional sample platforms such as polymer membranes and glass. The antibody immobilized capillary reactor is a very attractive sample platform for ELISA because of its low cost, compactness, reuse, and ease of regeneration. Moreover, an array of capillary reactors can provide high-throughput ELISA. In this report, we describe the use of an array of antibody-immobilized capillary reactors for multiplex detection of *E. coli* O157:H7 in our miniature biochip system. Side-entry laser beam irradiation to an array of capillary reactors contributes significantly to miniaturized optical configuration for this biochip system.

The detection limits of *E. coli* O157:H7 using ELISA and Cy5 label-based immunoassays were determined to be 3 cells and 230 cells, respectively. This system shows capability to simultaneously monitor multifunctional immunoassay and high sensitive detection of *E. coli* O157:H7.

The application of a biochip using the molecular beacon (MB) detection scheme has been reported [Culha et al, 2004]. The medical application of this biochip novel MB detection system for the analysis of the breast cancer gene BRCA1 was illustrated. The MB is designed for the BRCA1 gene and a miniature biochip system is used for detection. The detection of BRCA1 gene is successfully demonstrated in solution and the limit of detection (LOD) is estimated as 70 nM.

1.4. CONCLUSION

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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REFERENCES

- [1] J.P. Alarie and T.Vo-Dinh. *Polycyc. Aromat. Compd.*, 8:45, 1996.
- [2] S.L.R. Barker, R. Kopelman, T.E. Meyer, and M.A. Cusanovich. *Anal. Chem.*, 70:971, 1998.
- [3] M. Boncheva, C. Duschl, W. Beck, G. Jung, and H. Vogel. *Langmuir*, 12:5636, 1996.
- [4] C.N. Campbell, T. de Lumley-Woodyear, and A. Heller. *Fresen. J. Anal. Chem.*, 364:165, 1993.
- [5] D. Charych, Q. Cheng, A. Reichert, G. Kuziemko, M. Stroh, J.O. Nagy, W. Spevak, and R.-C. Stevens. *Chem. Biol.*, 3:113, 1996.
- [6] L.C. Clark, Jr. and C. Lions. *Ann. Acad. Sci.*, 102:29, 1962.
- [7] M. Culha, D.L. Stokes, G.D. Griffin, and T. Vo-Dinh. *Biosens. Bioelectron.*, 19:1007, 2004.
- [8] B.M. Cullum, G.D. Griffin, and T. Vo-Dinh. *Anal. Biochem.*, 1999.
- [9] D. Diamond. (ed.). *Chemical and Biological Sensors*. Wiley, New York, 1998.
- [10] J. Freudenberg, S. Schelle, K. Beck, M. vonSchickfus, and S. Hunklinger. *Biosens. Bioelectron.*, 14:423, 1999.
- [11] C. Galan-Vidal, J. Munoz, C. Dominguez, and S. Alegret. *Sensor. Actuat. B-Chem.*, 53:257, 1998.
- [12] J.J. Gooding and D.B. Hibbert. *Trac-Trend Anal. Chem.*, 18:525, 1999.
- [13] J.G. Hacia, S.A. Woski, J. Fidanza, K. Edgemon, G. McCall, S.P.A. Fodor, and F.S. Collins. *Nucleic Acids Res.*, 26:4975, 1998.
- [14] T. Huang, A. Warsinke, T. Kuwana, and F. W. Scheller. *Anal. Chem.*, 70:991–997, 1998.
- [15] D. Kroger, M. Liley, W. Schiweck, A. Skerra, and H. Vogel. *Biosens. Bioelectron.*, 14:155, 1999.
- [16] N. Isola, D.L. Stokes, and T. Vo-Dinh. *Anal. Chem.*, 70:1352, 1998.
- [17] E.A. James, K. Schmeltzer, and F.S. Ligler. *Appl. Biochem. Biotechnol.*, 60:189, 1996.
- [18] P.M. Kasili, J.M. Song, and T. Vo-Dinh. *J. Am. Chem. Soc.*, 126:2799–2806, 2004.
- [19] R. Koncki, G.J. Mohr, and O.S. Wolfbeis. *Biosens. Bioelectron.*, 10:653, 1995.
- [20] D. Kriz and K. Mosbach. *Anal. Chim. Acta*, 300:71, 1995.
- [21] I.V. Lamont, R.I. McConnell, and S.P. Fitzgerald. *Clin. Chem.*, 45:A102, 1999.
- [22] M. Malmquist. *Biochem. Soc.*, T 27:335, 1999.
- [23] T. McCormack, G.O'Keeffe, B.D. MacCraith, and R.O'Kennedy. *Sensor. Actuat. B-Chem.*, 41:89, 1997.
- [24] M. Minunni, M. Mascini, R.M. Carter, M.B. Jacobs, G.J. Lubrano, and G.C. Guilbault. *Anal. Chim. Acta*, 325:169, 1996.
- [25] Z.H. Mo, X.H. Long, and W.L. Fu. *Anal. Commun.*, 36:281–283, 1999.
- [26] J. Piehler, A. Brecht, G. Gauglitz, M. Zerlin, C. Maul, R. Thiericke, and S. Grabley. *Anal. Biochem.*, 249:94, 1997.
- [27] R.T. Piervincenzi, W.M. Reichert, and H.W. Hellenga. *Biosens. Bioelectron.*, 13:305, 1998.
- [28] T.E. Plowman, W.M. Reichert, C.R. Peters, H.K. Wang, D.A. Christensen, and J.N. Herron. *Biosens. Bioelectron.*, 11:149, 1996.
- [29] J. Polster, G. Prestel, M. Wollenweber, G. Kraus, and G. Gauglitz. *Talanta*, 42:2065, 1995.
- [30] Z. Rosenzweig and R. Kopelman. *Anal. Chem.*, 68:1408–1413, 1996.
- [31] U. Rudel, O. Geschke, and K. Cammann. *Electroanalysis*, 8:1135, 1996.
- [32] P. Sarkar and A.P.F. Turner. *Fresen. J. Anal. Chem.*, 364:154, 1999.
- [33] S. Sawata, E. Kai, K. Ikebukuro, T. Iida, T. Honda, and I. Karube. *Biosens. Bioelectron.*, 14:397, 1999.
- [34] A. Schmidt, C. Standfuss-Gabisch, and U. Bilitewski. *Biosens. Bioelectron.*, 11:1139, 1996.
- [35] S. Singh-Gasson, R.D. Green, Y. Yue, C. Nelson, F. Blattner, R. Sussman, and F. Cerrina. *Nat. Biotechnol.*, 10:974, 1999.
- [36] J.M. Song and T. Vo-Dinh. *Anal. Bioanal. Chem.*, 373:399, 2002.
- [37] J.M. Song, J. Mobley, and T. Vo-Dinh. *J. Chromatogra. B*, 783:501, 2003.
- [38] J.M. Song and T. Vo-Dinh. *Anal. Chim. Acta*, 507:115, 2004.
- [39] D.L. Stokes, G.D. Griffin, and T. Vo-Dinh. *Fresen. J. Anal. Chem.*, 369, 2001.
- [40] T. Thundat, P.I. Oden, and R.J. Warmack. *Microscale Thermophys. Eng.*, 1:185, 1997.
- [41] F. Tobalina, F. Pariente, L. Hernandez, H.D. Abruna, and E. Lorenzo. *Anal. Chim. Acta*, 395:17, 1999.

- [42] B.G. Tromberg, M.J. Sepaniak, T. Vo-Dinh, and G.D. Griffin. *Anal. Chem.*, 59:1226, 1987.
- [43] R.D. Vaughan, C.K. Sullivan, and G.C. Guilbault. *Fresen. J. Anal. Chem.*, 364:54, 1999.
- [44] T. Vo-Dinh. *Sensor. Actuat B-Chem.*, 51:52, 1998.
- [45] T. Vo-Dinh. *Proceedings of the 6th Annual Biochip Technologies Conference: Chips for Hits '99*. Berkeley, California, Nov 2–5 1999.
- [46] T. Vo-Dinh. *J. Cell. Biochem.*, 39(Suppl.):154, 2002.
- [47] T. Vo-Dinh, J.P. Alarie, N. Isola, D. Landis, A.L. Wintenberg, and M.N. Ericson. *Anal. Chem.*, 71:358–363, 1999.
- [48] T. Vo-Dinh, N. Isola, J.P. Alarie, D. Landis, G.D. Griffin, and S. Allison. *Instrum. Sci. Technol.*, 26:503, 1998.
- [49] T. Vo-Dinh, K. Houck, and D.L. Stokes. *Anal. Chem.*, 33:3379, 1994.
- [50] T. Vo-Dinh, B.M. Cullum, J.P. Alarie, and G.D. Griffin. *J. Nanopart. Res.*, (in press).
- [51] T. Vo-Dinh, G.D. Griffin, and K.R. Ambrose. *Appl. Spectrosc.*, 40:696, 1986.
- [52] T. Vo-Dinh, B.G. Tromberg, G.D. Griffin, K.R. Ambrose, M.J. Sepaniak, and E.M. Gardenhire. *Suppl. Spectrosc.*, 41:735, 1987.
- [53] W. Welsch, C. Klein, M. vonSchickfus, and S. Hunklinger. *Anal. Chem.*, 68:2000, 1996.
- [54] R.M. Wadkins, J.P. Golden, L. M. Pritsiolas, and F.S. Ligler. *Biosens. Bioelectron.*, 13:407, 1998.