

An Introduction to Biosensing for Biosafety

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Ministry of Environment
and Natural Resources



National Biosafety Framework
Development Project

**An Introduction
to
Biosensing
for
Biosafety**

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Preface

Biosensing can be simply defined as a technique, used for obtaining qualitative and/or quantitative measurements of biomolecular interactions in real time. This technique is important in its application to human systems as it ensures their safety in several ways, such as,

- in ensuring the environment they live in is free of toxic chemicals, particularly in the following respects.
 - the water bodies are not contaminated
 - the food consumed is free of deadly pathogens
- in providing a method to ensure that they are keeping in good health, or to detect the presence of diseases at early stages, which thus enables the necessary precautions to be taken as deemed appropriate to prevent or cure diseases.

An effort is made in this book to explain the basics of biosensing with emphasis on their application in the fields of medicine, food, industrial and environmental concerns. Thus the book consists of two sections, namely,

- (i) the basics of biosensing
- (ii) applications of biosensing

FOREWORD

Testing and detection of material hazardous to the environment and human health have become increasingly important and urgent in the modern world. With the rapid development of science, especially at the molecular level, we have witnessed the introduction of novel, powerful techniques, such as the recombinant DNA technique, which bring with them risks and concerns regarding their effects on the environment and human health. With every new invention, we humans have to find means of protecting ourselves from the very same inventions! But, fortunately, modern technology itself can be used to detect such risks and thereby help to preserve a healthy population in a pollution-free environment. Biosensing is one such modern technology. Prof. D.C. Bandara, who has had postdoctoral training in biosensing, has provided valuable information on this subject in this book, in a very simple manner to show how biosensing can be used to help protect ourselves from various hazards, including those posed by novel biological organisms and their products.

Prof. Athula Perera

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Ministry of Environment & Natural Resources

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We hereby also wish to express our appreciation of the presentations made by the 2004/2005 and the 2003/2004 M.Sc. (Biotechnology) students in their course of study, at the Postgraduate Institute of Agriculture (PGIA), University of Peradeniya, Sri Lanka , which provided some information for the preliminary studies in the development of this book.

We also wish to extend our thanks to Mr. Venura Herath, of the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, for his assistance in designing the cover page and graphics.

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Part 1

Basics of Biosensing

What is biosensing ?

Biosensing is a technique, used for obtaining qualitative and/or quantitative measurements of biomolecular interactions in real time.

Techniques for biomolecular analysis

There are two basic approaches adopted in understanding biological processes at molecular level. They are,

- (a) structural analysis
- (b) kinetic (functional) analysis

(a) Structural analysis

Structural analysis determines the atomic organization of biomolecules, either alone or in interaction with other molecules. Some techniques utilized for structural analysis are, X-ray, electron diffraction techniques, electron microscopy, NMR (Nuclear Magnetic Resonance) and sequence analysis. All these methods are largely static, having the ability to make only a snapshot recording of an interaction process frozen in time. These techniques thus do not reflect the actual process of biomolecular interactions which occur rapidly. This is the main disadvantage of adopting structural analysis techniques to study biomolecular interactions.

(b) Kinetic (functional) analysis

Availability of kinetic analysis techniques is relatively few and they possess serious limitations. These techniques include affinity chromatography, immunological techniques (ELISA) and spectrophotometric methods. They do not have the capability to record all the sequences of a particular reaction unless they occur in relatively long periods of time. Although spectrophotometric methods are relatively better in this respect than affinity chromatographic methods, they are limited to molecules with suitable absorbant or fluorescent groups.

The main disadvantages of these techniques are, the need to carry out biomolecule labeling and purification of interactants in relatively large quantities.

Biosensors

The problems that arise in adopting structural and functional techniques for biomolecular interaction analyses, could be overcome by employing biosensing techniques. Instruments that are used for biosensing are called biosensors.

A biosensor can be defined as, **an instrument that combines a biological recognition mechanism with a sensing device and transducer.**

The utilization of biosensors for biomolecular analyses is becoming popular, as they possess the following advantages.

- (i) They do not require any labelling (e.g. isotopes) of interactants.
- (ii) All stages in an interaction are monitored. In conventional methods detection is restricted to a single labelled component.
- (iii) Interactions are followed in real time. Therefore kinetic information is readily derived.
- (iv) In many cases, investigations can be performed without prior purification of components.
- (v) Very small amounts such as femtomoles can be determined.
- (vi) Sensitivity and accuracy are high.

Figure 1 represents the organization of basic components of a biosensor in general.

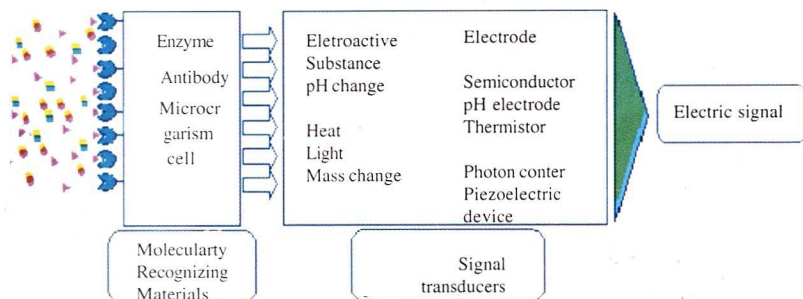


Figure 1. Basic components of a biosensor in general

The biological recognition mechanism involves biomolecular interaction of two interactants. They are referred to as the **ligand** and the **analyte**. The ligand is the interactant immobilized on the sensor chip surface. The analyte is the molecular species being measured in a biosensing application. Usually (but not always) it is the interactant injected in the free solution.

The transducer converts chemical signals generated on the active surface into electric signals, which in turn can be processed in a computer. The processed signal is presented as a function of time. A series of such signals make up a sensorgram. This can be used to derive valuable information regarding the biomolecular interaction, which can be useful in many applications.

Information obtainable with biosensors are,

- (i) Specificity or identity of an interaction – whether / which molecules interact?
- (ii) Concentration of an interactant – how many molecules are there?

- (iii) Affinity of two interactants – how strong is the interaction?
- (iv) Kinetics of an interaction – how fast is the interaction?
- (v) Cooperativity of an interaction – are there any allosteric effects?
- (vi) Relative binding patterns of interactants – are binding patterns for different species related?

There are different types of sensors such as optical, acoustical and electrochemical sensors. The principle behind each of these sensors is different. In this book, the principle behind optical biosensors will be discussed in detail. This will help to visualize the different principles of other biosensors, described in brief under the applications of biosensing in Part 2 of this book.

Optical Biosensors

Optical biosensors transduce the accumulation of mass at a surface into an optical signal. Coating the sensor surface with purified ligands confers the selectivity of these biosensors. They can detect a stable signal generated by binding as little as femtomoles of bulk phase interactant (analyte), to the immobilized ligand, over seconds to hours.

Many optical biosensors widely used today, are based on the principle of **Surface Plasmon Resonance (SPR)**.

Surface Plasmon Resonance (SPR)

In order to obtain SPR, an evanescent field should be generated, which is an exponential decaying wave at the sensor surface. It is generated when total internal reflection of the incident light occurs at the interface of two substances with different refractive indices, one with a high refractive index and one with a low refractive index. E.g. a glass-air interface. This incidence is represented in Figure 2.

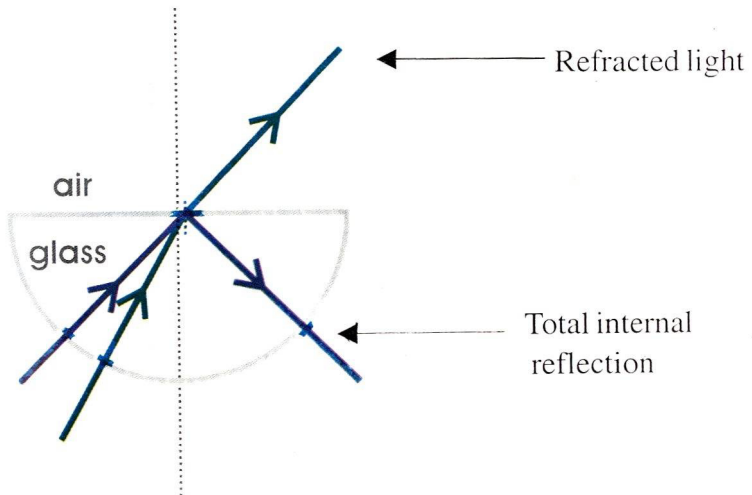


Figure 2. Total internal reflection

SPR occurs under certain conditions when a thin film of gold is present inside the evanescent wave. Therefore, usually the glass is coated with a thin film of gold. Figure 3 represents the occurrence of SPR.

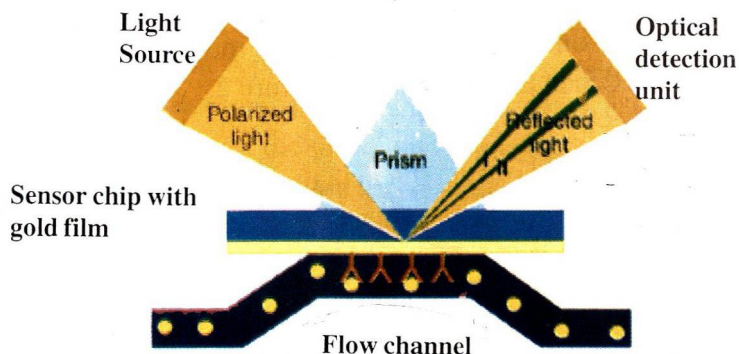


Figure 3. Surface plasmon resonance

When the incoming light is monochromatic and polarized, the free electrons of the metal will oscillate and absorb energy at a certain angle of incident light. The angle of incident light when SPR occurs is called the **SPR angle**.

SPR is detected by measurement of the intensity of the reflected light because, at the SPR angle a sharp decrease or 'dip' of intensity occurs. The SPR angle depends on the refractive index of the substance with the low refractive index close to the sensing surface. The refractive index near the sensor surface changes because of binding of biomolecules to the surface. The SPR angle is dependent on the amount of bound biomolecules. Value of the SPR angle changes linearly with the concentration of bound molecules.

Essential components of an optical sensor system

There are three main components essentially found in an optical sensor system.

- (i) the sensor chip
- (ii) the optical system
- (iii) a liquid handling system

(i) The sensor chip

The sensor chip consists of a biospecific surface where the interaction takes place. The ligand is immobilized on the sensor chip surface. It is also the place where the signal is generated and transduced.

Basically the sensor chip is a glass slide with a thin film of gold deposited on one side. Gold is chosen for its combination of chemical inertness and the ability to generate a good SPR response.

Different sensor chips are available for different purposes: general applications and specialized applications. However all these chips function in conformity to the same optical principle. The most generally applicable chip is the sensor chip CM5. It consists of three layers: glass, a thin gold film and a carboxy methyl dextran matrix, as shown in Figure 4. The matrix is bonded to the gold film through an inert linker layer. The chip SA5 is an example of a sensor chip used for specialized applications.

Significant characteristics of the surface matrix of the sensor chip are as follows.

- (i) Allows covalent immobilization of biomolecules using well characterized chemistry.
- (ii) Increases sensitivity by increasing the binding capacity of the surface.

- (iii) Provides a hydrophilic environment suitable for most interactions of biological interest.
- (iv) Provides a very low degree of non specific binding to the surface.

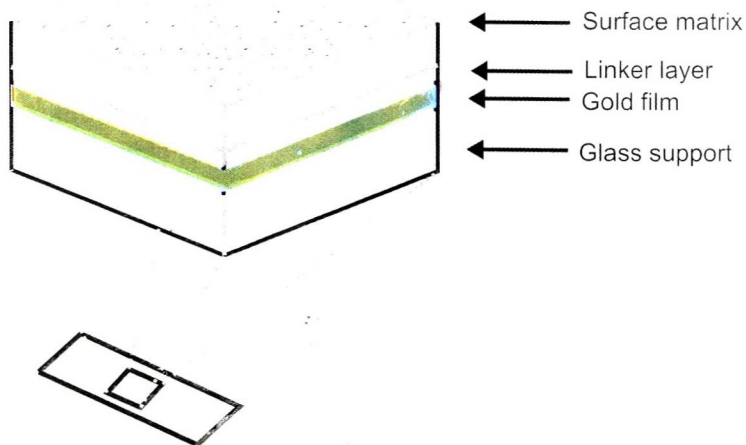


Figure 4. Arrangement of layers in sensorchip CM5

The sensor chip forms one wall of a micro flow cell where interactions are monitored. It is the matrix covered side of the sensor chip that forms this wall, which comes into contact with the solution being studied. The gold film is illuminated from the other side, through the glass. SPR arises through interaction of the light energy with the gold film, and monitors the surface concentration of biomolecules on the matrix side of the chip.

(ii) The optical system

In the optical system, the light source in most cases is a high efficiency diode emitting near infrared light. Light is focused on to the sensor chip in a wedge shaped beam giving a fixed range of incident angles. The SPR response is monitored by a fixed array of light sensitive diodes covering the whole wedge of reflected light. Computer interpolation routines automatically calculate the angle at which minimum reflection occurs (the SPR angle or the resonance angle) to a high degree of accuracy. The time resolution is set by the software responsible for analyzing the signals from the diode detectors.

(iii) The liquid handling system

In this system reagents, buffers and samples are supplied to the sensor chip surface through a precision liquid handling system. Therefore the system consists of pumps and automatic sample handling units. The **Integrated μ -Fluidic Cartridge (IFC)** is the major component of the liquid handling system. It is an integrated unit consisting of loops, channels and pneumatic valves making it possible for controlled volumes of liquid to be directed to flow cells. Operation of the IFC valves is automatically controlled through the instrument software.

The IFC is pressed into contact with the sensor chip by the docking mechanism of the instrument. Precision grooves in the IFC surface form 3 walls of the flow cell, while the sensor chip forms the fourth wall (Figure 5).

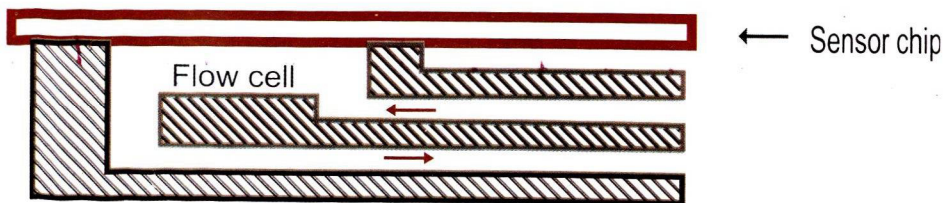


Figure 5. Integrated μ -Fluidic Cartridge (IFC)

The IFC construction and valve operation provide the facility of switching from sample to buffer instantaneously when required. In addition, very accurate control of the contact time of sample with the surface and analysis of rapid events within short intervals is also possible. All SPR measurements are carried out at controlled temperatures. This is because the refractive index, reaction kinetics and mass transport of the interacting molecules from bulk solution in flow cell to sensor surface, depend on temperature. Therefore the design of the instrumentation ensures that samples reach the measuring temperature during passage through the IFC.

Ligand immobilization

The ligand is the interactant immobilized on the sensor chip surface. Ligand immobilization can be either direct or indirect. Direct immobilization is done by covalent coupling of the ligand to the sensor surface directly. Indirect immobilization involves non covalent capture of the ligand by a covalently immobilized anchor as is illustrated in Figure 6.

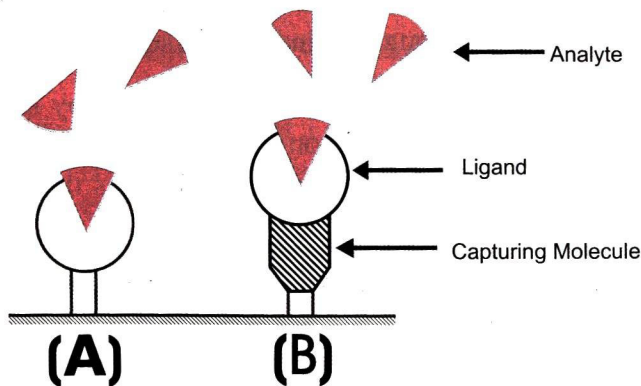


Figure 6. Direct (A) and indirect (B) ligand immobilization

Designing a real time experiment

The design of a real time experiment is dictated primarily by the purpose of the investigation. Some of the important factors to be considered before designing the experiment are:

- (i) Choice of ligand
- (ii) Choice of immobilization method
- (iii) Regeneration
- (iv) Preparing the surface
- (v) Buffer conditions
- (vi) Reproducibility of ligand immobilization

(i) Choice of ligand

If the investigation compares the interaction of several molecules with a common species, it is usually most convenient to use the common species as ligand.

The properties of an ideal ligand are as follows.

- **High specificity** : ensures a selective method even when the bulk solution is a complex mixture.
- **Regeneration ability** : the ligand should withstand conditions for regenerating the biospecific surface, so that one sensor chip flow cell can be reused in a series of measurements.
- **Purity** : the ligand should be as pure as possible, both for reproducible immobilization and to avoid non specific binding.
- **Size** : if the components are significantly different in size, since the response is proportional to the mass of interactant bound, it is better to immobilize the smaller component.

(ii) Choice of immobilization method

In direct immobilization, amine coupling is the most widely applicable coupling chemistry. However most macro molecular ligands have a diversity of potential attachment points with this method, which is disadvantageous. Therefore, if a homogeneous ligand population is important (e.g. kinetic determinations) it may be advantageous to use a coupling method where the attachment site is more closely defined.

Other coupling methods such as surface thiol, ligand thiol, streptavidin-biotin and aldehyde coupling, may also be necessary if the ligand contains reactive amines or other nucleophilic groups at the analyte binding site.

If a well defined chemical coupling site is not present in the ligand and cannot conveniently be introduced by modification, ligand attachment through a specific capturing molecule may

be a useful alternative. If so, the ligand may often be attached in a relatively well defined orientation, determined by the specific interaction with the capturing molecule. Use of capturing molecule can also enhance the purity of the ligand on the surface.

Regeneration with this technique often removes the ligand from the capturing molecule, so that each analyte cycle requires attachment of new ligand.

In ligand immobilization it is important to reduce non specific binding. Non specific binding includes,

- 1) binding of analyte to non specific sites on the surface
- 2) binding of non analyte molecule in the sample to surface of ligand.

(iii) Regeneration

Regeneration of the sensor chip surface is a unique feature of real time applications. Regeneration refers to removal of non covalently bound interactants from the sensor chip surface after an analysis so that the surface can be used for a new analysis cycle. Ideally regeneration leaves the ligand unaltered on the surface. The ability of the ligand to completely withstand the regeneration conditions should be considered when regenerating a surface.

Persistent non specific binding may lead to accumulation of material on the surface. Therefore, to be effectively regenerated they should be removed as well. Non specific accumulation of material in the matrix can, with time give rise to poor matrix penetration by the analyte. This reduces the response for a given concentration of analyte. Incomplete regeneration also introduces “memory effects”, since a fraction of surface bound analyte remains on the surface after each regeneration step.

(iv) Preparing the surface

The immobilized ligand is the basis for specificity in real time experiments. There are certain factors that need to be considered in deciding the best immobilization conditions.

The primary requirement is that the ligand immobilized must retain functional interaction with analyte. Coupling the ligand to the matrix provides favourable conditions in comparison with direct adsorption to surface. When molecules are bound to the matrix, they are essentially freely accessible in three dimensions and can be expected to behave as in an aqueous solution.

Binding properties of the surface must be suited to the intended range of analyte detection. This means optimization of the amount of immobilized ligand. Therefore, coupling a suitable amount of ligand is also important for semi quantitative or purely qualitative applications to ensure a clear “yes” or “no” answer. The nature of the application determines the best level of immobilized ligand, which will be determined experimentally.

The best level of immobilized ligand will also depend on the relative sizes of ligand and analyte. For a large ligand, a given level in resonance units (RU) will represent a smaller number of analyte binding sites giving a correspondingly smaller maximum binding capacity in RU. Similarly, a larger analyte will give a larger response than a small number of binding sites.

Theoretical maximum response values for some combinations of ligand and analyte molecular weights (MW) are given below in Table 1, where 1:1 stoichiometry is assumed.

Table 1. Maximum response values for combinations of ligand and analyte molecular weights.

<i>Ligand RU (baseline)</i>	<i>Ligand MW</i>	<i>Analyte MW</i>	<i>R_{max}</i>
5,000	200,000	50,000	1,250
5,000	100,000	100,000	5,000
5,000	50,000	200,000	20,000

One sensor chip flow cell can be used for a large number of analyses. However, repetition of the same experiment on different occasions requires that identical biospecific surfaces to be produced on different sensor chips. The properties of the sensor chip itself are highly consistent, and with suitable attention to experimental detail the immobilization process is reproducible with a coefficient of variation of <5% within sensor chip batches. This means when choosing immobilization conditions the amount of immobilized ligand should not be strongly affected by minor variations in pH, ionic strength, reagent concentration, etc.

It should be possible to regenerate the sensor chip surface for repeated analyses with the same flow cell. Real time experiments differ from many surface interaction techniques because the sensor chip can be regenerated in situ and used repeatedly for up to 50-100 analyses more.

(v) Buffer conditions

For most covalent immobilization methods, electrostatic preconcentration of the ligand in the surface matrix is important. Without preconcentration, immobilization is inefficient at low ligand concentrations. Preconcentration is critically affected by the pH and ionic strength of the coupling buffer.

A preliminary choice of pH for immobilization can be made if the pI (Isoelectric point) of the ligand is known.

e.g. if $pI > 7$, appropriate buffer pH is 6

Low ionic strength in the ligand solution favours electrostatic interactions between the surface matrix and ligand. Therefore, ionic strength should be kept as low as possible for the required buffering capacity.

The ideal ligand concentration for a given pH is the lowest value which gives maximum pre concentration. Usually a suitable concentration will be in the range 10-200 $\mu\text{g/ml}$.

(vi) Reproducibility of ligand immobilization

In order to ensure that ligand immobilization is reproducible, particular attention should be paid to the following aspects.

- Establishing conditions necessary for ensuring that the amount of immobilized ligand is least sensitive to small variations in coupling conditions.
- Ensuring that the coupling procedure is carried out at a pH value at which the immobilization yield is not steeply dependent unless absolutely necessary.
- Assuring that the dependence of immobilization yield is more or less independent on ligand concentration. If possible it is best to work in a plateau region where small variation in bulk ligand concentration have little effect.
- Other conditions being equal the yield of coupled ligand is often strongly dependent on ionic strength. Therefore work should be carried out at a carefully controlled ionic strength.
- Ensuring that the instructions for storage and use of reagents are meticulously followed. Reagents that have been stored in solution (even frozen) longer than the specific time should not be used.

Sensorgram

When an analyte is injected in a discrete pulse across a ligand surface, the resulting sensorgram can be divided into 3 essential phases namely the association, equilibrium and dissociation phases and is illustrated in Figure 7.

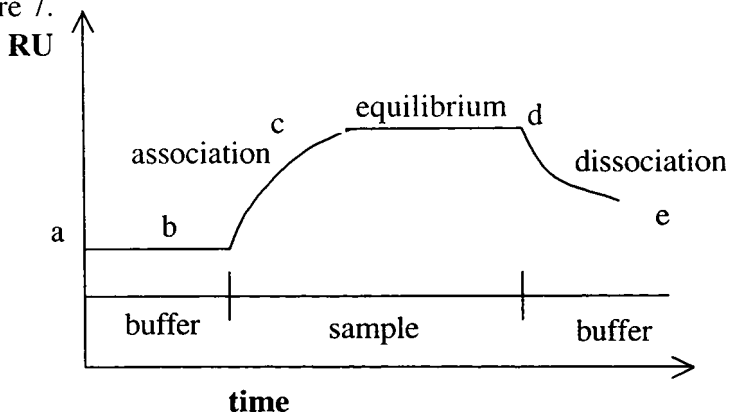


Figure 7. Sensorgram

The region between the 2 points 'a' and 'b' of the sensorgram, denotes the stable RU generated during buffer injection. Injection of the buffer is stopped at point 'b' and the sample is injected. The **association** of analyte with ligand during sample injection is shown by the curve "bc". While the sample injection is being continued an **equilibrium or a steady state** is reached, where the rate of analyte binding is balanced by dissociation from the complex. This is represented by the "cd" region of the sensorgram. At point 'd' sample injection is stopped and the buffer is reintroduced. The curve "de" shows dissociation of analyte from the surface during buffer flow.

The association and dissociation phases provide information on the kinetics of the analyte-ligand interaction (rates of complex formation and dissociation).

The equilibrium phase provide information on the affinity of the analyte-ligand interaction (strength of binding).

Kinetic determinations

Quantitative or semi quantitative

Factors influencing successful determination:

- For the association phase, the lowest level of immobilized ligand which is compatible with reliable measurements should be used. This will ensure that the binding rate is determined by the interaction kinetics and is not limited by mass transport processes in the sample solution.
- For the dissociation phase kinetic determinations, analyte needs to be bound as much as possible during sample injection, probably by using a relatively high analyte concentration.
- The early part of the dissociation phase should be used for measurements or comparisons. This will help to eliminate errors occurring due to rebinding of released analyte to the surface. If rebinding is a problem, it can be overcome by increasing the flow rate and including a soluble ligand in the eluent buffer.
- Quantitative kinetic measurements should be taken with a series of analyte concentrations. This will help to identify problems arising from heterogeneous systems.
- For equilibrium determinations, analyte concentrations used should give 20-80% saturation of the surface and it has to be assured that the sensorgram reaches a steady state during the sample injection.

Experimental considerations

1. Choice of ligand and analyte

In principle either of the interacting components can be immobilized. However, the factors that would influence the choice are as follows.

- A homogenous ligand population is important in measuring and interpreting rate constants. Therefore, it is advisable to immobilize the interactant for which a homogenous population is most easily ensured.
- If heterogeneity is suspected parallel determinations need to be performed with ligand and analyte roles reversed. Heterogenous ligand behaviour is easier to detect than heterogenous analyte.
- For accurate determination of rate constants measurements should be made at a range of analyte concentrations.
- In comparative kinetic investigations, immobilize the common ligand and vary the analyte. This will minimize effects of variations in immobilization and regeneration characteristics.

2. Choice of immobilization method

It is important to use the most specific coupling chemistry. Indirect immobilization (using capturing molecule) is often more advantageous. This is due to several reasons.

- Capturing the ligand on the surface serves as a significant purification step for the ligand.
- Specific binding of the ligand to the capturing molecule gives a uniform orientation of ligand molecules on the surface. The surface can be regenerated down to the capturing molecule after each cycle. This consumes more ligand
- but avoids possible deleterious effects of the regeneration conditions on ligand activity.

3. Amount of immobilized ligand

Using a low level of immobilized ligand reduces mass transport limitations. However, the amount of ligand used must be sufficient to give a reliably measurable response range for the analyte in question. In practical terms it should be kept within the framework of a measurable response, such as $R_{\max}$ 100-1000 RU.

Determination of concentration

Biosensing can be used for determining the concentration of both large and small molecules. With sufficiently selective ligand-analyte interaction, reliable measurements can be made of single components in complex mixtures such as serum. Since assays are based on specific binding of analyte to a ligand, the values obtained reflect the concentration of analyte which is active with respect to this binding.

There are three alternative approaches to measuring concentration as follows.

(1) Measure the amount of analyte bound after an arbitrarily fixed sample injection time. If required the analyte response can be enhanced by subsequent binding of a secondary interactant. Automated sample handling in the IFC ensures that injection times are accurately reproducible and therefore, the sensorgrams obtained should be reproducible as well. Reproducibility is one of the biggest advantages in biosensing experiments.

(2) Measure the rate of analyte binding at the beginning of sample injection. As long as the association is limited by mass transport, the binding rate is constant and is directly proportional to analyte concentration.

(3) Low molecular weight analytes which do not give a measurable direct response can be assayed using competitive techniques.

Requirements for concentration assay performance

The factors that need to be considered in setting up assay conditions are given below.

- (1) **Range:** the concentration range over which the assay will be used.
- (2) **Sensitivity:** the steepness of the calibration curve. i.e. response per unit concentration. The sensitivity may not be constant over the whole measuring range.
- (3) **Detection limit:** the lowest concentration that can be measured with the assay. This is determined largely by the signal:noise ratio for the assay as a whole.
- (4) **Precision:** precision of the assay is expressed as the coefficient of variation in response over a defined range of input concentration for repeated measurements on the same sample.
- (5) **Recovery:** this refers to the percentage recovery of analyte in standard addition experiments. If analyte molecules interact with some other species in the sample solution, recovery will be low, if resulting complex prevents binding to the immobilized ligand. However, recovery will be more than 100% if the complex can still bind to the ligand since the SPR response will be increased.
- (6) **Parallelism:** refers to measurements on the same sample at various dilutions. This is expressed as the measured value as a percentage of expected, after a defined dilution in appropriate diluent.
- (7) **Analysis time:** the total analysis time for the assay. The time for a complete analysis cycle is considered, including regeneration of the sensor chip surface.
- (8) **Sensor chip life time:** number of repeated analyses that can be performed on one single flow cell surface. This number will depend mainly on the stability of the immobilized ligand.

Analyte conditions

1. Analyte concentration

Determination of the association rate constants should be made at analyte concentrations well above the equilibrium dissociation constant. This will ensure that,

- equilibrium level of analyte binding is close to the maximum surface capacity.
- free ligand concentrations approach very low levels.
- binding is not limited by mass transport.

A range of analyte concentrations should be used for kinetic measurements. A range from 1 – 100 nM in 5-6 steps will cover the most typical rate constant values.

To determine the dissociation rate constants, using a high analyte concentration to saturate the surface as much as

possible before the dissociation phase will be advantageous. This minimizes mass transport limitations, potential rebinding effects, and also gives a high signal level.

2. Sample composition

Injection of a sample with a significant bulk contribution can obscure rapid changes at the very beginning of the association and dissociation phases. Also, sample dispersion can introduce errors in the rate determination. In order to minimize the change in bulk refractive index, careful preparation of eluent and sample is recommended.

3. Temperature

Temperature affects the determination of rate constants in two ways.

(i) Rate constants themselves are dependent on temperature. Therefore, comparative measurements must always be performed at the same temperature and measured constants should be related to a specific temperature.

(ii) SPR response is sensitive to temperature. Therefore, temperature fluctuations will cause baseline drifts. Since many measurements rely on a low SPR value, a stable baseline is very necessary.

The design of real time instrumentation ensures that samples reach the measuring temperature during passage through the IFC.

4. Flow rate

Flow rate for kinetic determinations should be,

- high enough to overcome mass transport limitations
- low enough not to waste sample

A flow rate of 10 $\mu\text{l}/\text{min}$ is recommended as a starting value for pilot experiments.

Useful measuring range

In order to determine rate constants, it is essential that the observed rate of binding or dissociation is not limited by mass transport of analyte to the sensor chip surface.

The mass transport is dependent on the diffusion coefficient of the analyte. Mass transport is slower if the analyte is large or the viscosity of the medium is high.

Equilibrium measurements

Equilibrium constants can be measured directly for interactions that reach equilibrium within a reasonable time. The primary requirement for this condition to be met is that dissociation is fast.

Part 2

Applications of Biosensing

Biosensing techniques are utilized in a broad range of applications. The fields and disciplines of application are very high and diverse and their selection is limited only by our imagination. Similarly the variety of the applications of biosensing which are employed to ensure biosafety, are also unimaginable. Even for the same application there can be a variety of biosensors that could be used which are based on different principles. E.g. a wide range of different biosensors is available to detect glucose level in blood.

In this section of the book a few out of the many different applications are discussed. They are mainly categorized into two disciplines as medical and environmental. Some biosensors however, can be applied in both disciplines, although they may be categorized under a particular discipline.

(a) Medical

(i) Cancer risk assessment

From literature it is evident that one of the causes of cancer is the DNA damage due to cancer causing pollutants called carcinogens. Inside the body, when the carcinogens get activated they can react with the DNA to form **DNA adducts**. DNA adducts are chemical compounds in which the carcinogen is attached to the DNA. If the natural defense system of the body is not capable of repairing the damage caused by carcinogens, it can lead to the development of an uncontrollably proliferating cell, which will result in cancer.

The researchers at the U.S. Department of Energy's Ames laboratory have developed a sensor chip which can detect DNA adducts found in urine. This is a highly sensitive and selective method that can be used to speed the cancer risk assessment.

In the development of the biosensor chip, initially a single-layer linker molecule is adsorbed to the bare gold. Then it is exposed to a drop of solution containing an antibody, specific to the DNA adduct of interest. It is possible to develop antibodies in the laboratory which are so selective so that they will preferentially bind a specific DNA adduct. The linker layer only serves as a coupling agent, binding the antibody to the chip. The chip can be used to detect the presence of an adduct in processed urine corresponding to the antibody immobilized on the chip. If the DNA adduct is present, it would bind to the antibody and fluoresce when scanned with a laser beam at low temperatures. The data gathered from the laser scanning is then used to produce a detailed fingerprint for adduct identification. This provides vital information for cancer risk assessment.

(ii) Monitoring and detection of diabetes

Detection of the glucose level and continuous glucose monitoring in a human being is possible by using biosensors. Thus biosensors are useful in fine controlling the blood glucose level of diabetic patients.

The most frequently used approach for sensing the glucose level in a body is by using a GOD (Glucose Oxidase) based electrochemical glucose sensor in the form of a needle. The principle behind this sensor is based on the following reaction.



Accordingly the quantity of H_2O_2 produced will be in direct proportion to the quantity of glucose. When coupled to an amperometric H_2O_2 electrode, the intensity of current measured shall be proportional to the quantity of H_2O_2 produced. Thus a useful indication of the glucose level can be obtained by using this instrument.

(iii) Diagnosis of genome mutations

Biosensing can be used to detect diseases caused due to genetic mutations. If the target DNA sequence is known (analyte), the sequence complimentary to it can be synthesized artificially and used as the ligand. This enables the sequence specific information to be obtained in a faster, simpler and less expensive manner compared to traditional hybridization assay methods.

The ligand actually is a molecular beacon with a complimentary sequence to the target DNA, and the general structure is shown in Figure 8.

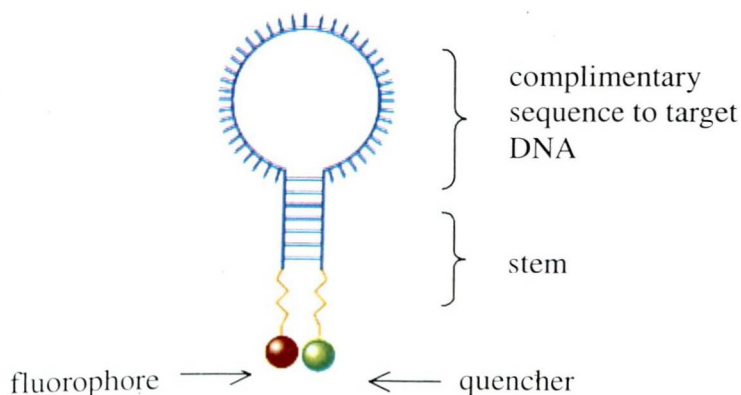


Figure 8. The ligand

The ligand is a single stranded oligonucleotide consisting of a probe sequence (loop) embedded within complimentary sequences that form a hairpin stem. To the two ends of the stem, a fluorophore and a quencher are linked. The emission spectrum of the fluorophore closely overlaps with the absorption spectrum of the quencher. Therefore, as long they are sufficiently close enough for the quencher to quench the fluorescence emitted by the fluorophore (upon exposure to a particular wavelength), fluorescence cannot be seen. In the presence of the target DNA, the loop (probe) hybridizes with it, forcing the fluorophore and quencher to move apart. As a result the quencher cannot quench the fluorescence emitted by the fluorophore. Thus the fluorescence can be seen. This is represented in Figure 9.

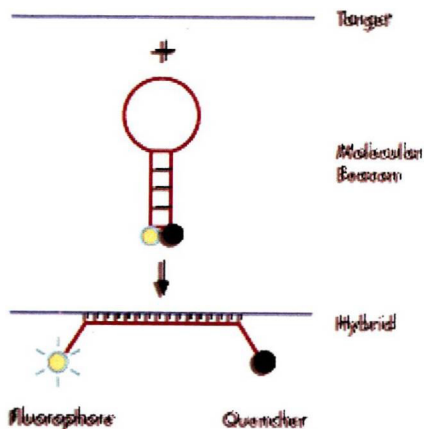


Figure 9. Hybridization of ligand and analyte

(iv) Detection of diseases by immunosensors

Diseases such as dengue fever, HIV, malaria and measles can be detected by using biosensors referred to as 'immunosensors'. The principle behind these biosensors is the antigen-antibody interaction. For every antigen, i.e. disease causing organism, there is an antibody that is very specific to the antigen that binds to it. The antibodies are immobilized on the surface, acting as the ligand. When the sensor is exposed to the intended medium (e.g. blood or other biological fluid), and if the antigen is present in the medium, it will be bound by the antibody to form larger, antigen-antibody complexes. This will change some physicochemical parameter (usually mass or optical parameter) at the transducer surface of the sensor and the change can be subsequently detected.

ELISA (Enzyme Linked Immuno Sorbant Assay) biosensors are a type of immunosensors, which are used to detect and amplify an antibody-antigen reaction.

(b) Environmental

(i) Pollutant detection in water by inhibition based biosensors

Mainly enzyme inhibition based biosensors are used for the detection of pollutants in water. The principle behind these biosensors is that, when a particular pollutant is known to inhibit a particular enzyme activity, the inhibition of the enzyme activity is taken as a measurement of the presence of the pollutant. Thus, in these biosensors, the interaction occurs between the following biomolecules.

- (a) specific chemical and biological agents (inhibitors)
- (b) biocatalyst (an enzyme, a polyezymatic sequence, and/or whole tissue)

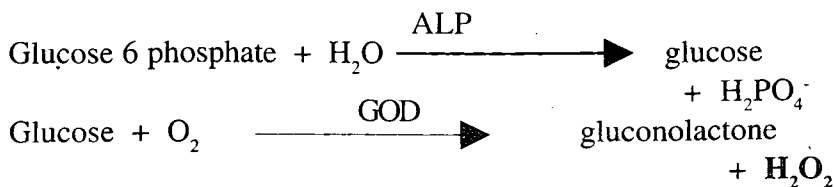
Some examples of inhibition biosensors developed for the analysis of environmental pollutants are given in Table 2.

Table 2. Some examples of inhibition biosensors

Inhibitors	Enzymes
Fluoride	Acetylcholinesterase
Atrazine	Tyrosinase
HCN	Peroxidase
H ₂ S, HCN	Cytochrome oxidase
Herbicides	Photosystem II
Dithiocarbamates	Aldehyde dehydrogenase

Pollutant detection can be discussed using 2,4-D amperometric biosensor as an example. 2,4-D is an auxinic herbicide with growth regulator activity, which is used for selective control of broadleaf weeds in cereal grain crops. 2,4-D is suspected to promote prostate and pulmonary cancer and also act as “hormone disruptors”, for their effects on the endocrine system. The wide use of 2,4-D in agriculture leads to contamination of ground and surface waters, including drinking water. Therefore, determining 2,4-D concentration in water samples, is an important consideration.

The biosensor used to detect 2,4-D involves two enzymes. The enzyme alkaline phosphatase (ALP) which is inhibited by 2,4-D and glucose oxidase (GOD). They catalyze the following reactions.



In the absence of 2,4-D the above enzymatic reactions will produce hydrogen peroxide (H_2O_2). In the presence of 2,4-D, since the enzyme ALP activity is inhibited, H_2O_2 will not be produced. Thus, the decrease in the production of H_2O_2 can be

used as a measurement of the concentration of 2,4-D present in the sample.

In actual practice the enzymes are coupled to an amperometric H_2O_2 electrode, in which, the current intensity measured is proportional to H_2O_2 production.

(ii) Detection of lead and other metal ions by DNA enzyme biosensors

DNA enzymes can be used to detect and quantify the metal ions present in a solution. They are actually catalytic DNA molecules that cleave other molecules such as other fragments of DNA in the presence of a metal ion. It is this property of DNA enzymes that enables the metal ions in solution to be detected and quantified. The number of substrate DNA cleaved by the DNA enzyme would vary with the concentration of the metal ions. If no metal ions are present no cleaving shall take place. The occurrence of cleavage can be detected by fluorescence detection, using fluorophores and quenchers. This is the principle behind using enzyme biosensors for detection of metal ions.

The university of Illinois at Urbana-Champaign has developed a biosensor based on this principle. The biosensor consists of a double stranded nucleotide substrate (DNA) to which is attached both a fluorophore and DNA enzyme fluorescence quenchers. The presence of metal ions induces a conformational change in the structure of the DNA enzyme. This causes the DNA enzyme to cleave the complimentary strand (the nucleotide substrate) bearing the fluorescent label. The label is activated by the release of the fluorophore and results in a 600% enhancement of the fluorescence signal. The intensity of fluorescence detected corresponds to the concentration of metal ion. The particular biosensor has high specificity for lead (Pb^{+2}) ions and is very sensitive as its detection range varies from 10 nM to 4 μM (2-800 ppb). Thus this biosensor is sensitive enough to detect the threshold level (15 ppb) set by the EPA (Environmental Protection Agency) for Pb^{+2} concentration in drinking water. This technology can be applied to quantify other metals, either beneficial (e.g. calcium, iron) or toxic (mercury, chromium, arsenic).

DNA enzyme biosensors can be used in medical laboratory diagnostics, clinical toxicology, developmental biology (e.g. calcium distribution), wastewater treatment, consumer household monitoring (e.g. tap water, pool, playground, paint, etc.), environmental monitoring, etc.

There are several advantages of using DNA enzyme biosensors.

- DNA enzyme is stable, extremely sensitive and selective.
- In addition to lead it can be used to detect a broad spectrum of metal ions such as Mg^{+2} , Ca^{+2} , Mn^{+2} , Zn^{+2} , Co^{+2} and Cu^{+2} .
- Detection of these metals using orthodox laboratory techniques require large and expensive lab equipment whereas the use of DNA enzyme technology permits on-site real-time detection at lower cost; portable equipment can be manufactured to be used in the field.
- Results can be visualized and quantified.

An instant which best illustrates the use of this technology is comparison with the current system available for detection of lead. According to the Current Center for Disease (CDC) the threshold for toxic lead levels in the body is 100 ppb. Although current laboratory equipment can measure at this sensitivity there are problems with background interference. In addition, the equipment is very large and bulky, requires operation by skilled personnel, and is not portable.

(iii) Detection of deadly food borne pathogen *Listeria monocytogenes*

A new biosensor has been developed by a team of food scientists at Purdue University, to detect the deadly bacteria *Listeria monocytogenes* in food. This biosensor is capable of detecting *L. monocytogenes* in less than 24 hours at concentrations as low as 1000 cells per milliliter of fluid. Further, this sensor is very selective and is able to recognize only the species *L. monocytogenes*, in a field in which other types of bacteria are also present.

Listeriosis is the illness caused by consuming *Listeria* contaminated foods like deli meats, cheese, smoked fish and hot dogs and as recorded has the highest rates of hospitalization and mortality than caused by any other foodborne illness. The mortality rate for people with listeriosis is very high so that it is vital to ensure that there is no *L. monocytogenes* at all in any ready-to-eat product. The bacteria classified as *Listeria* includes six different species, but only *L. monocytogenes* can infect humans. Therefore the selectivity exhibited by this sensor is important as it is able to detect only *L. monocytogenes*.

This new sensor is an optical biosensor. It is made up of a small piece of optical fibre – a clear, solid, plastic material that transmits light through its core. The fibre is coated with antibody molecules that specifically bind with *L. monocytogenes*. Therefore, when the fibre is placed in a liquid food solution, any *L. monocytogenes* present in the solution will stick to the fibre. The presence of *L. monocytogenes* is detected by using a second antibody. This antibody while having the ability to bind to *L. monocytogenes*, does also carry a molecule that produces a fluorescent glow when exposed to laser light. Thus, it acts as a flag, signaling the presence of *L. monocytogenes* when laser light is passed through the liquid.

Conventional methods used for detecting the presence of *L. monocytogenes* require a high concentration of pathogen cells (1 million to 10 million cells per milliliter of fluid) and rely on a process referred to as ‘enrichment’, where the sample believed to be contaminated is grown for a period of time in a nutrient broth to allow any pathogen cells present to multiply. This can take as long as seven days. Most of the foods susceptible to *Listeria* contamination are ready-to-eat products, which have already been processed. In processing, the bulk of microorganisms are bound to be eliminated rendering the processed product to have low levels of contamination. Thus the importance of this biosensor can be stressed due to its ability to detect *L. monocytogenes* rapidly even at low levels.

Thus in summation the selectivity, sensitivity and rapidity of this sensor shows a vast improvement over the other types of test kits currently available commercially. Therefore, this new biosensor may be termed as an important contribution in the field of food safety.

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