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General Introduction to Biosensors and Recognition Receptors

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1.1 Introduction to Biosensors

There are laboratory tests and protocols for the detection of various biomarkers, which can be used to diagnose heart attack, stroke, cancer, multiple sclerosis, or any other conditions. However, these laboratory protocols often require costly equipment, and skilled technical staff, and hospital attendance and have time constraints. Much cheaper methods can provide cost-effective analysis at home, in a doctor's surgery, or in an ambulance. Rapid diagnosis will also aid in the treatment of many conditions. Biosensors generically offer simplified reagentless analyses for a range of biomedical [1–8] and industrial applications [9, 10]. Due to this, biosensor technology has continued to develop into an ever-expanding and multidisciplinary field during the last few decades.

The IUPAC definition of a biosensor is "a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals." From this definition, we can gain an understanding of what a biosensor requires.

Most sensors consist of three principal components:

1) Firstly there must be a component, which will selectively recognize the analyte of interest. Usually this requires a binding event to occur between the recognition element and target.
2) Secondly some form of transducing element is needed, which converts the biochemical binding event into an easily measurable signal. This can be a generation of an electrochemically measurable species such as protons or

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H_2O_2, a change in conductivity, a change in mass, or a change in optical properties such as refractive index.

3) Thirdly there must be some method for detecting and quantifying the physical change such as measuring an electrical current or a mass or optical change and converting this into useful information.

There exist many methods for detecting binding events such as electrochemical methods including potentiometry, amperometry, and AC impedance; optical methods such as surface plasmon resonance; and piezoelectric methods that measure mass changes such as quartz crystal microbalance (QCM) and surface acoustic wave techniques. A detailed description of these would be outside the remit of this introduction, but they are described in many reviews and elsewhere in this book. Instead this chapter focuses on introducing the recognition receptors used in biosensors.

1.2 Enzyme-Based Biosensors

Leyland Clark coated an oxygen electrode with a film containing the enzyme glucose oxidase and a dialysis membrane to develop one of the earliest biosensors [11]. This could be used to measure levels of glucose in blood; the enzyme converted the glucose to gluconolactone and hydrogen peroxide with a concurrent consumption of oxygen. The drop in dissolved oxygen could be measured at the electrode and, with careful calibration, levels of blood glucose calculated. This led to the widespread use of enzymes in biosensors, mainly driven by the desire to provide detection of blood glucose. Diabetes is one of the major health issues in the world today and is predicted to affect an estimated 300 million people by 2045 [12]. The world market for biosensors was approximately $15–16 billion in 2016. In 2009 approximately half of the world biosensor market was for point-of-care applications and about 32% of the world commercial market for blood glucose monitoring [13].

Enzymes are excellent candidates for use in biosensors, for example, they have high selectivities; glucose oxidase will only interact with glucose and is unaffected by other sugars. Being highly catalytic, enzymes display rapid substrate turnovers, which is important since otherwise they could rapidly become saturated or fail to generate sufficient active species to be detected. However, they demonstrate some disadvantages: for instance, a suitable enzyme for the target of interest may simply not exist. Also enzymes can be difficult and expensive to extract in sufficient quantities and can also be unstable, rapidly denaturing, and becoming useless. They can also be subject to poisoning by a variety of species. Moreover, detection of enzyme turnover may be an issue, for instance, in the glucose oxidase reaction; it is possible to directly electrochemically detect either consumption of oxygen [11] or production of hydrogen peroxide. However in samples such as blood and saliva, there can be other electroactive

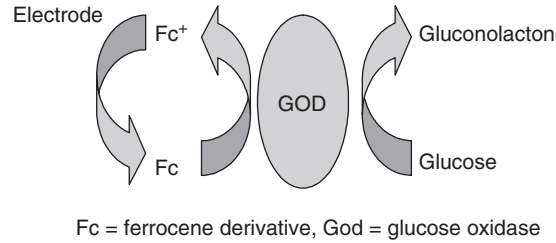

Figure 1.1 Schematic of a second-generation biosensor.

substances such as ascorbate, which also undergo a redox reaction and lead to false readings. These types of biosensors are often called "first-generation biosensors." To address this issue of interference, a second generation of glucose biosensors was developed where a small redox-active mediating molecule such as a ferrocene derivative was used to shuttle electrons between the enzyme and an electrode [14]. The mediator readily reacts with the enzyme, thereby avoiding competition by ambient oxygen. This allowed much lower potentials to be used in the detection of glucose, thereby reducing the problem of oxidation of interferents and increasing signal accuracy and reliability. Figure 1.1 shows a schematic of a second-generation glucose biosensor.

Third-generation biosensors have also been developed where the enzyme is directly wired to the electrode, using such materials as osmium-containing redox polymers [15] or conductive polymers such as polyaniline [16]. More recently nanostructured materials such as metal nanoparticles, carbon nanotubes, and graphene have been used to facilitate direct electron transfer between the enzyme and the electrode as described in later chapters. As an alternative to glucose oxidase, sensors based on glucose dehydrogenase have also been developed.

The techniques for glucose sensing using glucose oxidase can be applied to almost any oxidase enzymes, allowing sensors to be developed based on cholesterol oxidase, lactate oxidase, peroxidase enzymes, and many others. Sensors have also been constructed using urease, which converts urea to ammonia, causing a change in local pH that can be detected potentiometrically or optically by combining the enzyme with a suitable optical dye. Enzyme cascades have also been developed; for example, cholesterol esters can be determined using electrodes containing cholesterol esterase and cholesterol oxidase. Applications of enzyme-containing biosensors have been widely reviewed [16–18].

1.3 DNA- and RNA-Based Biosensors

DNA is contained within all living cells as a blueprint for making proteins, and it can be thought of as a molecular information storage device. RNA also has a wide number of applications in living things, including acting as a messenger between DNA and the ribosomes that synthesize proteins and as a regulator of

gene expression. Both DNA and RNA are polymeric species based on a sugar–phosphate backbone with nucleic bases as side chains, in DNA, namely, adenine, cytosine, guanine, and thymine. In RNA uracil is utilized instead of thymine. It is the specific binding between base pairs, that is, guanine to cytosine or adenine to thymine (uracil), that determine the structure of these polymers, in the case of DNA leading to a double helix structure (Figure 1.2) [19].

DNA sensors are usually of a format where one oligonucleotide chain is bound to a suitable transducer, that is, an electrode, surface plasmon resonance (SPR) chip, quartz crystal microbalance (QCM), and so on, and is exposed to a solution containing an oligonucleotide strand of interest [20]. The surface-bound oligonucleotide is selected to be complementary to the oligonucleotide of interest, and the bound and solution strands will undergo sequence-specific hybridization as the recognition event.

An in-depth review of DNA sensing is outside the scope of this introduction and has been reviewed elsewhere [20–24]; however, a few examples are given here. A method based on ruthenium-mediated guanine oxidation allowed selective electrochemical detection of messenger RNA from tumors at 500 zmol L^{-1} levels [25]. A sandwich-type assay using magnetic beads and fluorescence analysis utilized a complementary nucleotide to dengue fever virus

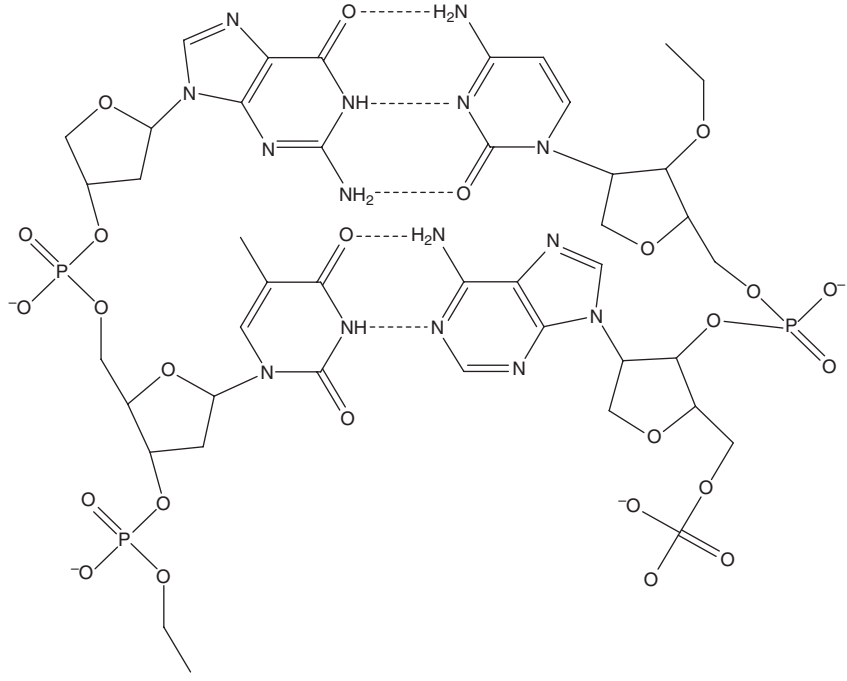

Figure 1.2 Schematic of interstrand binding in DNA.

RNA to allow detection at levels as low as 50 pmol L^{-1} [26]. Five different probe DNAs could be immobilized onto an SPR-imaging chip and simultaneously used to determine binding of RNA sequences found in several pathogenic bacteria such as *Brucella abortus*, *Escherichia coli*, and *Staphylococcus aureus* [27] for use in food safety.

1.4 Antibody-Based Biosensors

Antibodies are natural Y-shaped proteins produced by living systems, usually as a defense mechanism against invading bacteria or viruses. They bind to specific species (antigens) with an extremely high degree of specificity by a mixture of hydrogen bonds and other non-covalent interactions, with the binding taking place in the cleft of the protein molecule [28]. One major advantage of antibodies is that they can be "raised" by inoculating laboratory animals with the target in question; the natural defense mechanisms of the animal are to develop antibodies to the antigen. These antibodies can then be harvested from animals. A range of animals are used including mice, rats, rabbits, and larger animals such as sheep or llamas. Therefore, it is possible to develop a selective antibody for almost any target. This high selectivity led to first the development of the Nobel prize-winning radioimmunoassay [29] and then later the enzyme-linked immunosorbent assay (ELISA) [30], which is commonly used today to quantify a wide range of targets in medical and environmental fields.

Once developed the antibody can be immobilized onto a transducer to develop a biosensor, shown schematically in Figure 1.3. One issue is that when antibodies bind to their antigens to form a complex, no easily measured by-products such as electrons or redox-active species are produced. There

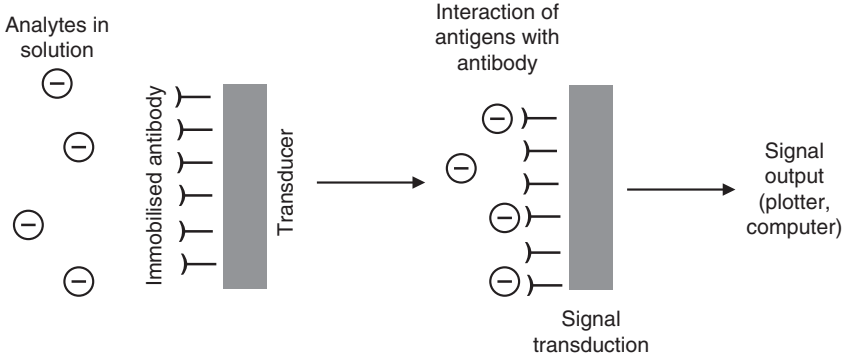

Figure 1.3 Schematic of an antibody-based immunosensor.

are several methods of addressing this drawback. For example, a sandwich immunoassay format can be used where an antibody is bound to the surface and an antigen bound to it from the solution to be analyzed. Development then occurs by exposing the sensor to a labeled secondary antibody, which binds to the antigen, and then the presence of the label is detected; this can be an enzyme or a fluorescent or electroactive species. Competitive assays where the sample is spiked with a labeled antigen and then the labeled and sample antigens compete to bind to the immobilized antibody are also used. However these require labeling of the antibody/antigen, which can be problematic, leading to loss of activity and requiring additional steps with their time and cost implications. Therefore, label-free detection methods have been widely studied that can simply detect the binding event directly without need for labeling. These include electrochemical techniques such as AC impedance, optical techniques such as SPR, and mass-sensitive techniques such as QCM [28].

Another issue is that the strong binding between antibody and antigen means that there is no turnover of substrate; the binding is essentially irreversible. In this case, the sensors are often prone to saturation and can only be used once. Although the antibody–antigen reaction can be reversed by extremes of pH or strongly ionic solutions, these can damage the antibody, leading to permanent loss of activity. However, if costs can be brought down far enough, the possibilities of simple single-shot tests for home use become possible. This led to the first commercially available immunoassay, the home pregnancy test, which detects the presence of human chorionic gonadotrophin (hCG). Initial tests simply detect its presence by showing a blue line, that is, pregnant or not pregnant; however later models incorporate an optical reader that measures the color intensity, thereby assessing the hCG level and giving an estimate of time since conception.

1.5 Aptasensors

Aptamers are a family of RNA/DNA-like oligonucleotides capable of binding a wide variety of targets [31] including proteins, drugs, peptides, and cells. When they bind their targets, the binding event is usually accompanied by conformational changes in the aptamer; for example, it may fold around a small molecule. These structural changes are often easy to detect, making aptamers ideal candidates for sensing purposes. Aptamers also display other advantages over other recognition elements such as enzymes and antibodies. They can be synthesized *in vitro*, requiring no animal hosts and usually with a high specificity and selectivity to just about any target from small molecules to peptides, proteins, and even whole cells [31]. The lack of an animal host means that aptamers can be synthesized to highly toxic compounds. Once a particular optimal aptamer for a certain target has been determined, it can be

commercially synthesized in the pure state and often displays superior stability to other biological molecules, hence their nickname "chemical antibodies."

Aptamers can be sourced by firstly utilizing a library of random oligonucleotides. It is possible that within this library a number of the oligonucleotides will display an affinity to the target, whereas most of them will not. They are then subjected to a process called systematic evolution of ligands by exponential (SELEX) enrichment. In this process, the library is incubated with the target and then bound molecules, that is, oligonucleotide/target complexes separated and the unbound species discarded. The bound oligonucleotides are then released from the target and then subjected to polymerase chain reaction (PCR) amplification. This then forms a new library for the process to begin again. Over a number of cycles (6–12) [31], the oligonucleotides with the strongest affinity to the target are preferred in a manner similar to natural selection. After a number of cycles, these aptamers are cloned and expressed. Figure 1.4 shows a schematic of this process.

Aptamers bind to their targets with excellent selectivity and high affinity, dissociation constants often being nanomolar or picomolar [32]. Like antibodies, aptamers can be utilized in a variety of formats; for small molecules there is usually a simple 1 : 1 complex formed with the target encapsulated inside the aptamer. However with larger analytes the aptamer binds to the surface of the target, and different aptamers can be isolated, which bind to different areas [31].

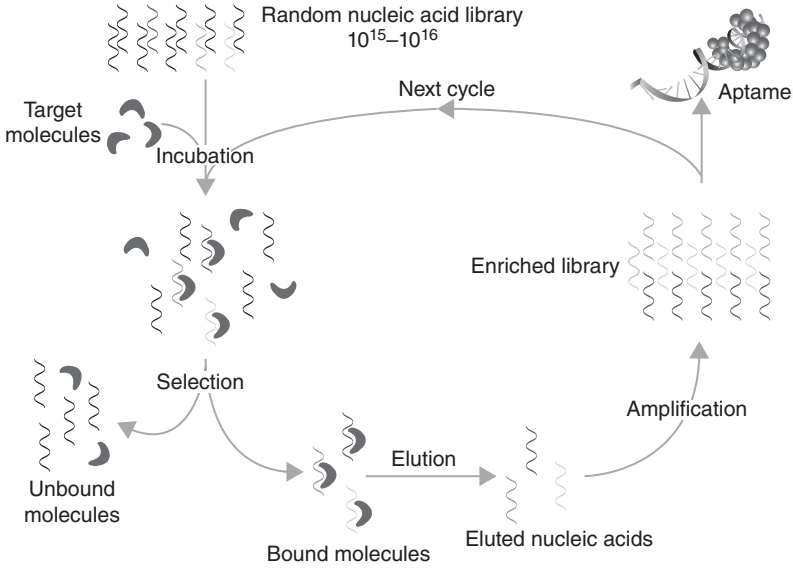

Figure 1.4 Scheme for the systematic evolution of ligands by exponential (SELEX) enrichment process. *Source:* Song et al. [31]. Reproduced with permission of Elsevier.

This allows for sandwich-type assays where two aptamers are used to enhance the biosensor response; there also exist mixed sandwich assays using an aptamer and an antibody.

One issue is that since aptamers simply form complexes with their counterparts, again there is no easily detectable product such as a redox-active species formed. However, the easy availability and stability of aptamers also allows their functionalization with labels such as enzymes, nanoparticles, fluorescent, or redox-active groups for use in labeled assays. Alternatively, label-free techniques such as AC impedance, SPR, and QCM can be used to detect binding events [31].

1.6 Peptide-Based Biosensors

Peptides are natural or synthetic polymers of amino acids and are built from the same building blocks as proteins. Since many proteins have the ability to bind targets with good selectivity and specificity, peptides of the correct amino acid sequence should be capable of doing the same [33]. Shorter peptides have a number of advantages over proteins; they will generally display better conformational and chemical stability than proteins and be much less susceptible to denaturing. Also they can be synthesized with specific sequences using well-known solid-phase synthesis protocols and can be easily substituted with labeling groups without affecting their activity. Especially popular is the labeling of one or both ends of the peptide with fluorescent groups [33].

These recognition receptors can be synthesized with a particular sequence or a library of peptides can be used to assess affinity to a particular target. For example, peptides can be made to specifically chelate certain metal ions even in the presence of other metal ions. Peptide-based sensors are especially effective systems for activity of certain enzymes such as proteases. Proteases can hydrolyze peptide bonds, and certain proteases are linked to many disease states. For example, matrix metallopeptidase-2 (MMP-2) and MMP-9 are thought to be important in a number of inflammatory and pathological processes as well as tumor metastasis [34–36]. Peptides can be used to assess proteinase activity. For example, quantum dots could be coated with peptides conjugated with a large number of dye molecules, fluorescence resonance energy transfer interactions occur between the dye molecules, and the dot, which quenches the dot fluorescence. When a proteinase is added, the peptide is hydrolyzed, the coating removed, and the dot fluorescence returned [37]. Activity of a variety of other materials such as kinases can also be assessed [33].

Libraries of short (<50 amino acids) peptides from random phage display can be screened against various targets as reviewed before [38]. Also *in silico* modeling of peptide strand interactions with targets of interest can be used to select possible receptor peptides, these can then be synthesized and assayed [38, 39].

One issue however is that immobilizing these onto a solid surface may lead to structural modifications, which remove its activity. Also peptide sequences that form the active sites of natural receptors can be synthesized and can retain the activity of the parent molecule.

1.7 MIP-Based Biosensors

Biosensors were initially made using biological molecules such as enzymes or antibodies; however, this led to issues such as cost, difficulty in purification and isolation, and stability. The use of semisynthetic materials such as aptamers and peptides that can be synthesized or selected has addressed this issue to some extent. However, another approach is to use totally synthetic materials that mimic the behavior of enzymes or antibodies. This has led to the development of molecularly imprinted polymers (MIPs), which although not biosensors *per se*, are a possible solution [40–42].

For manufacturing of MIPs, the analyte of interest (often biological in nature) is mixed with a variety of polymerizable monomers and some of these will interact with the analyte. Polymerization will then be initiated and a cross-linked polymer is formed containing entrapped analytes, which act as templates (Figure 1.5). Removal of the analyte will, if the polymer is sufficiently rigid, leave pores within the polymer, which not only match the template size and shape but also contain their internal surface groups, which will interact with the analyte [42–45]. Often this technique is combined with *in silico* modeling of the template interaction with a library of monomers, allowing selection of a monomer mixture that will interact strongly with the template [9, 10, 46]. MIPs display several advantages over biological materials; they have much higher stabilities and can be stored dry for months or years, synthesized in large quantities from readily available monomers, and used in nonaqueous solvents and over a range of temperatures [45].

A wide variety of protocols can be used. For example, inorganic polymers containing glucose were deposited onto a QCM by a sol–gel process, the glucose washed out, and the resultant system shown to act as a sensor, giving an

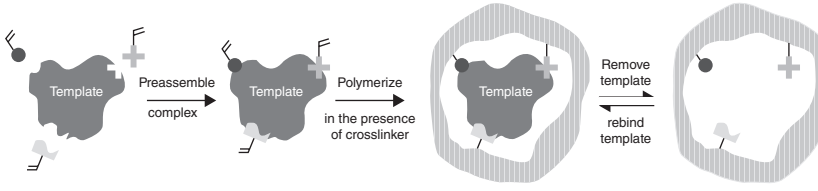

Figure 1.5 Schematic representation of the imprinting process. *Source:* Whitcombe and Vulfson [42]. Reproduced with permission of John Wiley & Sons.

increase in mass when exposed to aqueous glucose [47]. Polymers can also be deposited electrochemically onto electrode surfaces in the presence of a template. For example, poly(*o*-phenylenediamine) could be electrochemically deposited from template solutions onto a QCM chip to give sensors for atropine (with a linear range between 8×10^{-6} and 4×10^{-3} M) [48]. Much larger targets can also be used; for example, a number of enzymes can be incorporated into cross-linked polymers, then removed, and the resultant MIPs display strong binding affinities for those templates [49]. These types of system have even been successfully applied to the detection of viruses in tobacco plant sap using QCM chips [50].

Most of these MIPs have been utilized as solid films since the cross-linking reaction renders them completely insoluble. However, more recently methods of making nanoparticle MIPs, which are soluble, have come to the field [51, 52]. For example, nanosized MIPs toward a range of substrates could be synthesized and used in competitive ELISA assays, giving comparable or better performance than assays based on commercial antibodies with detection limits as low as 1 pM [50]. MIP-based biomimetic sensors have been successfully developed for viruses [51–53], toxins [9, 10, 54], and drugs [45, 46, 55] in recent years in the form of nanoparticles, which can be covalently immobilized on gold sensor chips. Moreover, regeneration of sensor surfaces using acidic and/or basic solutions is also possible which allows use the same sensor multiple times and decreases the required cost and time substantially. A comprehensive research on adenoviruses has compared the sensing efficiency of antibodies and these MIPs by employing SPR biosensors [52], which indicates the promising future of these recognition receptors for many important analytes. The recent years have also witnessed the implementations of MIPs in biosensors for the detection of disease biomarkers, which are covered in Chapter 12 with detailed examples.

1.8 Conclusions

In this chapter, we have described the major groups of recognition elements used in biosensors. Initial studies used enzymes because of their specificity, high turnover, and the fact that they often produce an easily measured product such as hydrogen peroxide. Antibodies also show high specificity; although in their case measurement of the recognition event can be more complex. One major issue with these biological receptors is their fragility; since purification, immobilization, storage, and labeling may all abolish their activity. This drawback has led to the development of semisynthetic and synthetic analogues of these biological species, such as peptides, aptamers, and MIPs. These demonstrate much higher stabilities and can be produced in greater quantities for almost any target. However, in many cases the sensitivity and selectivity of

these materials is still not as high as natural molecules. It can be concluded that the requirements of an assay may well determine the optimum recognition receptors to be used in any biosensor.

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