Synthetic Hybrid Biosensors

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Keywords

Receptor:
The biological recognition element used in the sensor.

Target:
The analyte of interest; the one which is to be quantified.

Biorecognition:
The process of identifying the target molecules through biocatalysis or bioaffinity reactions.

Transducer:
A device which converts the chemical signal from the biorecognition event into a quantifiable physical signal.

Immobilization:
The process of attaching receptor molecules to the transducer surface, without compromising its activity and selectivity.

Biosensors are analytical tools in which biological or biologically derived receptor molecules are used as recognition elements in conjunction with physico-chemical transduction mechanisms. Biosensors can be classified according to the bio-recognition process or the transduction mechanism employed. Bioaffinity sensors involve affinity reactions between the receptor and the target, while biocatalytic sensors employ the specific catalysis of the target analyte by the biological

molecule. Depending on the transduction mechanism, biosensors can be divided in four broad types: electrochemical; optical; piezoelectric; and thermal. Biosensors find application in fields ranging from clinical and point-of-care diagnosis, medicine and drugs, process industries, environmental monitoring to defense and biowarfare. Present biosensor research is focused on developing more compact and easy-to-use devices while retaining the efficiency and sensitivity.

1 Introduction

Biosensors are fascinating analytical tools that combine the specificity and sensitivity of biological processes with the physico-chemical transduction mechanism to provide bioanalytical measurements. The International Union of Pure and Applied Chemistry (IUPAC) defines a biosensor as 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 [1, 2]. In 1962, Leyland C. Clark was the first to elucidate the basic concept of the biosensor in his seminal report on ''enzyme electrodes'' [3], which he had built on his earlier invention of the oxygen electrode. Clark reasoned that the electrochemical detection of oxygen or hydrogen peroxide could be used for the analysis of a wide range of analytes that produce either oxygen or hydrogen peroxide on being acted upon by a specific enzyme(s) [4].

The field of biosensors can be divided into two broad categories of instrumentation: (i) sophisticated high-throughput laboratory instruments capable of delivering rapid and accurate measurements; and (ii) easy-to-use, portable devices for use by the non-specialists for decentralized, *in situ*, or home analysis [4]. Medical diagnostics – and in particular blood glucose sensors for diabetic patients – present the largest field of applications for biosensors, though they also find applications in food and process control, medicine, environmental monitoring, and defense and security. A biosensor may serve different analytical functions; in a clinical diagnosis it may just be required to determine whether a targeted analyte is above or below a certain threshold value, whereas in process control the sensor may be required to provide a continuous and precise feedback about the analyte. Hence, the sensor needs to be designed to meet the requirements of each and any application.

Based on the type of biological recognition process involved, biosensors can be allocated to two categories: (i) biocatalytic, which are typically based on the selective catalysis of biochemical reactions by enzymes; and (ii) bioaffinity, in which affinity interactions resulting in the formation of biocomplexes – such as antigen–antibody, the hybridization of complementary single-strand DNAs, protein–nucleic acids, and chemoreceptor–ligand – provide a very selective and sensitive mechanism for biosensing. Based on the transduction mechanism, biosensors can be broadly divided into four categories: electrochemical; optical; piezoelectric; and thermal. A combination of two transduction mechanisms can also be used to yield better sensitivity.

The aim of this chapter is to provide a basic knowledge of the various types of biosensor, and to outline the underlying principles and general design criteria, by providing specific examples.

2 Sensor Design

Figure 1 shows a general schematic of a biosensor, which will usually consist of three main components: the bioreceptor or recognition unit, which is used in conjunction with a transducer that converts the chemical information from the analyte–receptor interaction into an easily measurable and quantifiable signal which is then shown on a display unit. The sensor design also incorporates an associated electronic circuit or signal processor. In a biosensor, enzymes, cell organelles, tissues, microorganisms, antibodies and nucleic acids are the commonly used bioreceptors. Biologically derived materials such as aptamers and apoenzymes, or biomimetic materials such as molecularly imprinted polymers (MIPs) can also be used as bioreceptors. The biological reaction must usually take place in close vicinity of the transducer, so that the transducer can pick up most of the chemical information from the receptor–analyte interaction.

In order to create a viable biosensor, the biorecognition unit must be properly attached to the transducer surface, without affecting the former's activity. This process, which is known as *immobilization*, is the most critical step in the fabrication of any biosensing device. The choice of immobilization method depends on several factors, including the nature of the biological component, the type of transducer, the physico-chemical properties, and the environment in which the sensor is intended to be used. The most commonly used immobilization methods used are adsorption, covalent binding, intermolecular crosslinking, matrix entrapment, and membrane entrapment.

- **Physical adsorption** utilizes a combination of van der Waals forces, hydrophobic interactions, H bonding and columbic interactions to immobilize biological elements on the surface of the transducer. Many substrates, such as cellulose, collodion, collagen, silica gel, glass, alumina and hydroxyapatite are

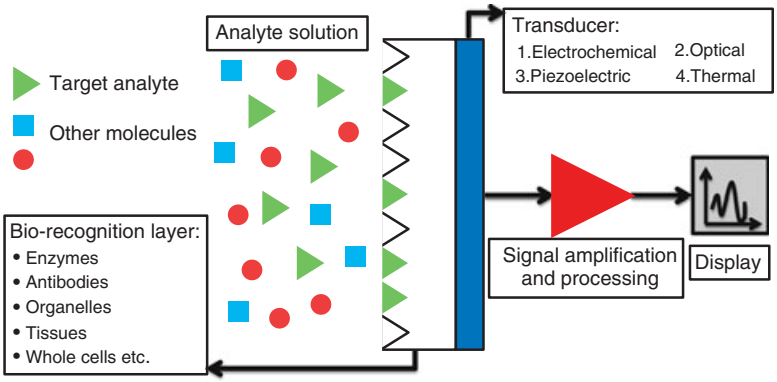

Fig. 1 Schematic representation of sensor design.

known to adsorb biomolecules. However, the interaction forces between the substrate and the immobilized elements are weak, and the latter may tend to be released over a period of time, leading to sensor dysfunction [5, 6].

- **Covalent binding** involves the formation of covalent bonds between the certain reactive groups of the biological element which do not play a role in the biorecognition process and the substrate surface, which is modified to have functional groups. Generally, the nucleophilic functional groups present in the amino acid side chain, such as amine, carboxylic acid, imidazole, thiol, and hydroxyl are used for the coupling reaction. Coupling requires mild conditions such as low temperature, low ionic strength, and pH in the physiological range. Covalent binding leads to a uniform surface coverage and helps to eliminate certain problems such as instability, aggregation, diffusion, and deactivation of the immobilized biocomponent [1].
- Bifunctional or multifunctional reagents such as glutaraldehyde, hexamethylene di-isocyanate, 1,5-difluoro 2,4-dinitrobenzene and bisdiazobenzidine-2,2'-disulfonic acid are used for immobilizing biocomponents through **intermolecular crosslinking**. The nonrigidity of the enzyme layer formed, the higher demands for amounts of biological material and the formation of multiple layers of enzyme, which negatively affects the activity, represent some of the disadvantages of this method. Moreover, larger diffusional barriers may delay interactions and increase the response time of the device [7].
- In **matrix entrapment**, the polymeric gel matrix precursors are polymerized in the presence of the biological elements to be entrapped. The most commonly used gels are polyacrylamide, polyvinyl alcohol, polycarbonate, cellulose acetate, starch, alginate, and silica gel. Matrix entrapment is usually not the preferred method of immobilization as it may lead to possible delays in response time due to a diffusional barrier to the analyte and the leakage of biological species during sensor operation, resulting in a loss of bioactivity.
- In **membrane entrapment**, enzyme solutions, cell suspensions or tissue slices can simply be encapsulated in analyte-permeable preformed membranes on the electrochemical transducer. Self-assembled monolayers (SAMs) and bilayer lipid membranes (BLMs) can also be used to encapsulate biological molecules and bind them to the transducer surface. A sol–gel method is used to immobilize biological molecules in ceramics, glasses, and other inorganic materials. Bulk modification of the entire electrode, for example, enzyme-modified carbon paste or graphite epoxy resin [8], magnetic interactions [9], and biotin–avidin binding [10, 11] are also effective methods of immobilization.

3 Electrochemical Biosensors

In the simplest of terms, an *electrochemical biosensor* can be defined as one which transduces or converts a biological event into a measurable, reproducible, and discrete electronic signal. Electrochemical biosensors combine the electrochemical transducers' sensitivity with the specificity of biological recognition processes involving biological elements such as enzymes, proteins, nucleic acids, antibodies, cells, or tissues [12]. Electrochemical biosensors provide easy fabrication, ease of operation,

portability, and entail low costs in manufacturing. An electrochemical study of a reaction will yield a measurable current (amperometry), a measurable charge accumulation or potential (potentiometry), a change in the conductivity of the medium (conductometry), or changes in capacitance and/or resistance of the medium (impedance spectroscopy). As electrochemistry is a surface phenomenon, electrochemical transduction does not require large sample volumes, and this provides for the effective miniaturization of biosensor devices. Electrochemical transduction helps in the speedy, continuous, real-time and inexpensive monitoring of many components in clinical laboratories and industries [13].

3.1 Amperometric Biosensors

The amperometric technique involves applying a fixed potential to the working electrode versus a reference electrode, and measuring the current produced as a result of the electrochemical reduction or oxidation occurring at the working electrode. This current is proportional to the concentration of the electroactive product, which is in turn proportional to the nonelectroactive substrate in the sample. However, the electrolysis current is limited by mass transfer rates. Amperometric biosensors provide an additional selectivity because the oxidation or reduction potential used is normally characteristic of a particular analyte [12]. Additionally, the fixed potential used in amperometry results in a negligible charging current, which minimizes the background signal. Simplicity and a low limit of detection make amperometric transduction a good choice for biocatalytic and bioaffinity sensors [14].

The electrochemical cell usually consists of a three electrodes system: (i) a working electrode made from conductive inert metals such as Pt, Au, or graphite, and at which the biochemical reaction involving the target analyte occurs catalyzed, in most cases by enzymes which are immobilized on the electrode surface; (ii) a counterelectrode, which is usually a Pt wire; and (iii) a reference electrode against which the potential measurement is made. However, if the current density is low ($<\mu A\,cm^{-2}$), a two-electrode system without the reference electrode can also be used; in fact, such a system is generally preferred in disposable sensors as long-term stability of the reference electrode is not required and the costs are lower [12].

The electrodes can easily be miniaturized to micron size, or even to nanometer size [15, 16], which results in low sample volume requirements for detection of the analyte. Recently, screen-printed electrodes (SPEs) with patterned microelectrodes have gained in popularity because of their low cost, ease, and speed of mass production [17]. Disposable SPEs have been used in immunochemical sensors and glucose sensors [18]. Interdigitated array electrodes consisting of two pairs of working electrodes made from parallel metal finger strips interdigitated and separated by insulating materials may serve as another good amperometric transducer [12, 19].

Amperometric biosensors are typically based on enzyme electrodes. The simplest design of an amperometric biosensor is the direct detection of either the increase of an enzymatically produced electroactive species or the decrease of a substrate of a redox enzyme. A typical example of this design is a glucose sensor based on using glucose oxidase (GOx) as the biorecognition element. The increase in

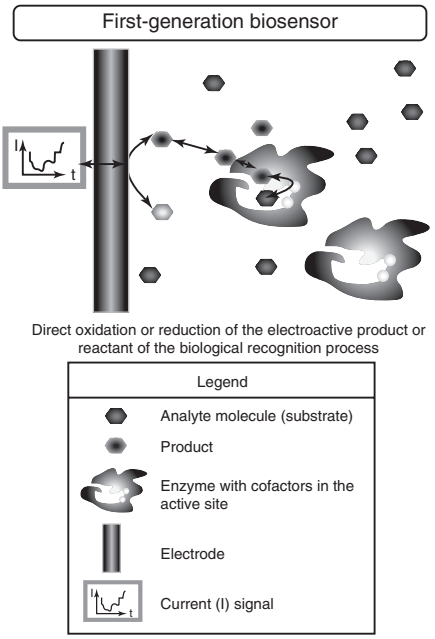

Fig. 2 "First-generation" amperometric biosensors. Reprinted with permission from Ref. [24]; © 2011, John Wiley & Sons.

concentration of the product, H_2O_2, or the decrease in concentration of the cosubstrate, O_2, is electrochemically monitored in order to quantify the glucose concentration [3, 20–23]. Such sensors are termed *"first-generation" biosensors* (Fig. 2). Unfortunately, the reproducibility of these biosensors is dependent on the concentration of oxygen, while the electrode potential is prone to interference [24].

The use of artificial redox mediators was introduced to overcome the operational problems associated with their first-generation counterparts. Cass *et al.* developed the first amperometric glucose biosensor based on ferrocene, a redox mediator [25]; such biosensors are termed *"second-generation" biosensors* (Fig. 3). Artificial redox mediators are small, soluble molecules capable of undergoing rapid and reversible redox reactions that

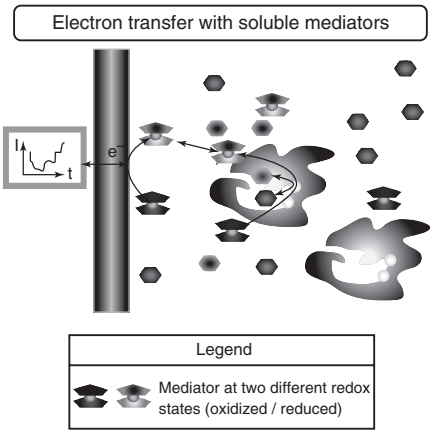

Fig. 3 "Second-generation" amperometric biosensors. Reprinted with permission from Ref. [24]; © 2011, John Wiley & Sons.

shuttle electrons between the active site of the enzyme and the electrode surface. These sensors are prone to leakage of free diffusing redox mediators from the electrode surface, which adversely affects their long-term operational stability. However, this does not affect their successful application in one-shot devices, such as those for the self-monitoring of glucose [13].

A better biosensor architecture can be realized through the immobilization of a redox enzyme on the electrode surface, in such a way that a direct electron transfer is made possible between the active site of the enzyme (where the catalytic reactions occur) and the transducer. Such a design obviates the use of freely diffusing redox mediators, and biosensors based on this design principle are termed *"third-generation" biosensors* (Fig. 4). A reagentless biosensor architecture can be realized by co-immobilizing the enzyme and the mediator at the electrode surface [24]. Third-generation biosensors have a greater stability and can be used for repeated measurements or continuous

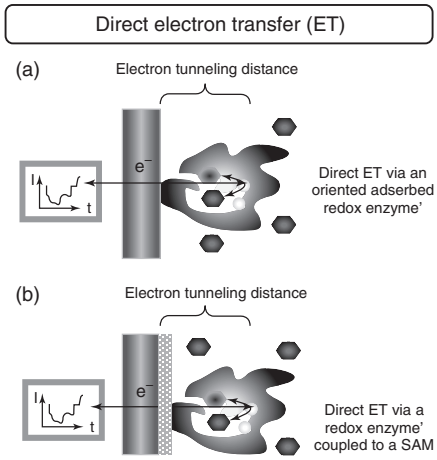

Fig. 4 "Third-generation" amperometric biosensors. Reprinted with permission from Ref. [24]; © 2011, John Wiley & Sons.

monitoring, as neither the enzyme nor the mediator needs to be added. Consequently, such sensors are self-contained and the cost of each measurement is reduced [12].

3.2 Potentiometric Biosensors

Potentiometric biosensors are based on the principle of measurement of charge accumulation at the working electrode compared to a reference electrode, under the conditions of negligible or zero current, and are governed by the Nernst equation:

$$E = \frac{E^{\circ} \pm RT}{n\mathrm{F}\log(a_{\mathrm{i}})} \quad (1)$$

where E is the measured potential of the cell, E° is the standard cell potential at temperature T, R is the universal gas constant, n is the number of moles of electrons transferred in the cell reaction, F is Faraday's constant ($\sim$96 500 C mol^{-1}), and a_i is the chemical activity of the species i.

A typical potentiometric device set-up consists of a reference and one working electrode in contact with the sample solution. Common electrodes used for potentiometric quantification are the glass pH electrodes and ion-selective electrodes (ISEs) for ions such as K^+, Na^+, and Ca^{+2} [26]. These sensors can be converted into biosensors by using biological elements such as enzymes capable of catalyzing reactions that involve analyte molecules to produce ions for which the sensor is designed. An immobilized enzyme layer adjacent to the working electrode catalyzes a biological reaction involving the analyte in which ionic species are either consumed or produced. A local equilibrium is established at the sensor interface and the membrane potential, developed due to the difference in concentration of the ions across the membrane, is measured. The ISE generates an electrical signal in response to the change in concentration of ionic species. Currently, three types of ISE are used in potentiometric biosensors:

- Glass electrodes for cations: these are made from a very thin hydrated glass membrane as the sensing element. A transverse electrical potential is developed due to the concentration-dependent competition between cations for specific binding sites. The selectivity of a glass electrode is determined by the composition of the glass. A common glass electrode is shown in Fig. 5.
- Gas electrodes, which are the usual glass pH electrodes coated with hydrophobic gas-permeable polypropylene or Teflon membranes selective for gases such as CO_2, NH_3, and H_2S. The diffusion of gases through the membrane causes a change in the pH of the sensing

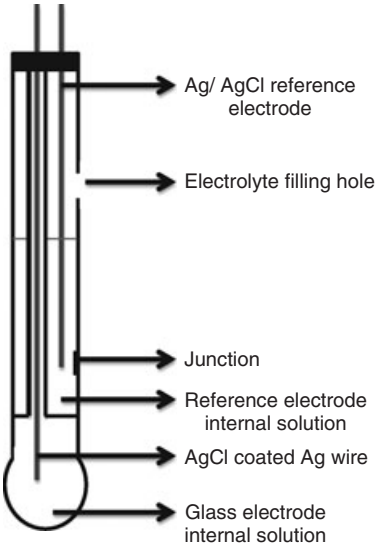

Fig. 5 Typical arrangement of a glass electrode.

solution between the membrane and the electrode, which is then determined.

- Solid-state electrodes which consist of a thin membrane of a specific ion conductor made from a mixture of Ag_2S and AgX, where X is a halide anion.

Although, amperometric transduction – given its good sensitivity and low limit of detection – is favored in the case of glucose biosensors, the details of a number of potentiometric biosensors have been reported [27, 28]. Potentiometric biosensors are known for their simplicity of operation, and their continuous measurement capability makes them interesting for environmental applications, especially for monitoring heavy metals and pesticides [13]. With limits of detection as low as 10^{-8} to 10^{-11} M, potentiometric biosensors are suitable for measuring low concentrations in small sample volumes as they do not chemically influence the sample [29].

The ion-selective field effect transistor (ISFET), a type of potentiometric device, was developed by Bergeveld and successfully combines solid-state integrated circuit (IC) technology with ISEs. The chemical-sensitive property of the glass membrane electrode is used in conjunction with the impedance-converting characteristics of the metal oxide semiconductor field effect transistor (MOSFET), in which the metal electrode (gate) is removed and its function is taken over by the sample solution under study (Fig. 6). This modified FET is capable of detecting changes in ion concentration when the gate is exposed to a solution containing ions. Many biosensors based on ISFET have been described since the first report of the enzymatic-modified ISFET (enzyme field-effect transistor; EnFET) for the determination of penicillin [30]. ISFET-based enzyme biosensors can also be used to detect and quantify heavy metal ions and organic pollutants, through the inhibitory action of such species on enzyme activity [31].

These biosensors have many advantages over other types of biosensor, notably miniaturization, high sensitivity, low cost and multianalyte detection potential [32]. Unfortunately, however, they still suffer from a variety of fundamental and technological problems, such as the impurity of the semiconductor layer and instability of the functional groups in the sensing layer [33]. On the other hand, ISFETs can be directly incorporated into the electronic signal processing circuitry [13], and this can lead to their integration in microsystems such as micro-total-analysis-systems (μ-TAS) and lab-on-a-chip (LoC) [33].

3.3 Conductometric Biosensors

Conductometric techniques rely on measurements of the change in electrical

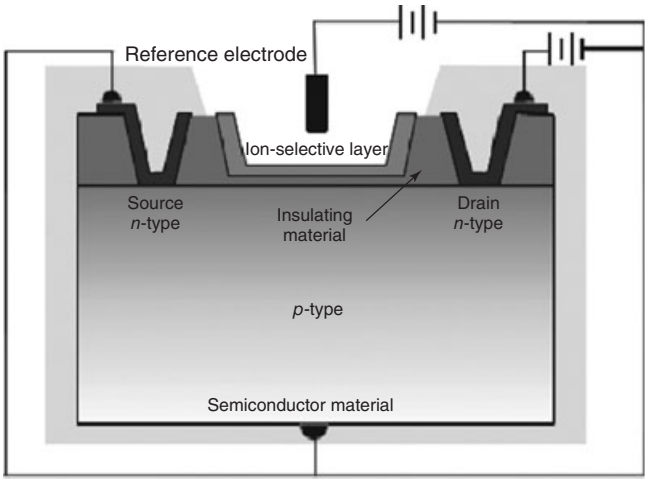

Fig. 6 Typical arrangement of an ISFET device.

conductivity of the sample solution due to the production of charged species, such as ions and electrons, during the course of a biochemical reaction catalyzed by an enzyme. For example, urease – which catalyzes the production of ionic species – can be used in combination with conductometric transduction. Conductance measurements have a lower sensitivity compared to other techniques [34], as conductance is sensitive to temperature, faradaic processes, double-layer charging, and concentration polarizations [13]. These effects can be minimized, however, by using an alternating current voltage for measurements [34]. This will result in a higher limit of detection and reduces potential interferences from variations in the ionic strength of the samples [13]. Conductometric techniques can be used to create inexpensive and disposable sensors; however, in order to obtain reliable measurements the ionic strength of the sample solution should undergo a significant change.

Conductometric biosensors have been used for environmental monitoring, as they provide an easy-to-use, accurate, selective, fast and cheap alternative to conventional methods of heavy metal determination, such as gas and liquid chromatography, spectrophotometry, and chemical and physical techniques which are time-consuming and require expensive instruments and skilled personnel. The presence of heavy metals can be determined using thin-film interdigitated planar conductometric electrodes, with enzymes such as GOx, butyric oxidase and urease having been used to detect Ag^{+}, Hg^{2+} and Pb^{2+} [13]. Conductometric biosensors have also been used to monitor the presence of organic pollutants and pesticides in the environment [35, 36].

4 Optical Biosensors

4.1 Conjugated Polymer-Based Biosensors

Conjugated polymers (CPs) are π-conjugated polymeric compounds in which the backbone is composed of alternating saturated and unsaturated bonds, while the backbone atoms are

sp^2-hybridized [37–41]. These sp^2 hybrid orbitals, which are bonded through σ bonds with the remaining out-of-plane P_z orbitals overlapping with the neighboring P_z orbitals, provide the movement of free electrons. Therefore, the p-orbital overlap is the origin of the emissive and conductive properties of CPs and provides unique optoelectronic properties under certain conditions. For example, CPs are highly conductive under chemically doped conditions and are good candidates for flexible electronic materials. The unique optoelectronic property of CPs has attracted much attention for use as effective optical transducers, with CPs emerging as the active materials for various applications including light-emitting diodes (LEDs), field effect transistors (FETs), light-emitting electrochemical cells (LECs), polymer actuators, plastic lasers, batteries, photovoltaic cells, and biomaterials for sensory applications.

Conjugated polyelectrolytes (CPEs) are π-CPs that have charged (anionic or cationic) side chains [42–46]. In this case, ionic groups such as sulfonate, carboxylate, phosphate and quaternary ammonium ion are introduced into the chemical structures of the CPs to change their polarity. These ionic functional groups usually prevent the CPs from aggregating in water, and also control their solubility. In particular, CPEs may be good candidates for biological applications because the excellent water solubility of the CPs is essential for their homogeneous use in aqueous media. The ability to control the water-solubility of CPs might be considered as a sensing mechanisms for CPEs to be exploited as biosensory materials [47]. A change in water solubility by adding the target analytes, and the subsequent conformational change of the CPs, lead to alterations in the fluorescence wavelength and intensity of CPs. Recently, hydrophobic CPs have also been prepared as nanoparticles in an aqueous environment and used as sensory materials [48, 49].

CPs are largely classified based on how they release the energy absorbed from excitation. A CP molecule in an excited state can lose either an emission of radiation (as fluorescence and phosphorescence) or radiationless transition, such as the intersystem crossing shown in the Jabnolski diagram (Fig. 7). The emission of radiation from the lowest vibrational level of the excited state S_1 to any of the vibrational levels of the ground state S_0 is termed *fluorescence* (fluorescence lifetime: 10^{-9} to 10^{-7} s). Although the population

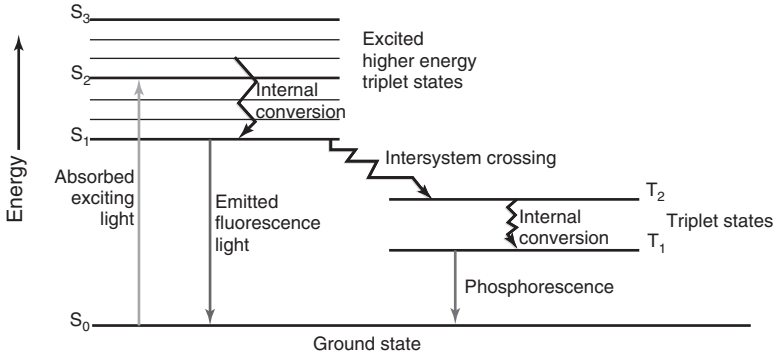

Fig. 7 Jabnolski diagram.

of triplet states by direct absorption from the ground state is insignificant, a more efficient process exists for the population of triplet states from the lowest excited state in CPs (intersystem crossing). If intersystem crossing has occurred, and the initial spin state is different from the final energy levels, then the emission energy may change; this is termed *phosphorescence*. Once intersystem crossing has occurred, the molecule undergoes a singlet–triplet process within the lifetime of an excited singlet state (10^{-8} s), and the life time of a triplet state is much longer than that of an excited state (ca. 10^{-4} to 10 s). This could be a good indication to judge the type of emission in CPs. However, fluorescence in CPs is usually statistically much more likely than phosphorescence, unless vibrational coupling between the excited singlet state and a triplet state causes intersystem crossing (this usually could occur at a very low temperature, <80 K) [39]. A similar type of emission termed *delayed fluorescence* has been identified which normally follows the fluorescence characteristic emission spectrum; however, the lifetime of delayed fluorescence in CPs is slightly shorter than that of phosphorescence as it is caused by a recombination of geminate electron hole pairs rather than triplet–triplet annihilation.

Fluorescence from CPs is very sensitive to any environmental changes around CPs. The optical properties of CPs undergo dramatic changes such as fluorescence amplification, quenching, or nonradiative energy transfer when the light is absorbed [50], and therefore the provision of mechanisms for optical changes in CPs allows their implementation in sensing applications. This appealing property of CPs provides a highly sensitive transduction mechanism by signal amplification of CP fluorescence, and also explains various detection modes. The signal-amplifying model of CPs was proposed by Swager and colleagues in 1995 [51, 52]. When a target analyte binds locally to a receptor on a CP repeat unit, the entire conjugated backbone is affected due to its one-dimensional wire-like optoelectronic property, such that the fluorescence of the entire polymer chain is altered. The wiring of chemosensory molecules in series provides a universal method by which to obtain signal amplification relative to single-molecule systems. CPs are ''molecular wires,'' as the key feature of a CP is that it can harness extended electronic communication and transport. However, the terms amplification and sensitivity enhancement only indicate when a single event – the binding of an analyte – in a supramolecular polyreceptor system produces a response larger than that afforded by a similar interaction in an analogous small monoreceptor system.

The photophysical properties of CPs are strongly related to their polymer structure, whether in solution and/or in solid state [50]. Changes in the chemical nature, effective conjugation length, intramolecular conformation and intermolecular packing will each have an influence on the color and intensity of fluorescence. The emission wavelength can be modulated through the design of backbone structure of CPs and by changing the charge density around the CP backbone. Side-chain modification along the backbone, using either electron-rich or electron-deficient functional groups, provides the emission wavelength. The effective conjugation length is also a critical factor in determining wavelength, with long chains generally showing a longer wavelength emission. However, the fluorescence wavelength of CPs is not influenced further when the

conjugation length of CP exceeds the ''effective'' conjugation length. Rather, the shorter lifetime of CPs and the exciton mobility – which may be limited by conformational disorder in solution – will prevent diffusion throughout the entire length of high-molecular-weight CPs.

Several detection modes in CPs have been actively developed for the sensing of chemical or biomolecules, including fluorescence turn-on (amplification) and turn-off (quenching), fluorescence color change, and visible color change (Fig. 8). In the turn-on mechanism, the fluorescence signal of CPs is inherently excellent but is completely and partially quenched due to the change in electron density along the CP backbone as a result of conformation changes or intramolecular packing. Target binding to CPs, and the associated conformational rearrangement of CPs or unpacking among the backbones, perturb the electronic state along the CP backbone and induce an enhancement in fluorescence. Although the polymer may be soluble in water, the fluorescence quantum efficiency of CPs in aqueous solution may be low due to their limited water-solubility and the resultant polymer aggregation. The use of a surfactant to improve CO solubility in water can also provide an improvement in signal turn-on, without affecting target binding and signal transduction by surfactants.

Another interesting turn-on sensor has been developed as a colorimetric CP sensor by using polythiophene derivatives. These CPs provide a color change and signal enhancement when a target is bound to the receptor such that their conformation is altered. As an example, cationic polythiophene derivatives will form a duplex with negatively charged single-strand DNA molecules, which results in polymer aggregation and, hence, fluorescence quenching. When a target single-strand DNA molecule is hybridized to the complementary receptor DNA, the DNA/DNA/polymer triplex will be less planar than when in the duplex conformation, and so will have a shorter conjugation length and different absorption characteristics. Target molecules detected using this system vary from small DNA molecules to large protein molecules.

In a turn-off system, a variety of mechanisms can result in quenching, such as Forster or Dexter energy transfer, static quenching, complex-formation between polymers and a target, and collisional

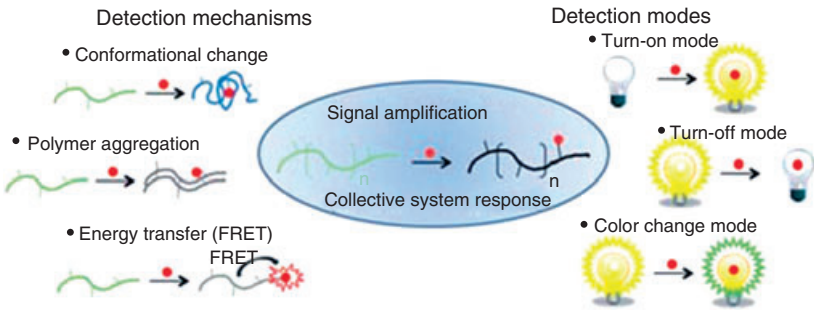

Fig. 8 Conjugated polymer based biosensors: detection mechanisms and modes. Reproduced with permission from Ref. [50]; © 2010, Royal Society of Chemistry. (http://pubs.rsc.org/en/Content/ArticleLanding/2010/AN/c0an00239a)

quenching [53, 54]. For these reasons, the fluorescence quenching of CPs is often dependent on environmental factors such as temperature and pressure. In most CP-based quenching systems, target binding induces the electronic state of CPs by an intermolecular aggregation of the polymer chains. Such aggregation is often due to hydrophobic effects induced by target analytes, and the addition of surfactants or a change in temperature can prevent CP aggregation. Quenching that occurs upon interaction with a specific molecular biological target forms the basis of active optical contrast agents for molecular imaging.

In a similar way, the fluorescence resonance energy transfer (FRET)-induced detection mode also begins with the fluorescence quenching of CPs that normally are used as energy donors [55–57]. FRET (also known as Forster energy transfer) is a dynamic quenching mechanism because energy transfer occurs while the CP donor is in an excited state. When a target analyte labeled with fluorescence acceptor molecules is bound to a target receptor and is located in the proximity of CPs (usually within 10 nm), the CP as the donor chromophore will transfer energy to an acceptor chromophore through dipole–dipole coupling. The efficiency of FRET depends on many physical parameters, including the distance between the donor and the acceptor, the spectral overlap of the donor emission spectrum and the acceptor absorption mechanism, and the relative orientation of the donor emission dipole moment and acceptor absorption dipole moment. The dominant factor among these CP-based sensors is the distance between the donor and the acceptor, because the efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the CP and the acceptor dye. Consequently, FRET-based CPs have been used as potent tools to measure distances and to detect molecular interactions in a number of systems, and are widely applied in biology and chemistry.

4.2 Surface Plasmon Resonance-Based Biosensors

Surface plasmon resonance (SPR) was first observed by Wood in 1902, but a complete explanation of the phenomenon was not provided until 1968, by Otto [58–63]. Since the first application of SPR-based sensors to biomolecular interaction monitoring by Liedberg *et al.* in 1983, the phenomenon of SPR has served as a fascinating detection tool for biosensor applications [64]. When polarized light is shone through a prism on a sensor chip, on top of which is a thin film of metal (usually gold or silver); the film will act as a mirror and reflect the light. On changing the angle of incidence, the intensity of the reflected light will pass through a minimum. At the angle of incidence when this occurs, the light will excite the surface plasmon so as to induce SPR and cause a dip in the intensity of the reflected light. The angle at which the maximum loss of reflected light intensity occurs is termed the *resonance angle* or *SPR angle*. The SPR angle depends on the optical refractive indices of the media at both sides of the metal. The SPR conditions can be changed, and the shift of the SPR angle is suited to provide information on the kinetics of target adsorption on the metal surface. A schematic of a biosensor device based on SPR is shown in Fig. 9.

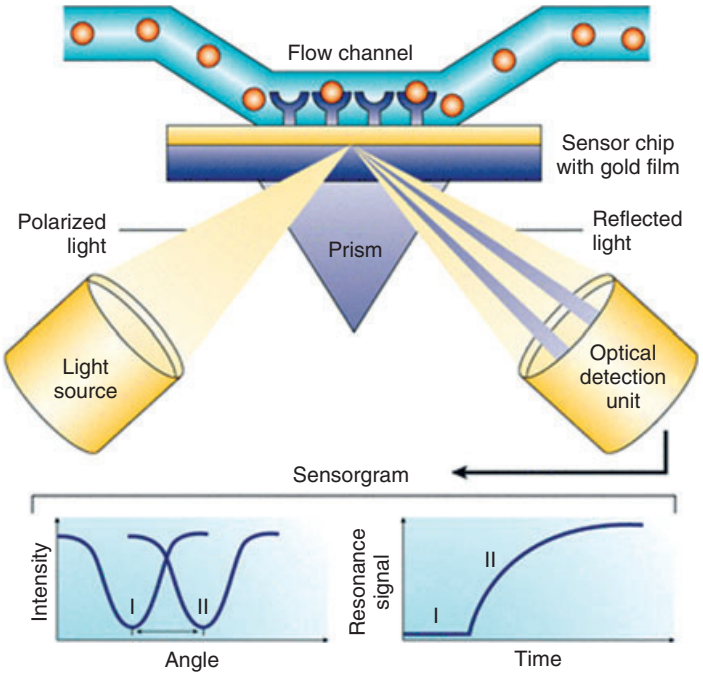

Fig. 9 Schematic of surface plasmon resonance (SPR)-based biosensor. Reproduced with permission from Ref. [65]; © 2002, Macmillan Publishers Ltd.

4.2.1 SPR Principles

SPR can be used to monitor changes in the refractive index in the near vicinity of the metal surface. When the refractive index changes, the angle at which the intensity minimum is observed will also change [66]. Hence, SPR not only provides an excellent means of measuring the difference between two states, but can also be used to monitor the intensity change in time-lapse. SPR sensors function in only a very limited vicinity or fixed volume at the metal surface, as the exponential decay of the evanescent field intensity in a typical SPR-based sensor presents practical blindness at distances beyond 600 nm from its surface. A process occurring within the first few tens of nanometers from the metal surface will result in a few-fold higher response than the same process performed at a distance of a few hundred nanometers. A signal observed at the penetration depth of the electromagnetic field is termed the *evanescent field*, and does not exceed a few hundred nanometers. The penetration depth of the evanescent field is a function of the wavelength of the incident light. In order to provide selectivity for the SPR sensor, its surface must be modified with ligands that are suitable for capturing the target compounds and which are permanently immobilized on the sensor surface.

SPR-based sensor applications are associated with specific properties: (i) field enhancement; (ii) surface plasmon (SP) coherence length; and (iii) the phase jump of the reflected light upon SP excitation. In field enhancement, calculation of

the electric field transmission coefficient based on Fresnel's equation for the interface shows that the electric field at the high refractive index side of the metal can be much smaller than that at the low index side of the metal layer. At very close to the SPR angle, the intensity can be enhanced by a factor of more than 30, a circumstance which explains much of the remarkable sensitivity that the SPR condition has for a changing dielectric environment. The metal thickness is also critical for SPR phenomena, and affects the biosensor's efficiency; for example, with an excitation wavelength of 700 nm the field enhancement is normally maximized when the gold layer is about 50 nm thick, but is decreased as the gold layer become either thicker or thinner.

SP coherence length implies that the field intensity of SPR decays with a characteristic distance $1/2k_x''$, where the metal's dielectric constant is complex and a complex propagation constant is $k_x = k_x''(\text{real parts}) + jk_x''$ (imaginary parts). For gold or silver (the most frequently used metals in sensory applications), the imaginary part of the dielectric constant increases with decreasing wavelength, and the SP propagation length decreases accordingly. Hence, the SP propagation length will become longer with increasing wavelengths used for the sensor studies. Finally, phase jump refers to the reflection event at an interface, that is generally accompanied by a phase jump of the reflected field. The phase of the reflected electric field undergoes a relatively large change around the SPR dip, and this is critical for sensing purposes. However, the absolute values found are of limited validity due to the complicated experimental set-up, though a phase measurement will provide an order-of-magnitude better sensitivity.

4.2.2 Surface Chemistry in SPR Technique

Detection processes in SPR between a target and a receptor are critical to provide a thorough understanding of all processes in living organisms [60]. Fast, selective and quantitative analyses, without a need for labels for optical biosensors, is the key to this situation. However, an elegant direct detection technique for label-free targets in bare SPR substrates provides a nonspecific binding of other components, as well as the desired specific targets of a biomolecular interaction. Another issue when using SPR detection without a relevant surface modification is the irreversible binding of numerous proteins and other biomolecules, as this can result in a failure to completely regenerate chip surfaces for reuse. In fact, after being used only a couple of times, the SPR substrates will be only partially regenerated, and 90% or more of their activity will be lost. Thus, it is clear that the surface energy and surface charge of the SPR chips must both be carefully modulated to obtain not only a minimum false signal but also maximum detection yields. In addition to a high signal-to-noise ratio, the ability to retrieve the biological activity of an immobilized ligand is essential when creating successful SPR-based sensors.

The transport of targets to the SPR chip surface by convection and diffusion has a profound effect on the signal. A correctly selected surface nanoarchitecture is extremely important to control the amount of immobilized ligand. The steric hindrance of target binding sites via a chemical immobilization process has a strong effect on the affinity of the ligand towards the target molecules. A sufficient spacing between the ligands can help to minimize any steric problems by controlling the ligand densities. The binding rate and

equilibrium constants can normally be determined based on a novel distribution analysis. A detailed characterization of the distribution of binding properties provides a useful tool for optimizing surface modifications to achieve an effective functionalization of biosensor surfaces with uniform high-affinity binding sites, and also for studying immobilization processes and surface properties. The surface charge may also influence the interaction kinetics and the extent of nonspecific interactions.

Controlling the hydrophilicity can achieve protection of the sensitive biomolecular ligands by the appropriate selection of a functional group. Nonspecific binding can be prevented by introducing a bioinert layer, while the most popular functional group is the carboxylate ion. The optimum thickness of the bioinert layer is based on the exponentially decaying strength of the evanescent field. A thickness >10 nm would reduce the sensitivity of the binding signal, whereas a thickness <1 nm would usually cause an inhomogeneous coating of the metal surface. Hence, the preferred thickness of the bioinert layer, as an adhesion-linking layer, should be 2–5 nm.

4.2.3 Surface Plasmon Fluorescence Technique

Among the various sensing principles proposed for biosensor studies, surface plasmon fluorescence spectroscopy has, in particular, found widespread application and has demonstrated its potential for the sensitive detection of targets in several examples. SPR provides a label-free detection principle, as only the presence of bound analytes will slightly alter the optical architecture at the sensor surface probed by the surface plasmon mode propagating along this metal/dielectric interface. Another reason for such rapid growth is that surface plasmon fluorescence-based detection principles present attractive sensitivities for the *in situ* and real-time monitoring of biological targets. In addition, several facile surface-modification protocols are available to obtain the required functionalization of the sensor surface for label-free detections.

As the intensity profile normal to the metal/dielectric interface decreases exponentially in the direction into the metal, this suggests that the analyte molecules must be brought as close to the metal surface as possible in order to place their chromophores into the highest possible optical field. Metal-enhanced fluorescence is wavelength- as well as environment-dependent; notably, the environment (e.g., the solvent) can affect the enhancement factors [67]. Other effects on chromophores in the excited state close to a metal surface such as gold, and which can lead to a quenching of fluorescence, should also be considered. The fluorescence subsequently decreases with close contact, most likely due to FRET at very close contact. At intermediate distances, however, an efficient back-coupling of the excitation energy from the vibrational relaxed–excited state of the chromophore to the metal substrate becomes the driving force for the excitation of a red-shifted plasmon mode that can re-radiate via the prism at its respective resonance angle; this effect can be used to enhance the fluorescence emission. Fluorescence emitted directly from chromophores is sufficiently separated from the substrate surface, but can still be enhanced within the enhanced optical field of surface plasmon mode. This combination of field enhancement and fluorescence detection has been applied to a range of chemical and biosensing studies [68–80].

4.3 Surface-Enhanced Raman Spectroscopy-Based Biosensors

Surface-enhanced Raman spectroscopy (SERS) or surface-enhanced Raman scattering is a surface-sensitive technique that enhances Raman scattering by molecules absorbed on rough metal surfaces [81]. Since the discovery in 1974 by Fleischmann *et al.*, that a high-intensity Raman scattering of small molecules could be achieved on an electrochemically roughened silver surface, the field of SERS has expanded dramatically due to improvements in technique that have resulted from advances in nanotechnology and improved instrumental capabilities [82]. Today, the SERS technique is becoming widespread and is encountering new and exciting horizons in analytical chemistry, biology and biotechnology, forensic science, and also in the study of artistic objects. Although the exact mechanism of SERS remains a matter of debate, and the mechanisms proposed experimentally have not been straightforward, two primary theories have persisted: (i) an electromagnetic theory based on the excitation of localized surface plasmons; and (ii) a chemical theory based on the formation of charge–transfer complexes [83–88]. As the chemical theory applies only to species which have formed a chemical bond with the surface, it cannot explain the observed signal enhancement in all cases. In contrast, the electromagnetic theory can be applied even to those cases where the specimen is absorbed only physically to the surface. Although the electromagnetic theory of enhancement can be applied regardless of the molecule being studied, it does not fully illustrate the magnitude of the enhancement observed in many molecules which have lone pair electrons and are bound to the surface. In this situation, the enhancement mechanism cannot be solely explained by involving surface plasmons. The chemical mechanism involves charge transfer between the chemically adsorbed species and the metal surface; in this case a spectroscopic transition – which takes place in the ultraviolet range and where the metal acts as a charge-transfer intermediate – can be excited by visible light.

An increase in the Raman signal on metal surfaces occurs due to enhancements in the electric field provided by the surface (Fig. 10). The light incident on the surface can excite a variety of phenomena in the surface, but the complexity of this condition can be simplified by surfaces with features much smaller than the wavelength of the light. For this explanation, one useful approximation to solve enhancement numerically – and which has been widely used in the literature – is the *electrostatic approximation*. In this case, the problem can be solved as in electrostatics, and the approximation corresponds then to ignoring the presence of the wave vector k. Therefore, the applied electric field does not have a wavelength; rather, it is a uniform field oscillating up and down with frequency. Although this approximation fails in many cases, it is not too difficult to imagine that the electrostatic approximation functions well when the size of the object is much smaller than the wavelength. This means that the electrostatic approximation will be valid mostly for objects of typical sizes in the range of about 10 nm or smaller. Another factor that affects the intensity in enhancement is the shape of the features. Objects with different shapes will have different resonances, and more than one resonance condition associated with a given shape. The local field intensity

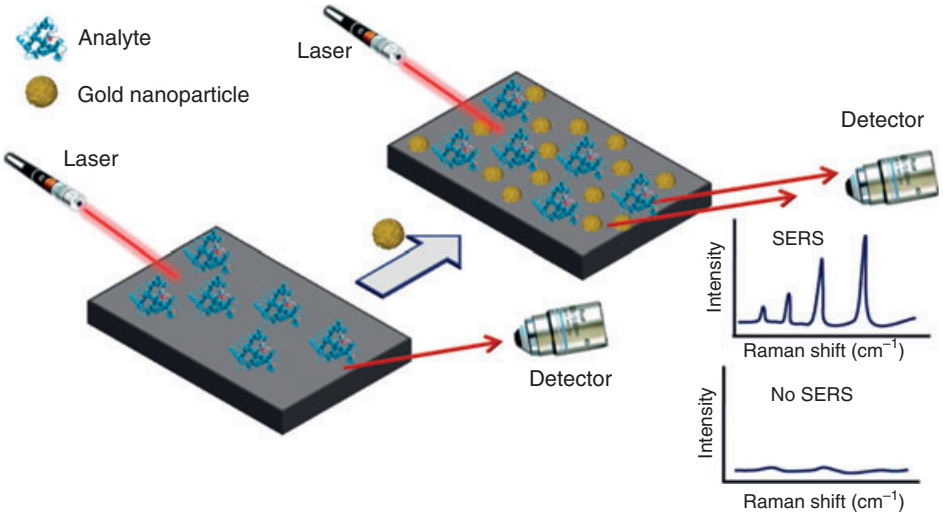

Fig. 10 Surface-enhanced Raman spectroscopy (SERS)-based biosensor.

enhancement factor at two different wavelengths is strongly position-dependent in most cases, and the direction of the polarization is vertical. Intensity enhancement in more complicated shapes than in the simplest cases of a cylinder or sphere can also be very high in some circumstances.

In electrostatic approximations for the calculation of field enhancement, size is not really important because the local field intensity enhancement factor will be the same as in the approximation. However, size is important if the objects are in the range of typical dimensions of ~30 to 100 nm. Generally, localized SPR red shifts as the size increases. SPR are also strongly damped as the size increases, mostly as a result of increased radiation losses. This results in a broadening of the resonance, such that the latter will eventually disappear for large sizes (typically 100 nm for dipolar localized surface plasmon in sphere, but possibly larger sizes for other geometries).

The choice of metal in SERS experiments is also critical for improving the results obtained. Generally, it is clear that silver outperforms gold, an advantage that can be tracked down to the higher absorption of gold at the frequencies where resonance occurs. However, the red shift induced by object interaction and shape and size effects can push the resonance in gold to the wavelength region (>600 nm). In this case, gold may be as good as silver, especially for bioapplications. Many biological applications of the techniques are based on near-infrared lasers (typical examples being diode lasers at ~750 or ~830 nm), and gold will be probably the most preferred plasmonic substrate.

5 Piezoelectric Biosensors

Piezoelectric biosensors are sensing devices which couple the bioaffinity recognition processes between the biological probe molecule and the target analyte molecule with the acoustic wave-based transduction mechanism, better known as the *piezoelectric effect*. The demonstration

of a linear relationship between the mass adsorbed onto the surface of the piezoelectric crystal and its resonant frequency [89] and the development of suitable oscillator circuits for their operation in liquid [90], led to their application in the field of biological sensing. Piezoelectric crystals can be combined with interfacial chemistry for the immobilization of biorecognition elements and macro- and micro-fluidic systems which enable a controlled contact of the analyte solution with the receptors [91], so as to yield efficient biosensing devices.

Certain solid materials (especially crystals lacking a center of symmetry) that demonstrate charge accumulation upon the application of mechanical stress or internal mechanical strain when subjected to an external electric field are said to exhibit the ''piezoelectric effect.'' In general, a mechanical stress that originates from a change in the mass of the adsorbed film on the piezoelectric crystal changes the resonance frequency of the crystal; this relationship is given by:

$$\Delta f = \frac{-2f_0 \Delta m}{A\sqrt{\rho\mu}} \tag{2}$$

where Δf is the change in resonant frequency (in Hz); f_0 is the resonant frequency of the crystal (in MHz); Δm is the change in mass (in g); A is the piezoelectrically active area of the crystal, between the electrodes (in cm^2); ρ is the density of the crystal (in $g\,cm^{-3}$); and μ is the shear modulus of the crystal (in $g\,cm^{-2}.s$).

The change Δf of the resonant frequency f_0 of the piezoelectric crystal is directly proportional to the change in mass, Δm. More specifically, the density, viscosity, elasticity, electric conductivity and dielectric constant of the sensing element can also undergo changes and, in turn, affect the piezoelectric transducer [92]. For an acoustically thin film, the mass change affects the transducer response [93, 94], whereas for an acoustically thick film, the film's viscous and elastic properties and geometric features also make significant contributions. Generally, the change in mass is central to the application of piezoelectric transducers in biosensors. However, there are instances when the ability of such transducers to quantify changes in shear modulus and viscosity has been exploited to fabricate efficient biosensors to study lipids and membranes. Figure 11 shows a typical piezoelectric transducer; here, the biosensing layer with the immobilized bioreceptors

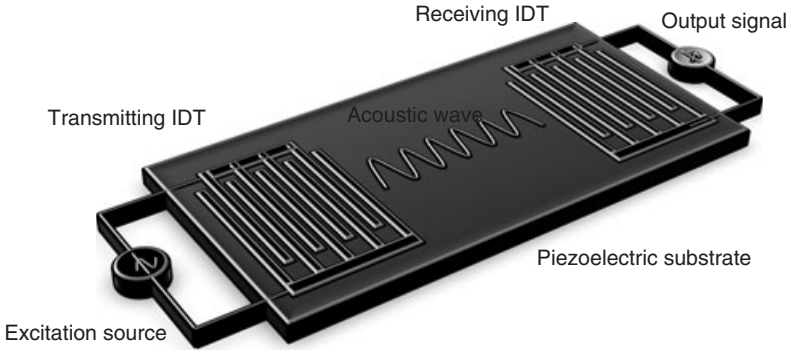

Fig. 11 Schematic of a piezoelectric transducer.

can be fabricated over the transducer surface. Piezoelectric biosensors have been employed in the label-free detection of a wide array of analytes ranging from proteins, oligonucleotides and DNAs, antigens, small molecules to viruses and bacteria. They have also been used widely to study protein–protein, protein–DNA, protein–peptide, peptide–peptide interactions, as well as interactions of carbohydrates with proteins, lipids, and other carbohydrates.

In general, for biosensor applications the piezoelectric material should be capable of operation in the liquid media. For efficient operation in the liquid medium, the acoustic waves must be either shear horizontally polarized, or their phase speed should be lower than the speed of sound propagation in the liquid [95]. The dielectric constant of the material should match that of the medium in which the device is to be used, in order to prevent a capacitive short-circuit of the electric field at the interdigital transducers (IDTs) [96]. Quartz, lithium niobate ($LiNbO_3$), potassium niobate ($KNbO_3$), lithium tantalate ($LiTaO_3$), and langasite (lanthanum gallium silicate) are some of the most commonly used piezoelectric crystals for the fabrication of acoustic devices. Knowledge of the specific properties of these different types of acoustic wave devices, such as the mechanical displacement of acoustic waves, the spatial distribution of mechanical and electrical fields, susceptibility to spurious coupling modes, and the sensitivity to temperature and pressure, is also important for the design of an efficient biosensor [92].

Depending on the acoustic wave-guiding mechanism, acoustic wave devices can be divided into three categories: (1) bulk acoustic wave (BAW) devices, in which the wave propagates unguided through the volume of the material; (2) surface acoustic wave (SAW) devices, in which the wave propagates, guided or unguided, along a single surface of the material; and (3) acoustic plate mode (APM) devices, in which the acoustic waves are guided by reflection from multiple surfaces [93]. The SAW and APM devices can be grouped together as surface-generated acoustic wave (SGAW) devices.

5.1 Bulk Acoustic Wave (BAW) Sensors

The BAW sensors, better known as thickness shear mode (TSM) devices or quartz crystal microbalance (QCM) devices, have traditionally been the choice of transducers for biosensors [97]. In the QCM bulk wave devices, the acoustic wave travels unguided through the entire volume of the piezoelectric substrate, resulting in vibration of the complete substrate. The displacement is maximized at the surface of the crystal, which makes the devices sensitive to surface interactions [98]. A typical BAW device consists of a piezoelectric crystal sandwiched between two electrodes that are generally produced by vapor-depositing Au or Pt onto the electrode surface. An electric field applied between the electrodes results in the mechanical oscillation of a standing shear wave across the bulk of the crystal at its natural resonant frequency. The frequency of the vibration depends on the properties of the crystal (size, density, cut, and shear modulus), and also on the properties of the phases adjacent to it [99]. This frequency changes when the target analyte molecules become attached to the bioactive layer that has been immobilized on the piezoelectric substrate, and this constitutes the output signal from the sensing device pertaining to the analyte. The sensitivity of these devices is limited by the thickness of the piezoelectric crystal. For

higher sensitivity, a higher resonant frequency is required; this can be achieved by reducing the thickness of the crystal, but thinner crystals are more fragile and difficult to handle. The QCM devices have been well investigated for the past 50 years, and have subsequently become a mature, commercially available, robust, and affordable technology [100, 101]. Typically, frequencies between 5 and 30 MHz are used.

The BAW sensors can generally be operated in two ways. In the first (*"dip-and-dry"*) method, the reaction between the analyte and the immobilized biorecognition element takes place in the solution phase, while the analysis and quantification occur under the gas phase [99]. The method involves measuring the vibrational frequency of the piezoelectric quartz crystal (PQC) before dipping the device in the analyte solution for a stipulated time. The device is then rinsed to remove any nonspecifically bound molecules, dried, and the vibrational frequency is measured again. Shons *et al.* [102] described the first PQC biosensor in 1972, while Grande *et al.* [103] discussed some of the complications of dip-and-dry methods, such as solvent retention. Unfortunately, the dip-and-dry method does not provide any real-time analysis. The second method involves solution-phase sensing for which the contact cell is configured in a flow or batch mode and a peristaltic or syringe pump is used to introduce the test solution into the cell [99]. Solution-phase sensing allows for real-time analysis.

Previously, BAW sensors have been used for the detection of viruses, bacteria and other cells. Lee *et al.* [104] demonstrated sensitivity comparable to an enzyme-linked immunosorbent assay (ELISA) for the detection of cattle bovine ephemeral fever virus. The application of QCM biosensors in microbiology can be categorized into three areas: the direct detection of a microbe or spore; the detection of an associated antigen or toxin; and the study and characterization of biofilm formed by a microbe [91]. Biosensors for a large number of bacteria, and for the toxins produced by them, have been reported. QCM sensors have been used successfully to monitor and quantify some key processes in biofilm formation and colonization in real time. QCM sensors have also been used to determine proteins, small molecules such as drugs, hormones and pesticides, and nucleic acids.

5.2 Surface-Generated Acoustic Wave (SGAW) Sensors

SAW and APM devices can be grouped together as SGAW devices as both involve the generation and detection of acoustic waves at the surface of the piezoelectric crystal by means of IDT [94]. Since, the acoustic wave is confined to the surface of the crystals, these devices are not affected by the crystal thickness [98]. SAW devices operate at higher frequencies than BAW devices which, in principle, may lead to higher sensitivities because the acoustic wave penetration depth in the adjacent media is reduced [105]. In a typical configuration, an electrical signal is converted at the input IDT into a polarized transverse acoustic wave traveling parallel to the substrate surface. The amplitude and/or velocity of the wave are affected by any coupling reaction at the surface. The output IDT at the opposite end picks up the acoustic wave and converts it back to an electrical signal; any attenuation of the wave is then reflected in the output signal (Fig. 12). Depending on the piezoelectric substrate material, the crystal cut, the positioning of IDTs on the substrate, plate thickness and

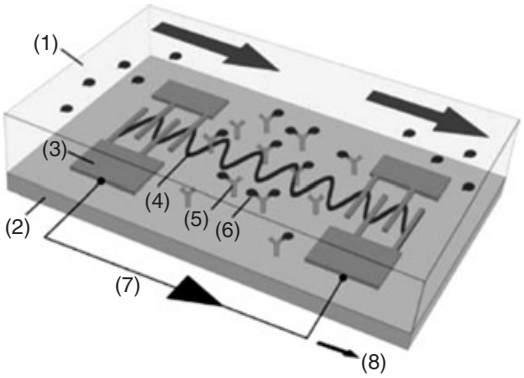

Fig. 12 Surface-generated acoustic wave (SGAW) sensor set-up. The arrows at the top indicate the flow of the liquid sample (1) in which the sensor is immersed. The elements of the SGAW biosensor are a piezoelectric crystal (2), IDTs (3), the surface acoustic wave (4), and immobilized antibodies (5) corresponding to the analyte molecules (6) in the sample. The driving electronics (7) operate the SAW biosensor and generate changes in the output signal (8) as the analyte binds to the sensor surface. Reproduced with permission from Ref. [107]; © 2008, Springer Science and Business Media.

wave guide mechanism, different operational modes of SGAW such as shear horizontal surface acoustic wave (SH-SAW), surface transverse wave (STW), Love wave, shear horizontal acoustic plate mode (SH-APM), and layer-guided acoustic plate mode (LG-APM) can be achieved [95]. For a better understanding of these SGAW modes, an excellent review is provided in Ref. [95]. SGAW devices can be manufactured using IC microfabrication or central metallica-oxen semiconductor (CMOS) techniques, which allows for the integration of a signal processing unit in the sensor architecture itself [95, 106].

The basic set-up of a SGAW-based biosensor consists of a piezoelectric transducer with an immobilized biospecific layer coupled to a driving electronic circuit and integrated with a sample flow mechanism driven by a peristaltic or syringe pump. Different SGAW techniques have been used according to the sensitivity and operational requirements. Love wave sensors are the most sensitive, with an operating range of 80–300 MHz and mass sensitivity of 150–500 $cm^2\ g^{-1}$ [108, 109]. When Gizeli *et al.* [110] reported the first biosensor based on Love waves in 1992, the device consisted of a quartz crystal with poly(methylmethacrylate) (PMMA) wave guide layer and immunoglobulin G (IgG) immobilized on the surface as the probe. Among others, a STW device is reported to have a sensitivity of 100–200 $cm^2\ g^{-1}$ and an operating frequency of 30–300 MHz [108, 109]. Respective values for SH-APM are 20–50 $cm^2\ g^{-1}$ [93, 108, 109] and 25–200 MHz, and for LG-APM are 20–40 $cm^2\ g^{-1}$ [108, 109] and 25–200 MHz.

6 Thermal Biosensors

Thermal biosensors function by monitoring the change in temperature due to the enthalpy changes associated with any biochemical reaction, and as such

are independent of the optical or electrochemical properties of the biocatalyst (usually enzyme), substrate or product. The invention of the enzyme thermistor (ET), which couples flow injection analysis (FIA) with an immobilized biocatalyst and a heat-sensing element [111], led to a surge in investigations into the design and applications of such biosensors. Their versatility and superior operational stability make thermal biosensors useful for such diverse applications as clinical analysis, food analysis, industrial process monitoring and environmental monitoring.

Any biochemical reaction is accompanied by the evolution or absorption of heat. The change in temperature (ΔT) of the system can be defined in terms of the enthalpy change associated with the reaction and the heat capacity of the system as: total heat evolved or absorbed during the reaction is given by:

$$\Delta T = \frac{-n_p \cdot \Delta H}{C_S} \tag{3}$$

where n_p is the total number of moles of the product, ΔH is the molar enthalpy change associated with the reaction and C_S is the total heat capacity of the system.

The enthalpy changes associated with biochemical enzymatic reactions are usually in the range of 10 to 200 kJ mol^{-1}, and are adequate to determine substrate concentrations at clinically interesting levels [112]. The molar enthalpy changes for some common enzyme-catalyzed reactions are listed in Table 1. The total enthalpy change is the sum of enthalpy changes associated with individual reactions. Thus, the measurement can be improved by coimmobilizing two enzymes – for example, oxidases with catalases – using a high-protonation enthalpy buffer such as tris(hydroxymethyl) aminomethane (TRIS) in the case of proton-producing biochemical reactions [112], using organic solvents which have lower heat capacities than aqueous solvents [113], or by the enzymatic recycling of the substrate where the net enthalpy change in each cycle adds to the overall enthalpy change [114, 115]. An enthalpy change of 100 kJ mol^{-1} is sufficient for the detection of analyte concentrations down to 5 µmol l^{-1}.

Tab. 1 Molar enthalpy changes for some common enzyme reactions [112].

Enzyme	*Substrate*	*Enthalpy change (ΔH; kJ mol^{-1})*
Catalase	Hydrogen peroxide	100
Cholesterol oxidase	Cholesterol	53
Glucose oxidase	Glucose	80–100
Hexokinase	Glucose	75*
Lactate dehydrogenase	Sodium pyruvate	62
NADH dehydrogenase	NADH	225
β-Lactamase	Penicillin G	115*
Trypsin	Benzoyl-L-arginine amide	29
Urease	Urea	61
Uricase	Urate	49

*In Tris buffer (protonation enthalpy: -47.5 kJ mol^{-1}).
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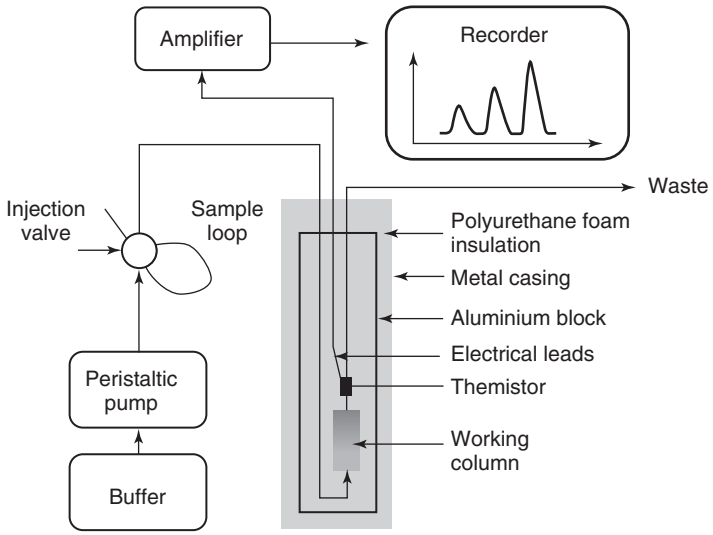

Fig. 13 Schematic of an enzyme thermistor. Reproduced with permission from Ref. [112]; © 2012, Elsevier.

The conventional thermometric device consists of a working column with the enzyme immobilized on a supporting matrix, and a thermal transducer (usually a thermistor) placed in the vicinity of the column. A schematic of a flow injection analysis enzyme thermistor (FIA-ET), a commonly used thermal biosensor, is shown in Fig. 13. The broad design includes an external jacket for insulation, a working column with immobilized enzymes, an indirect placement of the thermistor to prevent fouling, a heat exchanger prior to the working column to avoid temperature fluctuation, and a peristaltic pump to drive the buffer and analyte solution through the system. Nonspecificity is inherent in calorimetry, since all enthalpy changes in the reaction contribute to the measurement. However, this problem can be overcome by having a split-flow arrangement in which the test solution passes through two different columns. Typically, an active column and a reference column containing only the support matrix or, in some cases inactivated enzyme, is often used to minimize the effects of nonspecific enthalpies arising from nonenzymatic reactions [112]. On the other hand, the fabrication of miniaturized thermometric biosensors has become possible due to advances in the field of IC technology and the micromachining of liquid filters, microvalves and micropumps [113]. Miniaturized devices are suitable for portable use because of their high sensitivity, small size, modest buffer consumption, and good operational stability [112, 116–119].

Enzymes are the most commonly used biorecognition element in a thermal biosensor; however, in cases where the isolation of a pure enzyme is not possible, whole cells, organelles or tissue slices present a good alternative, although they may lack specificity and may also respond to some interfering compounds; MIPs may represent an alternative choice of receptor. Correct immobilization of the biorecognition element on the support matrix is important to maintain a

good catalytic activity of the former. The supporting matrix chosen should also be mechanically stable to withstand physical stress, to allow good flow properties [112], and should not interfere with the enzymatic reactions. Controlled pore glass (CPG), Sepharose CL-6B or CL-4B, Eupergit [112], reticulated vitreous carbon (for hybrid thermal-electrochemical sensors) [115, 120–122] and ceramic hydroxyapatite [123] are the most commonly used matrix materials. In general, a large excess of enzyme is immobilized on the support matrix to ensure correct operational stability.

Enzyme thermistors (ETs) have been used to determine a wide range of analytes, such as ethanol, glucose, oxalate, ascorbate, cellobiose and sucrose, and penicillin. Thermal biosensors have been used for the selective measurement of fructose in the presence of glucose [124] and to determine levels of urea in adulterated milk [125]. ETs have also been used for clinical monitoring; for example, a semi-continuous blood glucose monitoring ET device [126] and a cholesterol ET sensor [127] have been described. ETs have also been used for off-line as well as on-line monitoring of bioprocesses such as fermentation [128–130]. Heavy-metal ions such as Hg^{2+}, Cu^{2+} and Ag^{+} can be monitored using ETs via their inhibitory actions on urease, while pesticides can be determined by their inhibitory actions on the enzymes acetylcholinesterase and butylcholinesterase. Apoenzyme-based ETs can be used to monitor heavy-metal ions up to submillimolar levels [112]; an example is that of Cu^{2+} concentrations in human blood sera, which were measured using immobilized ascorbate oxidase [131] or galactose oxidase [132].

MIP-based thermistors have been used for the label-free characterization of MIP binding and catalysis [133, 134]. Thermometric transduction has also been coupled to an ELISA to yield a thermometric enzyme-linked immunosorbent assay (TELISA) [135] that can be used to determine the presence of hormones, antibodies and other biomolecules in complex matrices such as fermentation broth, blood samples and hybridoma cell media [112]. Although the sample capacity and sensitivity of TELISA is lower than for other established techniques (such as radioimmunoassay), it offers a faster monitoring and can be employed where rapid results are desired.

7
Microarrays

A microarray is a high-throughput, two-dimensional screening array that is located on a glass slide or a silicon thin-film cell and can be used to assay large quantities of biological materials. The concept and methodology of microarrays were introduced in 1983; since then the technologies of DNA, protein, peptide, tissue, cellular, carbohydrate and even phenotype microarrays have become highly sophisticated and the most used worldwide [136].

7.1
DNA Microarray

A microarray usually contains picomoles of a specific sequence as probes, and this enables many genetic tests to be executed in parallel, simultaneously. As such, DNA microarrays have dramatically accelerated many types of investigation [137–143]. The core principle of DNA (or RNA) microarrays is based on a hybridization between

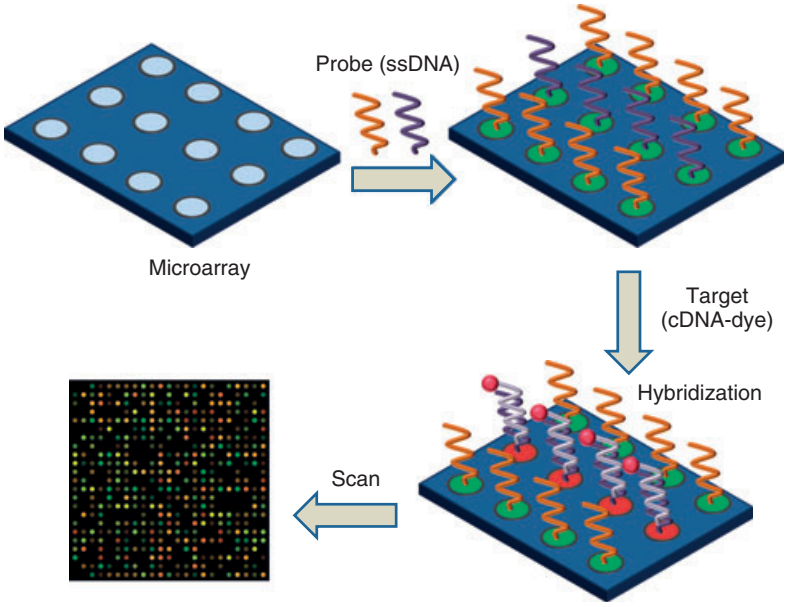

Fig. 14 DNA microarray.

complementary base pairs by strong hydrogen bonds; the total number of fluorescently labeled target sequences that will bind to a probe sequences will depend on the amount of target sample and thus provide quantitative information relating to the target (Fig. 14). The amount of target samples to be detected is generally limited, however, and additional steps such as the polymerase chain reaction (PCR) and target labeling with fluorescent dyes can be burdensome steps. Consequently, much effort has been made to increase the detection signal by using highly fluorescent probing materials such as CPs or inorganic quantum dots, and to develop label-free detection methods in microarrays in order to avoid a cumbersome labeling step [55, 56, 144–150].

Whilst a ''traditional'' solid-state array involves a collection of orderly microscopic spots called *features*, each with thousands of probes attached onto a surface, an ''alternative'' bead array is a collection of microscopic polystyrene beads, each with a specific probe and a mixture of two or more dyes which do not interfere with the fluorescence of the dyes used on the target sequence. Depending on the number of probes, the types of scientific questions being asked and the cost, DNA microarrays can be manufactured in different ways. Some of the most-often used technique for DNA microarray manufacture include printing with fine-pointed pins onto glass slides, photolithography using pre-prepared masks or dynamic micro-mirror devices, ink-jet printing, and electrochemistry on microelectrode arrays. DNA probes can also be synthesized directly onto a microarray substrate (*in situ*) or attached (spotted) via surface engineering by a covalent bonding such as silane, lysine, or amide chemistry.

Applications of DNA microarrays include gene expression profiling, comparative genomic hybridization, GeneID, chromatin immunepreciptation on chip,

DamID, single nucleotide polymorphism (SNP) detection, exon (junction) arrays, fusion gene microarray, and tiling arrays.

7.2 Protein Microarray

A protein microarray (protein chip) is a high-throughput method used to track the interaction of large numbers of proteins in parallel, and to determine their function [151–157]. One critical disadvantage of DNA microarrays lies in the fact that the quantity of mRNA in the cell often does not reflect the expression level of the corresponding proteins because proteins – unlike DNA or mRNA – are functional in cell response. Protein microarrays have enabled research groups to study the biological interactions at the cell level. The protein technology was relatively easy to develop as it is based on previously developed DNA microarray technology. Similar to the DNA microarray, the chip consists of a support surface such as a glass slide, nitrocellulose membrane or bead, while the probe molecules are typically labeled with fluorescent dyes. Nowadays, protein microarrays have replaced cumbersome techniques such as two-dimensional gel electrophoresis or chromatography, which are not suited to the analysis of low-abundance proteins and are time-consuming and costly. Protein microarrays can be applied to proteomics, protein functional analysis, antibody characterization, disease treatment development such as antigen-specific therapies for autoimmunity, cancer, allergies, for diagnostics such as tests for antigen–antibody interaction, the discovery of new biomarkers, and the monitoring of disease states.

The surfaces of protein microarrays must meet the sophisticated requirements of immobilizing protein probes, notably to prevent protein denaturation and to provide a relevant surface polarity at which the binding reaction can occur. There is also a need to prevent the nonspecific binding of other proteins, and to minimize the creation of false signals from the background noise. Immobilizing agents vary from layers of inorganic aluminum or gold to organic polymers, polyacrylamide gels, or small functional moieties such as amines, aldehyde and epoxy. Occasionally, thin-film technologies such as physical vapor deposition (PVD) and chemical vapor deposition (CVD) are also used to apply the coating to the support surface. Protein array methods include ink-jetting, robotic spotting, piezoelectric spotting, a drop-on-demand, and photolithography [158–162]. The probe molecules may be antigens, antibodies, aptamers, protein-mimicking peptides, or full-length proteins. Recently, an *in-situ*, on-chip synthesis of proteins directly from DNA, using cell-free expression systems called DAPA (DNA array to protein array), PISA (protein in situ array) or NAPPA (nucleic acid programmable protein array), was introduced as the proteins in an arrayal surface are highly sensitive and easily deteriorate, whereas DNA molecules are more stable over time and better suited to long-term storage.

The detection methods employed included fluorescence labeling, as well as affinity, photochemical or radioisotope tagging. For label-free detection, SPR, carbon nanowire sensors (where detection occurs via changes in electronic conductance) and microelectromechanical system (MEMS) cantilevers can be used. However, these systems are ill-suited for high-throughput screening and need to undergo further development before their future use.

8 Conclusions

During recent years the field of biosensors has undergone an evolutionary phase with ever-increasing demands for efficient, sensitive and robust sensors in the fields of clinical diagnostics, medicine and drugs, process control, and environmental monitoring. In this chapter, attention has been focused on the most basic principles of biosensory sciences, and has hopefully proved valuable to the reader. Electrochemical biosensors are the most widely used sensing devices, given their high efficiency and ease of operation. Optical techniques such as SPR and SER have also found widespread use in research and development, while biosensors based on conducting polymers are beginning to open up a new field of hand-held biosensors that involve internal transduction mechanisms and can respond to analyte recognition through color changes. While acoustic resonance devices seem to be a good choice for bioaffinity sensors, with some noteworthy advances having been made in this field, thermal biosensors do not yet appear to have made any serious practical impact.

During the past decade, the development of biosensors has been greatly spurred by advancements made in the materials sciences. For example, nanostructured metal oxides have been shown to provide an effective immobilization of biomolecules with desired orientation and conformation, resulting in better sensing characteristics [163]. While silver and gold nanoparticles are widely used as electrochemical labels in amperometric immunoassays, metal quantum dots have been used as multilabels for affinity reactions [4]. Carbon nanotubes (CNTs) have also proved to be a material of choice for electrode fabrication, due to their semiconductive behavior and high porosity [164]. Indeed, amperometric biosensors comprising a CNT-modified electrode have shown an enhanced reactivity of NADH and hydrogen peroxide at the electrode [4]. Graphene, with its one atom-thick single graphitic layer, has attracted much scientific interest due to its unique physico-chemical properties such as high surface area and excellent thermal and electric conductivities. Given its excellent electron transport properties and high surface area, functionalized graphene is expected to help in the direct electron transfer between the electrode substrate and enzymes, and thus aid in the design of mediator-free biosensors with potentially better sensing parameters. Whilst conducting polymers have been used successfully as a material for the immobilization of biomolecules, as well as providing enhanced electron transfer properties, they have also been shown to function as stand-alone sensors as their emission properties are influenced by their molecular environment.

With the principles of biosensing and transduction mechanism having been well established, attention in the field of biosensors has now been focused on miniaturization, and this has resulted in smaller, more sensitive, and more easily affordable devices. Microchip technology has helped to concentrate electronic circuits onto a single chip through embedded ICs. However, in spite of the technological innovations and improvements, the miniaturization of these devices poses technical challenges as they lack sensitivity, long-term stability and robustness for their intended applications. Nonetheless, newer technologies such as silicon microsensors, fiber-optic biosensors and cell-on-chip sensors are increasingly being investigated [165].

Further developments in the field of biosensors will most likely be effected by the emergence of personalized medicine, as escalating healthcare costs continue to force the development of a new generation of wearable, integrated and less-invasive sensors [4]. In addition to healthcare and clinical diagnostics, industrial processes and environmental monitoring will also continue to press for more efficient, sensitive and robust biosensors. Moreover, with the increasing risks of biological and chemical warfare, security and biodefense will also require new, innovative and efficient biosensors to meet these challenges.

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