Chapter 2
Affinity Biosensors

2.1 Biosensor

Biosensor is an analytical technique that has been developed since 1962 by Clark and Lyons. It is a device which incorporates a biological sensing element with a signal transducer, to give a sensing system specific for the target analyte (Cooper and Mcneil, 1990). Enzyme, antibody, nucleic acid, cell receptor, whole cell or tissue can all be used as biological sensing element. The transducer can be electrochemical, optical, piezoelectric or calorimetric (Tran Minh, 1993; Eggins, 1996). Biosensors are characterized by features, such as, repeatability, sensitivity, specificity, stability, linear range and response time (Hock, 1997). Application fields for biosensors include health care, food and drink industry, process industry, environmental monitoring, and security (Turner, 1997).

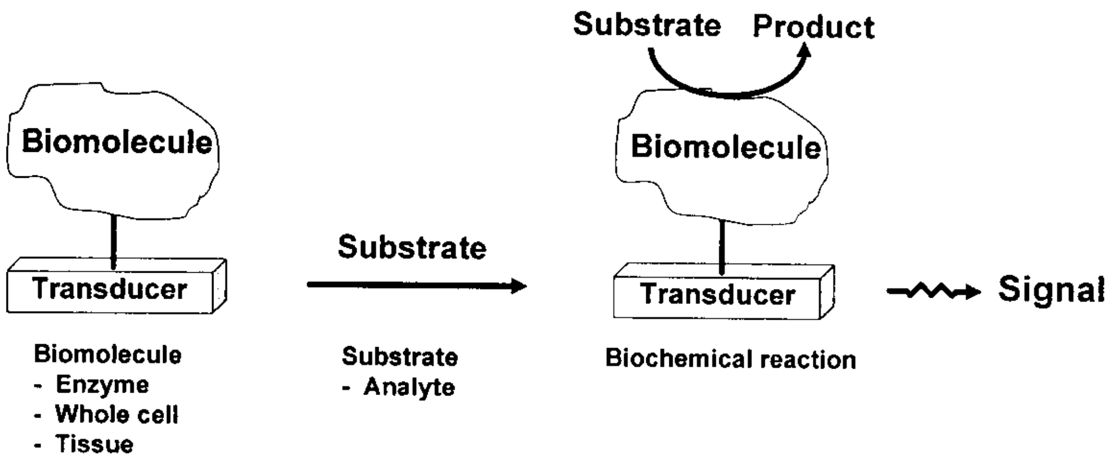
Biosensors can be divided into two main categories; catalytic and affinity biosensors (Figure 2.1). The biological sensing element used in catalytic biosensors are enzymes (mono-or multi-enzyme), whole cells (micro-organisms, such as bacteria, fungi, yeast), and tissues (plant or animal tissue slice) and this has been a subject of several reviews (Byfield and Abuknesha, 1994; Dong and Chen, 2002; Gerard *et al.*, 2002; Luppa *et al.*, 2001; Orazio, 2003). In catalytic biosensors the biological sensing element converts substrate molecules into product molecules (Figure 2.1a). The change in solution property, consumption of substrate or the products of the reaction, is detected. The transducer converts the change into a quantifiable electrical signal. Electrochemical, piezoelectric, optical and calorimetric transducers are frequently cited in the reviews (Byfield and Abuknesha, 1994; Dong and Chen, 2002; Orazio, 2003; Thévenot *et al.*, 1999).

The other category, affinity biosensors, is based on binding interactions between the immobilized biomolecules and the analytes of interest (Mattiasson, 1984; Taylor, 1991). In their purest form affinity biosensor detect the binding event itself and readout is proportional to biomolecule-analyte binding (Figure 2.1b). The

biological elements used in affinity biosensors include for example antibody, receptor and single stranded DNA and these have been reviewed by several authors (Byfield and Abuknesha, 1994; Dong and Chen, 2002; Gerard *et al.*, 2002; Lin and Ju., 2005; Luppa *et al.*, 2001; Orazio, 2003). The main advantages of these kinds of biosensors are the wide range of affinities available, thus, expanding the number of analytes that can selectively be detected. Affinity biosensors are a way to provide scientists and clinicians to develop the new method for many fields such as clinical diagnostics and environmental control (Gizeli and Lowe, 1996).

2.2 Types of Affinity biosensor

Affinity biosensor exploit selective binding of certain biomolecules toward specific target species. The biomolecular recognition process is governed primarily by the shape and size of the receptor pocket and the ligand of interest (the analyte). The high specificity and affinity of these biochemical binding reactions lead to highly selective and sensitive sensing devices (Wang, 2000). Affinity biosensors can be classified according to the biological sensing element into DNA biosensors, receptor biosensors and immunosensors.



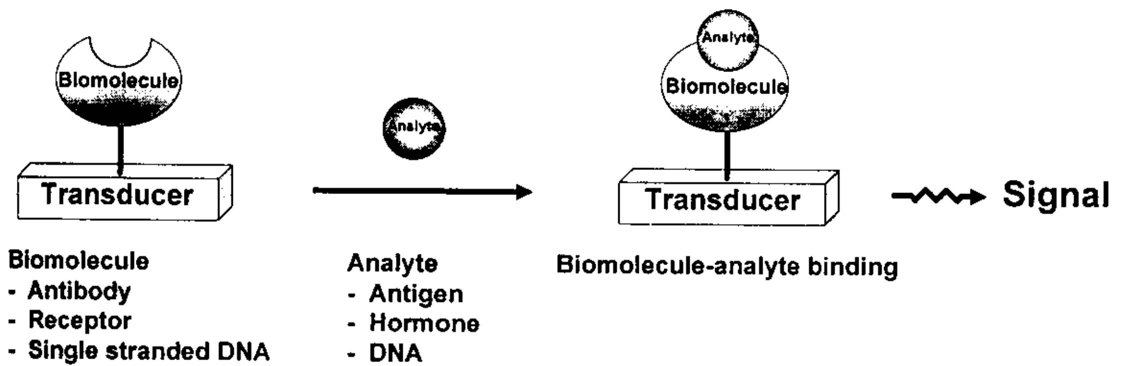


Figure 2.1 Major biosensor types.

2.2.1 DNA biosensors

Deoxyribonucleic acid, DNA, is the molecule that encodes genetic information. It is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. The four nucleotides in DNA contain the bases, adenine (A), guanine (G), cytosine (C), and thymine (T). In nature, base pairs form only between A and T and between G and C, thus, the base sequence of each single strand can be deduced from that of its partner. Most biosensors for DNA are based on the process of hybridization, the matching of one strand of DNA with its complementary strand. DNA hybridization biosensors can be applied in various research fields and for routine clinical, forensic, food and environmental analyses. This technique commonly rely on the immobilization of a single-stranded (ss) DNA molecule onto a transducer surface to recognize, by hybridization, its complementary target sequence DNA (Gooding, 2002; Mannelli *et al.*, 2005; Paleček *et al.*, 1998; Wang, 1998; 2002). Once hybridization is complete, label specific for binding to the hybridized sequence, for example, fluorescent (Yguerabide and Ceballos, 1995), radioisotopes (Su *et al.*, 1994), enzyme (Campbell *et al.*, 2002; Mikkelsen, 1996), or electrochemical label (Liu *et al.*, 2005; Peng *et al.*, 2005; Wong *et al.*, 2005; Yau *et al.*, 2003; Zhu *et al.*, 2004) was applied and the signal was detected.

Hybridization has also been measured directly, without the use of a label, using several transducers to convert the hybridization event into a measurable electrical signal, *i.e.*, optical transducer to detect the change in optical properties (Csáki *et al.*, 2003; Erickson *et al.*, 2003; Piunno *et al.*, 1995; Isola, *et al.*, 1998; Steinem, *et al.*, 2004), piezoelectric transducer to detect the change in frequency of the crystal which depends on the change in mass of the crystal (Eun *et al.*, 2002; Okahata *et al.*, 1992; Tombelli *et al.*, 2000) and electrochemical transducer to detect the potential, current, or capacitance change at the surface of the transducer (Berggren *et al.*, 1999; Gautier *et al.*, 2005; Mikkelsen, 1996; Piro *et al.*, 2005; Thompson *et al.*, 2003; Wang *et al.*, 2001a; 2001b; 2003).

2.2.2 Receptor biosensors

Molecular receptors are cellular, typically membrane, proteins that bind specific chemicals (ligands) in a manner that results in a conformational change in the protein structure. The conformational change triggers a cellular response, for example, opening an ion channel or secreting an enzyme (Byfield and Abuknesha, 1994; Subrahmanyam *et al.*, 2002). Receptor-based biosensors have been developed for several target analytes such as, viruses (Bertucci *et al.*, 2003; Sissoëff *et al.*, 2005), antibodies (Hagay *et al.*, 2006; Kamel, *et al.*, 2005), DNA (Lucas and Harding, M.M. 2000; Tosteson *et al.*, 2001), drugs (Bertucci *et al.*, 2003; Butala *et al.*, 2004), toxin (Brooke *et al.*, 1998; Ji *et al.*, 2002) and low-molecular weight compound (Luck *et al.*, 2003).

The binding of an analyte to an immobilized receptor can be measured indirectly by labeling one of the components. This allows competitive binding techniques to be used and the introduction of other methods of detection. For example, a fluorescent label can be detected via the evanescent wave at the surface of an optical fiber (Rogers *et al.*, 1989; 1991b; Ogert *et al.*, 1992) and an enzyme label can be detected potentiometrically (Rogers *et al.*, 1991a; 1992). The binding of an analyte to an immobilized receptor can also be measured directy by using an appropriate transducer such as, optical (Bertucci *et al.*, 2003; Brooke *et al.*, 1998; Butala *et al.*, 2004; Hagay *et al.*, 2006; Ji *et al.*, 2002; Kamel, *et al.*, 2005; Luck *et al.*, 2003; Stenlund *et al.*, 2003), piezoelectric (Ko and Park, 2005; Sung *et al.*, 2005; Wu, 1999), and electrochemical transducers (Hui *et al.*, 2003; Nilolelis *et al.*, 2005; Sugawara *et al.*, 1997; Tein *et al.*, 1997; Tosteson *et al.*, 2001).

2.2.3 Immunosensors

Immunosensors are defined as analytical devices that detect the binding of an antigen to its specific antibodies or antibody fragments by coupling the immunochemical reaction to the surface of a device known as a transducer (Gizeli and Lowe, 1996; Luppa *et al.*, 2001; Wang, 2000). Several reviews have appeared in the literature that examine the potential of immunosensors as systems for clinical analysis (Aizawa, 1994; Lin and Ju, 2005; Luppa *et al.*, 2001: Orazio, 2003) and

environmental monitoring (Dond and Chen, 2002: Marco *et al.*, 1995: Marty *et al.*, 1998).

The most reported affinity biosensors are based on immunological reactions involving the recognition of the antigen (Ag) by the antibody (Ab) binding site to form the antibody-antigen (Ab-Ag) complex. This will be the main emphasis of this work and the basics of antibody-antigen interaction will first be briefly described.

2.2.3.1 Antibody

Immunosensor relies on the highly selective nature of molecular recognition systems to measure the amount of antibody or antigen in a sample. Antibodies are immunoglobulins which are produced by the body in response to antigens. Immunoglobulins fall into five classes, IgG, IgA, IgM, IgD, and IgE (Cunningham, 1998; Gizeli and Lowe, 2002; Hermanson *et al.*, 1992). These classes are structurally related glycoproteins that differ in size, charge, amino acid composition, and carbohydrate content. They consist of two heavy and two light chains held together by disulfide bridges (Figure 2.2). Each heavy chain has three constant and one variable domain, and the light chains have one constant and one variable domain. It is the variable domains that gives rise to the antibody specificity and constitute the antigen binding sites of the antibody.

Antibodies are typically represented schematically as Y-shaped structures (Figure 2.2). The antibody consists of two identical Fab-parts hinged to an Fc-part. The variable region of the Fab fragments are where the amino acids are organized to produce a binding site for the specific antigen, two binding sites per antibody (Cunningham, 1998; Gizeli and Lowe, 2002; Hall, 1990; Hermanson *et al.*, 1992). The Fc-part has no real function when antibodies are used as recognition elements for affinity biosensor, but it can be used for site-directed immobilization (Hermanson *et al.*, 1992; Lu *et al.*, 1996). The structure, which represents the most commonly used antibody in sensor design is schematically shown in Figure 2.2. In biosensors based on antibodies the analyte is either the corresponding antigen of the antibody used or a part of it. An antibody-antigen interaction is characterized by two major properties that may be exploited for sensing or detection purposes (Byfield and

Abuknesha, 1994):

(i) It has a very high affinity constant; up to 10^{-18} M of antigen can be detected in certain laboratory assays based on antibodies.

(ii) It has low cross-reactivity; relatively minor changes in the structure of a given antigen can render it almost invisible to an antibody produced in response to the original unmodified structure.

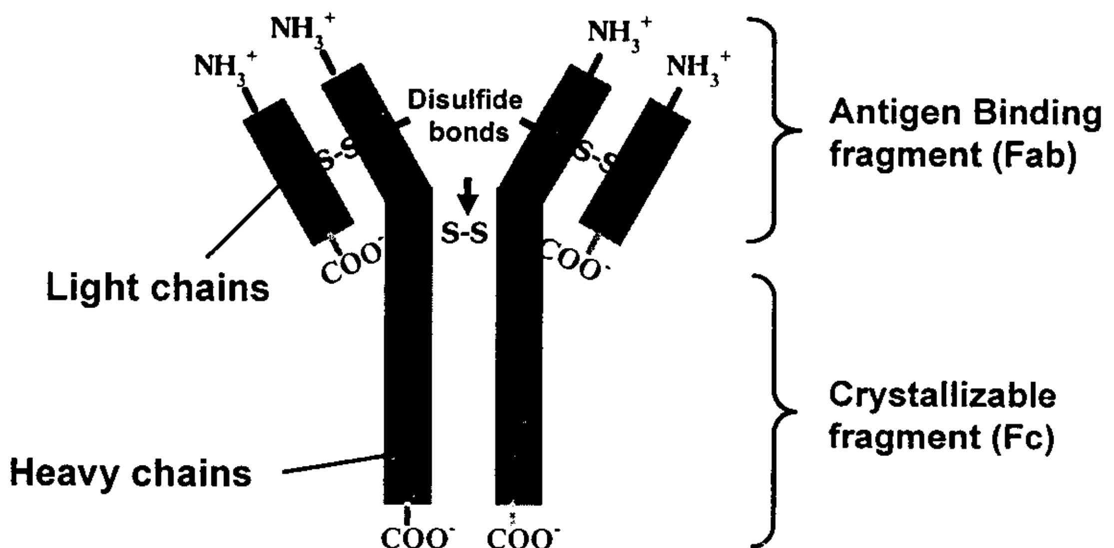


Figure 2.2 Structure of IgG antibody.

2.2.3.2 Binding forces

An antigen (Ag) will interact with an antibody (Ab) raised to one of its antigenic determinants (portion of structure to which antibodies are produced) with a high binding affinity. The strength of the interaction is dependent on the complementarily of the fit of the antigenic determinant to the binding site of the antibody, the binding forces present in the Ab-Ag complex are non-covalent forces, such as electrostatic interactions (major contribution), hydrogen bonding,

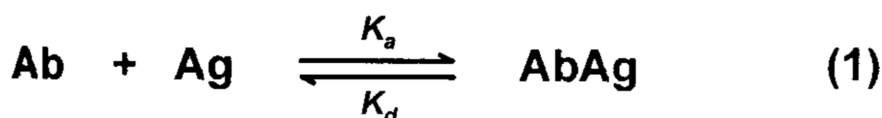
hydrophobic interactions and Van der Waals interactions (Byfield and Abuknesha, 1994; Gizeli and Lowe, 2002; Rabbany *et al.*, 1994).

Electrostatic interactions can be either attractive or repulsive forces between charged molecules or dipole-dipole interactions between highly polar molecules. In proteins, the polar amine and carbonyl groups of the polypeptide backbone lead to permanent dipoles. Polar and charged residues of the side chains also contribute to the dipoles. Hydrogen bonds are considered a subset of electrostatic interactions. They occur between a highly electronegative proton donor and an unbound pair of electrons on a highly electronegative proton acceptor. The amine groups constitute a proton donor, and carbonyl groups function as a proton acceptor. These hydrogen bonds and electrostatic interactions contribute to binding strength, and in aqueous solutions they are the predominant contributors to intermolecular stabilization (Buckingham, 1993; Rabbany *et al.*, 1994)

Van der Waals forces occur between weaker dipoles than electrostatic interactions. Electric fields of nearby molecules induce the temporary dipoles responsible for these forces. Although these interactions are relatively weak, the cumulative force from several interaction can contribute up to 50% of the total binding strength. Hydrophobic interaction are repulsive forces that occur between nonpolar molecules and water. Driven by entropy, nonpolar regions act to exclude water and thus attain lower, more favorable energy levels. If these nonpolar regions exist at a reaction site, achievement of thermodynamic stability leads to intermolecular stabilization and increased binding strength (Rabbany *et al.*, 1994).

2.2.3.3 Kinetics

The fundamental thermodynamic principle governing antibody-antigen interactions in solution is expressed by;



$$Ab + Ag \underset{K_d}{\overset{K_a}{\rightleftharpoons}} AbAg \qquad (1)$$

Where Ab represents free antibody, Ag represents free antigen, AbAg is the antibody-antigen complex, and K_a and K_d are the association and dissociation rate constants, respectively. The equilibrium constant, or the affinity, is given by:

$$K = \frac{K_a}{K_d} = \frac{[AbAg]}{[Ab][Ag]} \quad (2)$$

In solution, both association and dissociation are relatively rapid. The association rate is affected by the diffusion of reactants and the probability that a collision will result in a binding event. Dissociation is determined by the strength of the antibody-antigen bond, and the thermal energy available for the activation energy needed to break the bond. The equilibrium constant for monoclonal antibodies is typically 10^5 to 10^9 $Molar^{-1}$ (Eggins, 1996; Wang, 2000).

2.2.3.4 Labeled immunosensors

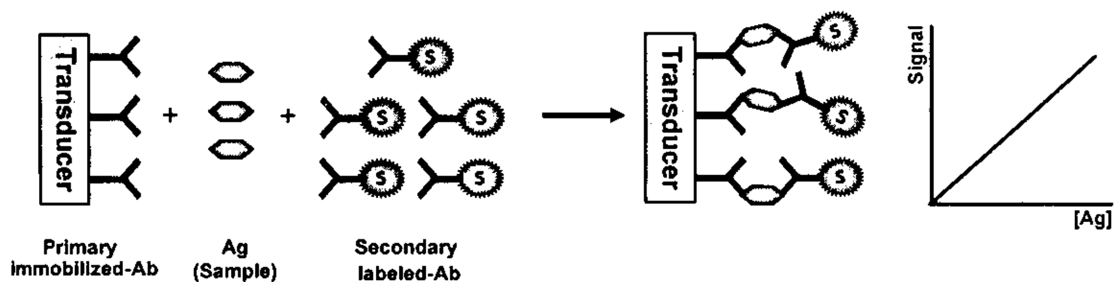
Immunosensors can be divided into two main categories; labeled and label-free (direct) (Byfield and Abuknesha, 1994; Ghindilis *et al.*, 1998; Gizeli and Lowe, 1996; Luppa *et al.*, 2001; Orazio, 2003). Labeled immunosensors are derived from the immunoassay technology. To be able to detect the interaction, one of the immunoagents has to be labeled of a signal-generating component. A labeled component is therefore used to generate a signal which enables quantification of the amount of bound Ag relative to unbound Ag. The most common types of a signal-generating components are fluorescent molecules (Arefyev *et al.*, 1990; Cummins *et al.*, 2006; Ho *et al.*, 2006; Lepesheva *et al.*, 2000; Oswald *et al.*, 2000; Rowe *et al.*, 1999; Wolfbeis, 2000) or enzyme *e.g.*, catalase, horseradish peroxidase, glucose oxidase, laccase (Chen *et al.*, 2005; Ghindilis *et al.*, 1991; 1992; González *et al.*, 2005; Ionescu *et al.*, 2005; Kaku *et al.*, 1993; Kalab and Skladal, 1995; Mirbabibollahi *et al.*, 1990; Romero *et al.*, 1998; Skladal and Kalab, 1995).

The two widely used principles for labeled immunosensors are sandwich-type and competitive-type sensors as shown in Figure 2.3. Sandwich-type

sensors are applicable for measuring large antigens. Such sensors utilize an antibody that binds the antigen (analyte), which then binds a signal-generating component-labeled second antibody. After removal of the nonspecifically adsorbed label, the concentration of the antigens (analyte) is determined by measuring the fluorescence signal (Ho *et al.*, 2006; Rowe *et al.*, 1999) or the production of colored or electroactive products by the enzyme when its natural substrate is added to the solution (Ghindilis *et al.*, 1997; Ionescu *et al.*, 2005; Krishnan *et al.*, 1995; Liu *et al.*, 1991; Thoss *et al.*, 1996; Vetcha *et al.*, 2002). In sandwich sensor case, the signal is proportional to the analyte concentration (Figure 2.3(a)).

In competitive-type sensors, the sample antigen (analyte) competes with a signal-generating component-labeled antigen for antibody-binding sites on a sensing probe. After the reaction is complete, the sensor is washed to remove unreacted components. The probe is then placed in a solution containing the substrate for the enzyme, and the product or reactant of the biocatalytic reaction is measured (Bier and Schmid, 1994; Chen *et al.*, 2005; González *et al.*, 2005; Miura *et al.*, Romero *et al.*, 1998; 1993; Yokoyama, *et al.*, 1995; Zacco *et al.*, 2004) or measured the fluorescence signal (Cummins *et al.*, 2006; Lepesheva *et al.*, 2000). In this case, the signal is inversely proportional to analyte concentration (Figure 2.3(b)). Signal generation is significantly facilitated, but this type of immunosensors is associated with other problems such as, expensive, time-consuming and makes real-time measurements impossible (Berggren *et al.*, 2001; Yagiuda *et al.*, 1996).

a.) Sandwich sensor



b.) Competitive sensor

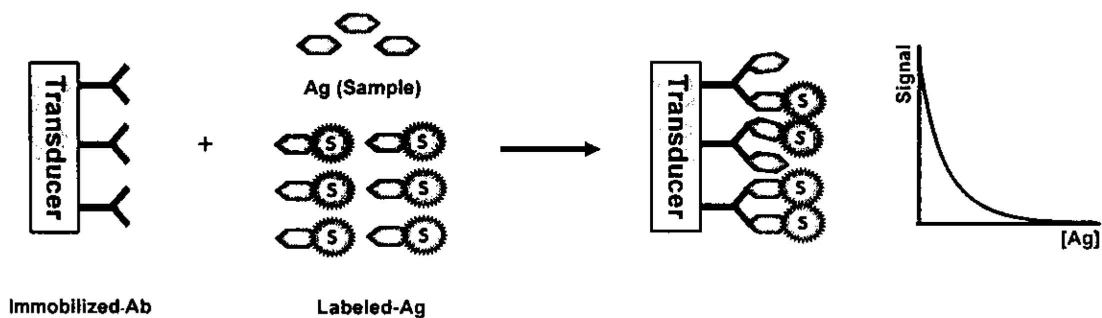


Figure 2.3 Two main types of labeled immunosensos. a). sandwich-type, b). competitive-type sensors. The signal is proportional to analyte concentration in a sandwich sensors, and inversely proportional to analyte concentration in a competitive sensors.

2.2.3.5 Label-free immunosensors

Direct or label-free immunosensors are based on measurement of changes in any physical property as a result of antigen-antibody complex formation (Figure 2.4). Generally the surface of a transducer is modified by either antigen or antibody. Antibody-antigen complex formation then leads to a change in surface properties which can be detected as an optical change (Calakos *et al.*, 1994; Chapple *et al.*, 1998; Corr *et al.*, 1994; Pei *et al.*, 2000; 2001; Polymenis and David Stollar, 1995; Rao *et al.*, 1999), mass change (Chu *et al.*, 1995; Konig and Gratzel 1994; Konig and Gratzel 1995; Lu *et al.*, 2000; Shen *et al.*, 2005; Su *et al.*, 1999; Suri *et al.*, 1995; Tajima *et al.*, 1998; Yang and chen, 2002) and electrochemical change (Berggren and Johansson, 1997; Berggren *et al.*, 1998; 2001; Bontidean *et al* 1998; Hu *et al.*, 2002; 2005; Hedström *et al.*, 2005; Ramanaviciene and Ramanaviciene, 2004; Taylor *et al.*, 1991; 2004a; 2004b; 2004c; Yagiuda *et al.*, 1996).

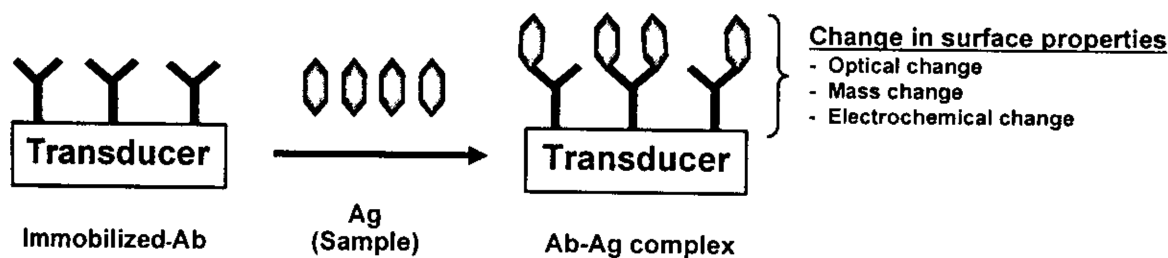


Figure 2.4 Label-free immunosensors.

2.3 Detection principles of label-free immunosensors

Label-free immunosensors rely on the detection of the physical changes during the immunocomplex formation. Such changes have been measured using optical (surface plasmon resonance, SPR), piezoelectric (quartz crystal microbalance, QCM) and electrochemical methods.

2.3.1 Optical transducer

Antibody-antigen interactions at a surface are accompanied by changes in layer thickness, refractive index, light absorption in the layer, refractive behavior of the incident light and light scattering. All of these physical changes can be related to antigen concentration and have been used for measurements with optical labeled-free immunosensors. Surface plasmon resonance is presently the most widely used detection technique (Johansson, 2004).

Surface plasmon resonance (SPR) immunosensor is a real-time, label-free, optical detection method for studying the interaction of soluble analyte with immobilized ligand (Mu *et al.*, 2001; Yang and Ngo, 2000). In principle, the SPR-immunosensor measures the mass concentration change caused by binding of an antigen (or antibody) to the corresponding antibody (or antigen) immobilized on the sensor surface (Patel, 2002). The SPR phenomenon occurs when plane polarized light is reflected from a gold film deposited on a glass support (Liedberg, 1983). Photons react with the free electron cloud in the metal film at a specific angle, the SPR-angle, and cause a drop in the reflected light. Refractive index changes near the surface give rise to a shift of the resonance angle. The shift is directly proportional to the mass increase and mass concentration can thus be measured (Stenberg *et al*, 1991). The analysis principle is summarized in Figure 2.5.

SPR technique has been widely studied in the fields of kinetics analysis, concentration measurements of biomolecules and molecule recognition involving protein-protein or protein-DNA (Calakos *et al.*, 1994; Pei *et al.*, 2000; 2001; Polymenis and David Stollar, 1995; Rao *et al.*, 1999) and receptor-ligand interactions (Corr *et al.*, 1994) as well as the examination of structure-function relationship of antibacterial synthetic peptides (Chapple *et al.*, 1998). However, SPR

method has a major disadvantage for bioanalytical applications. It is difficult to detect low concentration or low molecular mass analytes (Dong and Chen, 2002; Orazio, 2003).

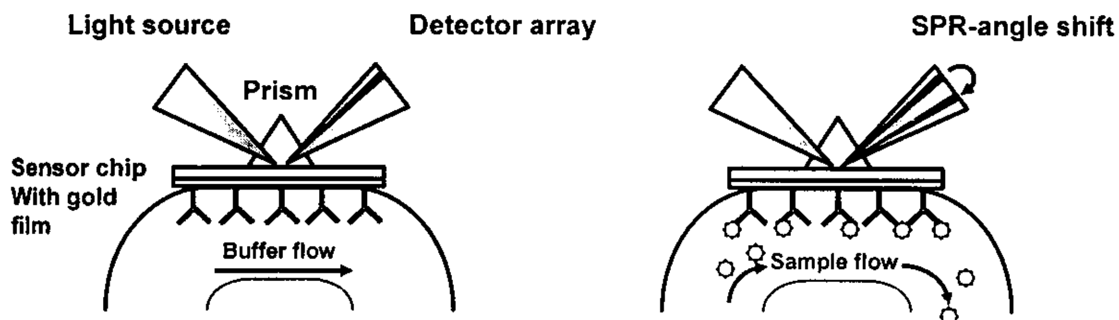


Figure 2.5 Surface plasmon resonance (SPR) biosensor principle. Binding of biomolecules to the surface increase the refractive index, which induces shift of the SPR-angle. The shift is directly proportional to the mass increase (Adapted from Johansson, 2004).

2.3.2 Piezoelectric transducer

The quartz crystal microbalance (QCM) is an ultra-sensitive weighing device that utilizes the mechanical resonance of piezoelectric signal-crystalline quartz (Hook and Rudh, 2005). QCM consists of a thin quartz disk with electrodes plated on it (Figure 2.6). As QCM is piezoelectric, an oscillating electric field applied across the device induces an acoustic wave that propagates through the crystal and meets minimum impedance when the thickness of the device is a multiple of a half-wavelength of the acoustic wave. A QCM is a shear mode device in which the acoustic wave propagates in a direction perpendicular to the crystal surface. Deposition of thin film on the crystal surface decreases the frequency in proportion to the mass of the film.

A typical acoustic device consists of a piezoelectric material (polished quartz plate) with metal transducers (*e.g.*, gold) on its surfaces which are used to send acoustic wave into the material at ultrasonic frequencies (one to hundreds of megahertz). The crystal orientation, thickness of the piezoelectric material, and geometry of the metal transducer determine the type of acoustic wave generated and the resonance frequency (Paddle, 1996). A change in weight on the crystal can be determined by measuring the shift in resonating frequency, wave velocity, or amplitude. The frequency shift of the piezoelectric crystal is proportional to mass change

$$\Delta F = \frac{C_Q f^2 \Delta m}{A} \qquad (3)$$

Where ΔF is the change in fundamental frequency, C_Q is the sensitivity (which for quartz = 2.26×10^{-6} cm^2 g^{-1}), f is the resonant frequency of the crystal, A is the area of the crystal, and Δm is the mass change deposited (Collings and Caruso, 1997).

Piezoelectric immunosensors were successfully used for assays of many different analytes, such as, viruses (Konig and Gratzel 1994; 1995), bacteria (Yang and chen, 2002), protein (Chu *et al.*, 1995; Lu *et al.*, 2000; Shen *et al.*, 2005; Su *et al.*, 1999), insulin (Suri *et al.*, 1995) and IgA in saliva (Tajima *et al.*, 1998).

The advantage of piezoelectric immunosensors lies in the direct measurement of immunointeraction. In this case, no label is necessary and no additional chemicals are used. However, non-specific binding can in some cases be a critical issue in piezoelectric immunosensors. In the absence of any label, it may result in difficulty in distinguishing between actual immunointeraction and changes owing to non-specific binding of other components of the reaction media to the surface of piezoelectric crystal. It also should be noted that significant mass changes can result owing to formation of immunocomplexes of relatively high molecular weight analytes. Small antigens (haptens) forming immunocomplexes with antibody-modified surfaces do not produce enough mass change for piezoelectric detection.

Hence, the main area of application of piezoelectric immunosensors is in the assay of relatively high molecular weight analytes (Ghindilis *et al.*, 1998).

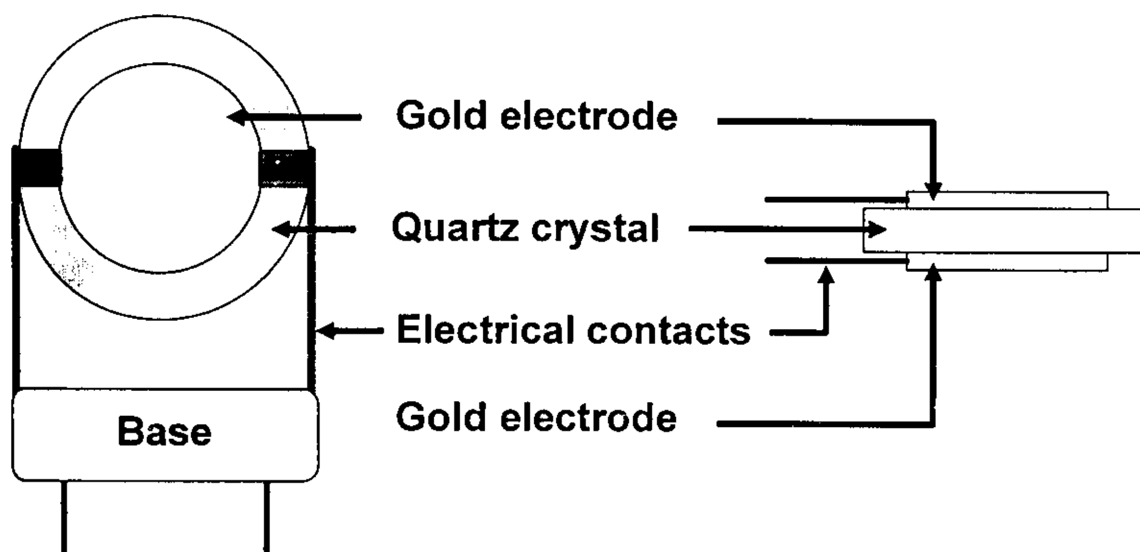


Figure 2.6 Quartz crystal microbalance.

2.3.3 Electrochemical transducer

Electrochemical transducers offer good possibilities for sensitive detection of target analyte (*i.e.*, antigen, protein, sugar). Electrochemical detection of immunointeraction are used for both label-free (Berggren *et al.*, 1998; 2001; Bontidean *et al.*, 1998; Fu *et al.*, 2004; Hu *et al.*, 2002; 2005; Hedström *et al.*, 2005; Jung *et al.*, 2005; Ramanaviciene and Ramanaviciene, 2004; Stefan and Bokretsion, 2003; Taylor *et al.*, 1991; Tang *et al.*, 2004b; 2004c; Zhang *et al.*, 2005; 2005) and labeled immunosensors (Grant *et al.*, 2003; Killard *et al.*, 1999; Kim *et al.*, 2001; Liu *et al.*, 2000; Liu and Ju, 2005: Padeste *et al.*, 1998; Sarkar *et al.*, 2002; Sun *et al.*, 2001). However, indirect immunosensors suffer from several disadvantages such as high cost, time-consuming, and not applicable when real-time measurements are required. Therefore, label-free immunosensors are preferred and this is the subject of a review in the next chapter.