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New biosensors for microbiological analysis of food

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14.1 Introduction

In a recent review on chemical sensors[1] the guest editors A.B. Ellis and D.R. Walt wrote: '*Chemical sensing is a critical aspect of life. Our ability to sense the environment is essential for gustation, vision, reproduction, olfaction and auditory and tactile stimulation*'. This is particularly true in the area of food analysis and food technology where there is an increasing demand for simple, inexpensive and reliable tests to control food and food samples. Recently, highly publicised outbreaks of severe foodborne illnesses, most notably the recent outbreaks of bacterial infections, has underscored the importance of rigorously testing raw materials and finished food products for the presence of pathogenic bacteria. This increased awareness has prompted significant interest in the development of more effective methods for pathogen detection.[2]

The analysis of foods for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality. Conventional bacterial testing methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in food. These traditional methods are very sensitive and inexpensive, but require several days to generate results because they rely on the ability of microorganisms to multiply to produce visible colonies. For example, the current official method for enumerating salmonella bacteria in food (ISO/DIS 6579, 2001) involves a series of sequential cultural steps and is time-consuming, taking more than 4–5 days for the detection. In general these steps include pre-enrichment to allow the resuscitation and multiplication of sublethally damaged salmonella cells; selective enrichment to increase the ratio of salmonella to the competitor organisms; isolation on selective agar medium to enable the recognition of salmonella colonies while

suppressing the growth of the background microflora; followed by biochemical and serological confirmation if presumptive positive salmonella colonies develop.

Over the past decade, control of food safety has been carried out mainly by product testing rather than process control. The main problem with doing endproduct testing is the high number of samples to be examined before one can decide on the safety of the product batch, especially when pathogens are expected to be heterogeneously distributed in the batch. Moreover, endproduct testing detects only failures and does not identify causes. HACCP (Hazard Analysis Critical Control Point) is now generally accepted as the most effective system to ensure food safety.[3] With the implementation of the HACCP system, for control of the process line, the demand for rapid, sensitive and accurate methods to detect foodborne pathogens is increased. In particular, tests that can be completed within minutes or hours would enable processors to take quick corrective actions when pathogens are detected.[4]

Some new technologies are very sensitive but analysis time is lengthy. For example, the polymerase chain reaction (PCR) can be used to amplify small quantities of genetic material to determine the presence of bacteria. The PCR method is extremely sensitive, but requires pure samples and hours of processing and expertise in molecular biology.[5,6] Moreover this technique is centralised in large stationary laboratories because complex instrumentation is required. In recent years, intensive research has been undertaken to decentralise such tests so that they can be performed virtually anywhere and under field conditions.[7] Hence the development of portable, rapid and sensitive biosensor technology is well suited for this purpose.[8,9,10] The importance of biosensors results from their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex samples with a minimum of sample pre-treatment.[11–14] Biosensors for bacterial detection generally involve a biological recognition component such as receptors, nucleic acids or antibodies in intimate contact with an appropriate transducer.[15–18] Depending on the method of signal transduction, biosensors may be divided into five basic groups: electrochemical, optical, piezoelectric, thermal and magnetic. Several bioassays for pathogenic microorganism identification employ these transducers only as detectors in a biosensing device in which the biological materials are immobilised on a solid phase, but are not in intimate contact with the transducer.[19,20]

In this chapter we describe, firstly, the principles of the transducers used in biosensor technology, and then some new biosensors and biosensing devices developed for the detection of the most important pathogens in food (such as *Salmonella*, *E. coli*, *Staphylococcus aureus*), algal toxins, aflatoxin and some indicators of microbial spoilage. Also a brief description of the important area of DNA biosensors is included.

14.2 Transducers used in biosensors and immunosensors

Table 14.1 reports some examples of transducers commonly used in bio- and immunosensor construction and assembly. These transducers are amenable to the immobilisation of a biological component so that it is in intimate contact with the sensor. They must convert the selective biological event (catalytic or binding) into a directly displayed response or to a signal, which is then processed by a microprocessor.

Table 14.1 Transducers commonly used in biosensors

Transducer	Example
Electrochemical	
– Amperometric	Clark oxygen electrode, mediated electrode systems
– Potentiometric	Redox electrodes, ion-selective electrodes, field effect transistors, light addressable potentiometric sensors
– Conductimetric	Platinum or gold electrodes for the measurement of change in conductivity of the solution due to the generation of ions
Optical	Photodiodes, waveguide systems, integrated optical sensors
Acoustic	Piezoelectric crystals, surface acoustic wave devices
Calorimetric	Thermistor or thermopile

14.2.1 Electrochemical transduction[21]

Amperometric detection

Amperometry utilises the measurement of current at a fixed potential and classically involves a three-electrode system, although this is often reduced to two electrodes in practical devices. An applied potential between a working and reference electrode is chosen so that the species of interest is either oxidised or reduced at the working electrode. This causes a transfer of electrons resulting in a current, which is directly proportional to the concentration of analyte at the electrode surface over a wide dynamic range. Electrodes are commonly made of inert metals such as platinum or gold, or carbon, either in the form of graphite, glassy carbon or pyrolytic graphite, as solid or as a paste. These electrodes are commonly used to detect chemical compounds produced or consumed by catalytic or binding reactions.

Oxygen electrodes are based on a gold or platinum cathode, separated by an insulating material from a large silver anode. The two electrodes are housed in a plastic jacket with an electrolyte solution. Contact is made to external solutions (samples) only through a gas-permeable membrane. The cathode is pressed against the gas-permeable membrane to form a thin-film solution between the membrane and the tip of the electrode, which will lead to a rapid response. The cathode (working electrode) is kept at −700 mV versus the silver anode (reference electrode). The overall reaction which describes the reduction of oxygen to water is:

$$O_2 + 4H^+ + 4e \rightarrow 2H_2O$$

Because oxygen is involved in many reactions, a variety of biosensors based on oxygen consumption have been developed for the detection of bacteria or their metabolic products. A diagram of the oxygen electrode is shown in Fig. 14.1.

Hydrogen peroxide electrode

Hydrogen peroxide is the product of many biological processes and this has stimulated the development of amperometric probes for this analyte. Generally, hydrogen peroxide electrodes are constructed using a platinum anode polarised at about 0.6–0.7 V versus a silver/silver chloride electrode. To facilitate use directly in real samples, selectivity is often enhanced by means of a cellulose acetate membrane acting as a barrier towards species with relative molecular mass higher than 100 Daltons. At the platinum anode the hydrogen peroxide is oxidised according to the reaction:

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e$$

A diagram of the H_2O_2 electrode is shown in Fig. 14.2.

Amperometric probes with mediators

A significant extension of the utility of amperometric probes has been obtained by the use of electron transfer mediators. These are low molecular weight redox couples which shuttle electrons from the redox centre of an enzyme to the surface of an indicator electrode. As an example the flavin group of the enzyme glucose oxidase FAD reacts with glucose according to the reaction:

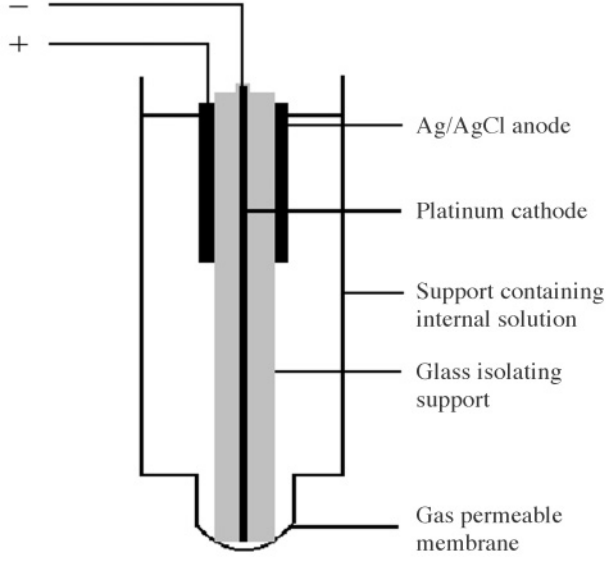


Fig. 14.1 Scheme of an oxygen electrode probe.

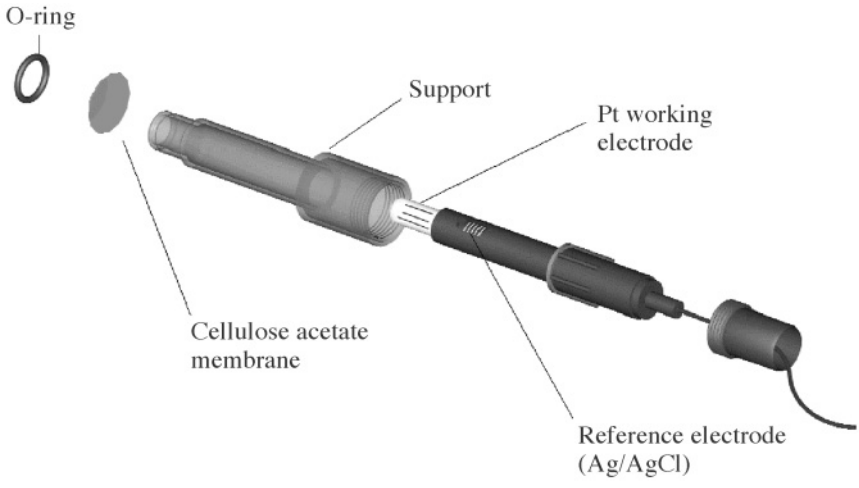


Fig. 14.2 Assembly of an H_2O_2 probe.

$$\text{glucose} + \text{FAD} + H_2O_2 \rightarrow \text{gluconic acid} + FADH_2$$

Normally *in vitro,* $FADH_2$ reacts with oxygen to reform FAD + H_2O_2, but if a mediator which competes with oxygen is present, the reaction proceeds as follows:

$$FADH_2 + \text{Mox} \rightarrow \text{FAD} + \text{Mred} + 2H^+$$

where Mox is the oxidised form of the mediator and Mred is its reduced form.

At the electrode surface the reduced form is reoxidised:

$$\text{M red} \rightarrow \text{M ox} + \text{ne}$$

It is necessary to select a mediator which does not react with oxygen in the reduced form if it is to be used in an amperometric sensor. One of the most successful classes of mediator compounds is based on ferrocene and its derivatives. These have been used for the construction of the first disposable glucose biosensor[22] and amperometric biosensors of this type now dominate the world of home blood glucose monitoring for people with diabetes.

Amperometric sensors based on NADH oxidation have also been proposed since this compound is a cofactor for more than 250 NAD-dependent dehydrogenase enzymes, according to the following reaction:

$$SH_2 + NAD^+ \rightarrow S + \text{NADH} + H^+$$

where SH_2 is the oxidation form of the enzymatic substrate and S is its reduced form. The NADH can be monitored through its reoxidation to NAD^+ at a suitable transducer. Graphite electrodes are the most frequently used transducers to monitor these reactions and to determine many compounds present in foods.

14.2.2 Optical transduction

The simplest form of optical transducer relies on optical fibres used as a waveguide to carry light to and from a reaction area.

Fibre optic sensors

Numerous types of fibre-optic chemical sensors can be useful as transducers to monitor catalytic reactions resulting either directly from the activity of microorganisms or to detect indicators or markers via an immobilised enzyme. Examples include the oxygen, pH, carbon dioxide and ammonia optodes.

Oxygen optodes

The feasibility of measuring oxygen partial pressure via the dynamic quenching of indicator fluorescence by molecular oxygen was first described by Kautsky and Hirsch in 1935.[23] The first oxygen sensor based on this principle was described by Lübbers and Opitz in 1975.[24] Pyrenebutyric acid (PBA) dissolved in dimethylformamide (DMF) was held in place between a quartz slide and a Teflon membrane. Oxygen from the analyte solution diffused through the Teflon membrane into the DMF to quench the fluorescence of PBA. The system was improved by dissolving the oxygen-sensitive dye into silicone rubber membranes to achieve mechanically stable sensing layers that were placed at the end of a fibre-optic probe as can be seen in Fig. 14.3. This optode was steam sterilisable. In a further refinement, oxygen measurements could be performed in the temperature range from 300 to 500 K.

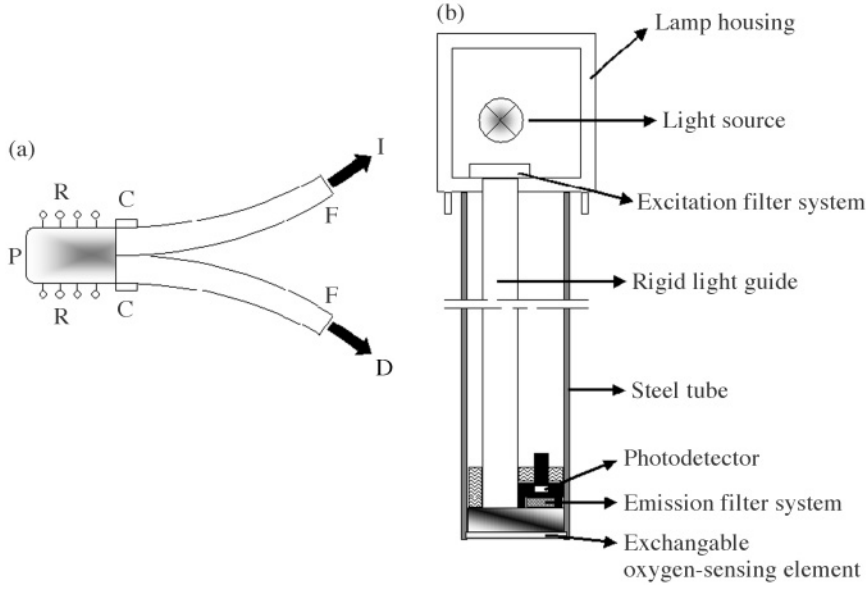


Fig. 14.3 (a) Schematic diagram of a fibre-optic chemical sensor. **F**: optical fibres, **C**: cladding, **I**: input radiation, **D**: detected readiation, **R**: reagent immobilised onto polymer, **P**: polymer grown on fibre tip. (b) Cross-section of an oxygen optode.

Limitations in the performance of these optodes include photobleaching of the dyes, the migration of the dye molecules from the silicone membrane into the analyte solution and the required excitation wavelength of 350–400 nm, which makes them incompatible with glass or plastic fibres. Using a metal-organic ruthenium complex as an oxygen indicator, some of these limitations were overcome. The resulting oxygen optode showed an excitation maximum at 460 nm, which makes possible the use of blue light-emitting diodes (LED) as light sources.

pH optodes

pH optodes are based on the changes in the optical properties of an acid/base indicator. This principle has been widely used in colorimetric titrations and in developing pH paper strips. A miniaturised fibre-optic pH sensor was described in 1982[25] in which phenol red was immobilised onto 10 μm polyacrylamide gel microspheres and packed into a cuprophane hollow fibre fixed on two plastic optical fibres. One fibre guided light to the immobilised indicator while the other guided, reflected and scattered light back to a detector. Hydrogen ions can pass through the cuprophane membrane and cause a change in the reflectivity of phenol red. Another type of miniaturised reflection-based pH optode was presented in 1987, which showed an enlarged dynamic range. Fluorescence-based pH optodes with a large dynamic range have also been reported.[26]

Optodes for carbon dioxide and ammonia

Almost all fibre-optic sensors for carbon dioxide are based on sensing pH changes in a buffer solution encapsulated in a gas-permeable membrane. The membrane also prevents ionic species, including protons, from entering the internal buffer. Gaseous carbon dioxide or ammonia diffuses through the membrane material and changes the pH of the internal buffer. This in turn causes a change in the optical properties of the dissolved indicator according to the following fully reversible reactions:

$$CO_2 + H_2 \rightleftharpoons OH^+ + HCO_3^-$$

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

To facilitate miniaturisation of the sensor, a fibre-optic carbon dioxide sensor without a gas permeable membrane has been described.[27] It consisted of a single fibre connected to a small capillary tube. The fluorophore (dichlorofluorescein) was fixed at the distal end of the fibre either by immobilisation onto a glass bead, which was glued to the fibre, or with a liquid plug of indicator/buffer solution brought into the capillary tube. The surface of the capillary was hydrophobic and a gas bubble at the tip of the capillary acted as a gas-permeable membrane to protect the pH indicator from ionic species. To avoid the need for both the internal buffer solution and the gas-permeable membrane, the pH-sensitive dye (fluorescein) was dispersed in polyethylene glycol and then incorporated in a membrane. This resulted in very short response times; for

example 10 sec to reach 90 per cent of the full response. Both these new sensor developments have limitations. The first suffers from photobleaching and the second is humidity sensitive because of the swelling of polyethylene glycol in water. Consequently this sensor can only be used to sense carbon dioxide at constant humidity.

Fibre-optic ammonia sensors are based on similar pH effects. In place of the internal bicarbonate buffer an ammonium chloride buffer is used. With carbon dioxide there is an increase in proton concentration, that is, a decrease in the pH of the internal buffer. With ammonia, hydroxyl anion is formed resulting in an increase in pH. Obviously the two gases would interfere if both were present in the same sample solution.

Surface plasmon resonance

An important optical transduction system used for affinity sensors is Surface Plasmon Resonance (SPR).[28] A surface plasmon is an electromagnetic wave which propagates along the surface of a metal. Optical excitation of a surface plasmon can be achieved if a light beam undergoes total internal reflection at the surface of a glass substrate onto which a thin metal film has been deposited. With the correct choice of metal, at the appropriate thickness, surface plasmon resonance is obtained at a certain angle of incidence of the light beam, which is observed as a sharp minimum of reflectance intensity. This minimum reflects the angle at which an evanescent wave can couple with the electron plasma of the metal. This angle is very sensitive to variations in the refractive index of the medium outside the metallised surface within a few hundred nanometres. Absorption of large molecules to biological receptors, such as antibodies, immobilised on the metal surface will change the local refractive index and therefore the resonant angle. The usual mode of operation is to measure the increase of reflected light as the system goes out of resonance. A diagram of an SPR system is shown in Fig. 14.4.

14.2.3 Piezoelectric/acoustic transducers

Piezoelectric transduction

Piezoelectric transducers[29] are conventionally used to measure small masses of material in applications such as the vacuum deposition of metals. However, they can be readily adapted to detect microorganisms by coating the active surface with the appropriate antibody. Piezoelectric materials are able to generate and transmit acoustic waves in a frequency dependent manner. The resonant frequency for optimal wave transmission is highly dependent on the surface of the crystal.

The deposition of foreign material on the surface changes the resonant frequency in air according to the Sauerbrey equation:

$$\Delta F/F = -\Delta m/Apt$$

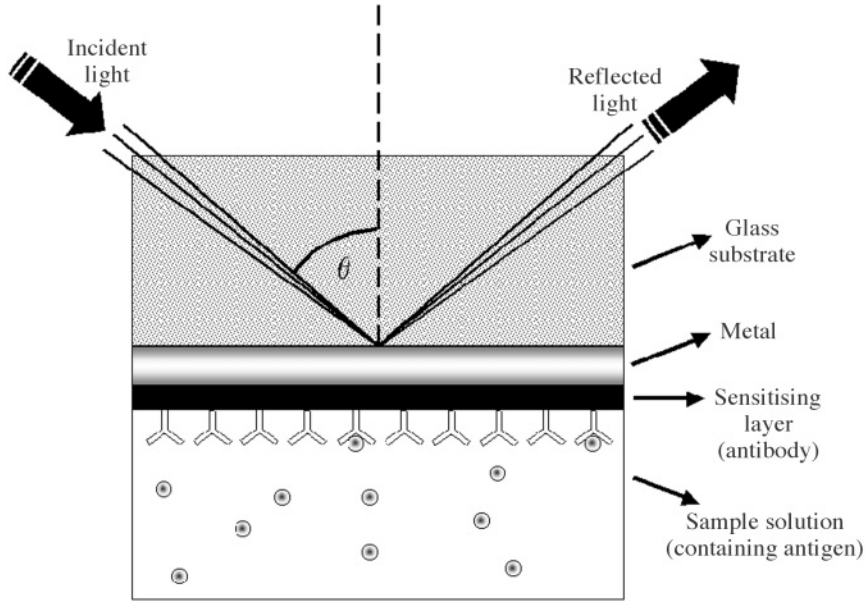


Fig. 14.4 Schematic diagram of the SPR (surface plasmon resonance) device for the measurement of minute changes in the refractive index. The device can be configured as a highly sensitive gas sensor or as an immunosensor by the immobilisation of sensitising layers on the device.

where: ΔF = frequency change (Hz), F = resonant frequency (Hz), Δm = surface mass change (g), A = crystal area covered by the adsorbed materials (cm^2), p = density of the crystal (g cm^{-3}), and t = thickness of the uncoated crystal (cm).

As can be seen, the basic concepts of piezoelectric transduction are relatively simple, but the use of piezoelectric devices in liquids is a relatively new concept, and the precise mechanism of operation is still not fully characterised. Moreover, the simple mass dependency which applies in gaseous phase operation does not apply and visco-elastic effects prevail. Piezoelectric instrumentation has been available for many years, and is not excessively expensive. Likewise, simple piezoelectric sensors are available at relatively low cost, but at present costs are still considerably higher than for electrochemical transducers.

Surface acoustic wave (SAW) devices

Rather than employing the bulk wave piezoelectric microbalance model as the basic transducer, an alternative approach is to use surface acoustic waves (SAW).[30] In SAW devices, two sets of interdigitated electrodes are deposited on a piezoelectric crystal surface as transmitter and receiver, respectively. Radiowave frequencies applied to the transmitter electrodes produce a synchronous mechanical stress in the crystal, which produces an acoustic wave

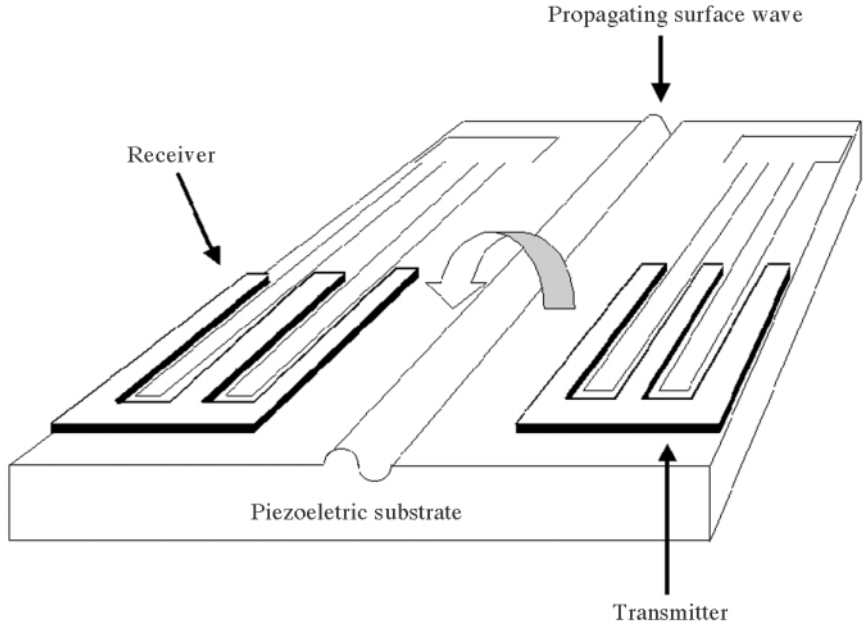


Fig. 14.5 Model of a surface acoustic wave (SAW) device.

with both longitudinal and vertical shear components. This Rayleigh surface wave (it was Lord Rayleigh who, at the end of the last century, theoretically treated acoustic wave propagation along the surface of a semi-infinite isotropic medium) propagates along the surface of the piezoelectric crystal and is received at the second set of electrodes, where mechanical vibrations are translated back to an electrode voltage (Fig. 14.5).

14.2.4 Calorimetric transduction[30]

The heat from exothermic reactions can be detected as a temperature change by one of the many types of conventional thermometers, thermistors or thermopiles (arrays of thermocouples). This approach provides a near-universal transducer since so many biological reactions are exothermic. An added attraction is that it is also independent of the properties of the sample being treated. A drawback is that external temperature fluctuations need to be shielded from the sensor. The conventional approach has involved adiabatic flow through, resulting in bulky, expensive systems. Recently, the use of advanced silicon microfabrication technology has improved the performance of thermal sensors such that smaller, less expensive devices can be made.

All the transducers described above are widely used in biosensor technology. Some examples of their application to sense the most important pathogens or their direct products in foods are reported in the following sections.

14.3 Biosensors used to detect *Salmonella*

A significant part of human infectious pathology is due to the consumption of foods carrying pathogenic bacteria and viruses. Among these *Salmonella* plays a primary role and is currently considered the most common cause of toxinfection in industrialised countries, where contamination is associated particularly with raw meat, poultry and eggs.[31,32] According to European legislation *Salmonella* must be absent in established amounts of food product (1 g or 25 g, depending on the kind of product). Since the control of salmonellosis requires action at all levels of the transmission chain according to the application of the HACCP system, there is a strong need for rapid, sensitive and reliable methods of detection. Numerous biosensors and biosensing systems based on electrochemical, optical and piezoelectric transducers have been developed to meet this need.[15,16,19,33-36] Only some examples are reported in this section.

In 1997, Ye *et al*. developed a piezoelectric Flow Injection Analysis (FIA) biosensor for the detection of *S*. Typhimurium.[15] This kind of biosensor has the potential to provide direct label-free detection of bacteria. Anti-salmonella antibody was immobilised onto a coated gold electrode on a quartz crystal surface using polyethylenimide-glutaraldehyde (PEG). The working principle of this piezoelectric biosensor is illustrated in Fig. 14.6; only salmonella cells bind to the specific antibodies and consequently the mass of the crystal increased while the frequency of oscillation decreased proportionally. Five different

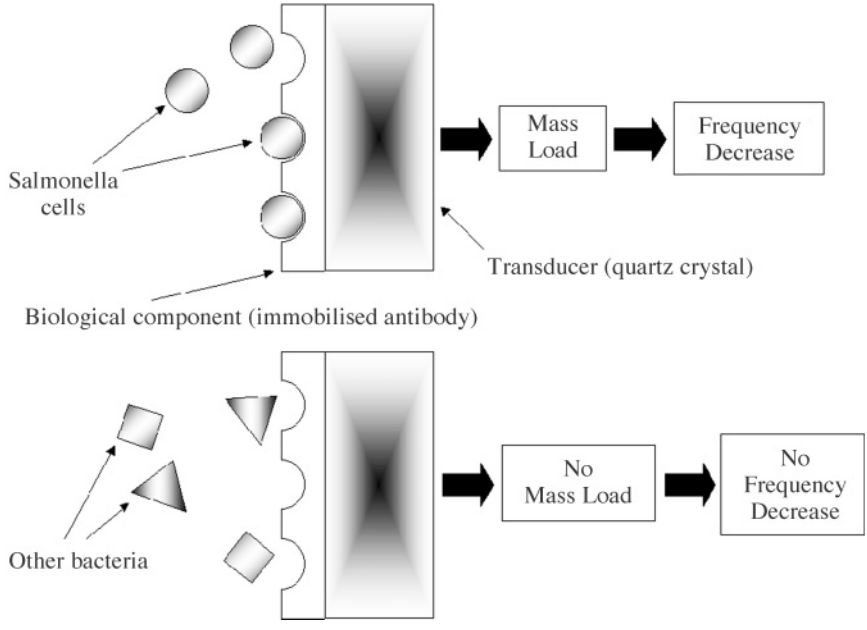


Fig. 14.6 Working principle of the salmonella piezoelectric biosensor: only salmonella cells bind to the specific antibodies (immobilised on the crystal surface) and consequently the mass of the crystal increased while the frequency of oscillation decreased.

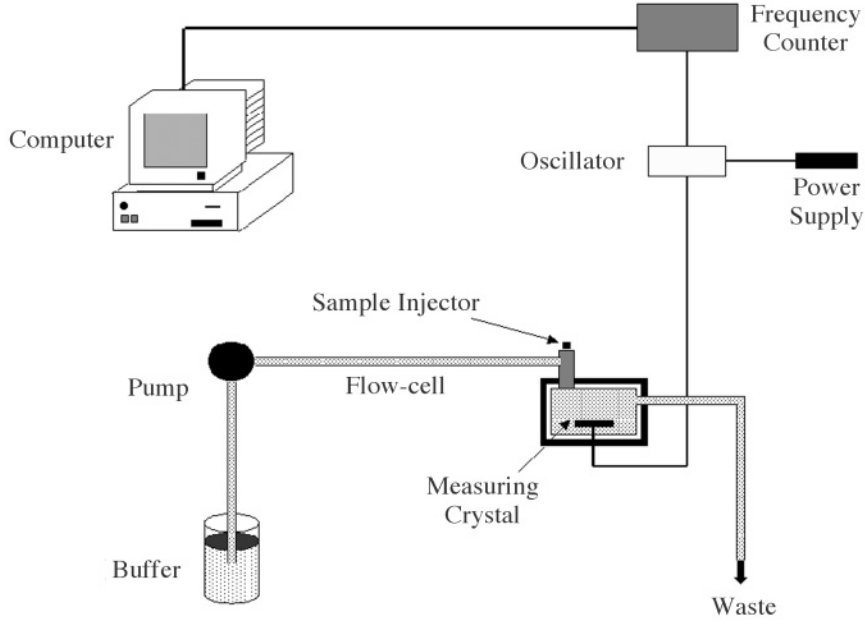


Fig. 14.7 Schematic diagram of the piezoelectric biosensor system.

sensing crystals and two reference crystals (treated as sensing crystals but without attaching the antibodies) were prepared for each salmonella concentration. The reference crystal was used at the beginning and end of each assay. The antibody coated crystal was inside a flow cell and 0.05 M phosphate buffer, pH 7.2 was pumped through for use as a FIA system (Fig. 14.7). The buffer flow rate was 0.15 mL/min. After stabilisation of the oscillation frequency, 60 μL of a *S*. Typhimurium cell suspension (10^5–10^9 cells/mL) was injected and pumped into the flow cell. To allow better interaction between antibodies and cells, the pump was stopped 1 min after injection and the frequency base-line was recorded as F1. After 25 min of antibody-cell interaction, another frequency was read as F2. The crystal was then replaced with a fresh crystal and the analysis repeated five times. The frequency decrease of the crystal was reported as $\Delta Fs(\Delta Fs = \mathrm{F1}s - \mathrm{F2}s)$ for the sensing biosensor or $\Delta Fr(\Delta Fs = \mathrm{F1}r - \mathrm{F2}r)$ for the reference. The average frequency decrease of reference crystals was < 5 Hz and was used as background signal. The actual frequency decrease ΔF was calculated by subtracting the background signal from the frequency change of the biosensor and the average decrease in frequency of five sensing crystals was related to the concentration of the injected *S.* Typhimurium cells. The biosensor had responses of 23–47 Hz in 25 min, with $R^2 > 0.94$ for *S*. Typhimurium concentrations of 5.3×10^5 cells/mL to 10^9 cells/mL.

More recently Babacan *et al.*[16] have developed a similar piezoelectric biosensor system for the detection of *S.* Typhimurium, in which specific

antibodies were immobilised onto the quartz crystal surface through a protein A procedure (to produce a better orientation of antibodies). The sensor had responses of 5 to 65 Hz in 30 min with $R^2 = 0.94$ for *S*. Typhimurium concentrations of 10^7 cells/mL to 10^9 cells/mL. Both piezoelectric FIA systems had poor sensitivity and were not applied to food samples. The major disadvantage of piezoelectric technology is the regeneration of the crystal surface. This last problem may not be important if small crystals can be manufactured at low cost so that disposable transducers are economically feasible.

Recently immunomagnetic beads (IMB) have been used in immuno-electrochemical assays for the detection of *S*. Typhimurium.[36] This technique combines the selectivity of antibody-coated superparamagnetic beads with the rapidity and sensitivity of electrochemical detection in a format termed enzyme-linked immunomagnetic electrochemistry (ELIME). Heat-killed *S*. Typhimurium were sandwiched between antibody-coated magnetic beads and an alkaline phosphatase-conjugated antibody. With the aid of a magnet, the beads were localised onto the surface of disposable graphite ink electrodes in a multi-well plate format. The multi-well electrode/magnet scheme is illustrated in Fig. 14.8. As shown, the individual strip electrodes are placed between a magnet and

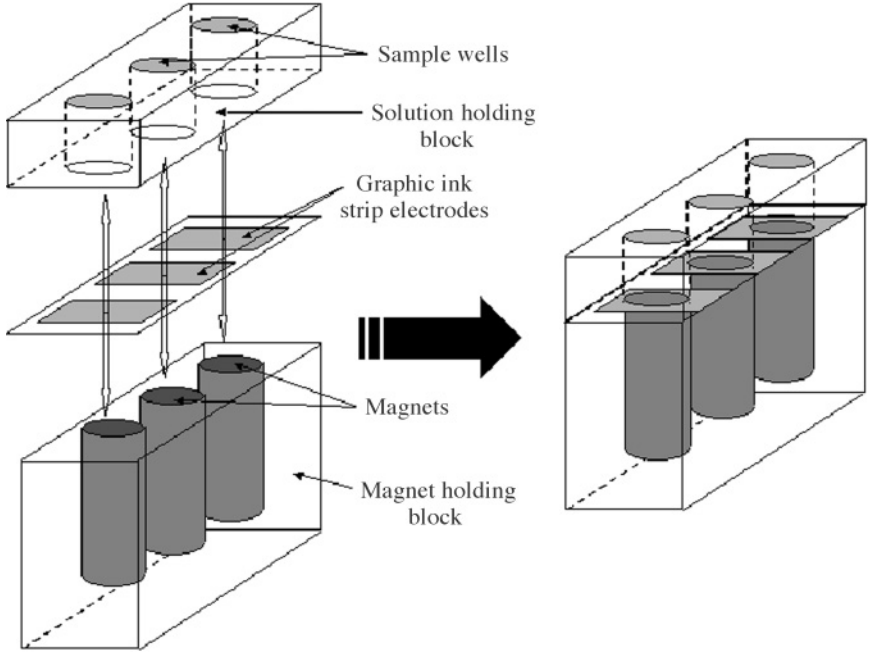


Fig. 14.8 Multi-well electrode/magnet assembly. The double-headed arrows indicate positioning of the solution and magnet underneath separate sample wells. The individual strip electrodes were effectively sandwiched between a magnet and a sample well with the graphite ink exposed to and forming the bottom of each sample well. Only three of the 12 sample wells are shown.

a sample well, with the graphite ink exposed to and forming the bottom of each sample well. Only three of the 12 sample wells are shown in the diagram.

The ELIME procedure was performed as follows: 20 μL of antibody-coated magnetic beads were placed in 1.5 mL polypropylene microcentrifuge tubes, 1 mL of heat-killed *S*. Typhimurium was added and the mixture was incubated by shaking for 30 min. The tubes were placed into the magnetic tube holder for 3 min in order to trap IMB against the wall of the tubes and the liquid was removed by aspiration. The IMB were resuspended by gentle vortexing and incubated by shaking with 1 mL of alkaline phosphatase-conjugated antibody anti-salmonella (diluted 1:500 in 1% blocker casein) for 30 min. The IMB were again separated using the magnetic tube holder for 3 min and the liquid was removed. After two washing steps the IMB were resuspended in 0.2 mL of TBS (tris-buffered saline) and transferred to the multi-well electrode/magnet. After 2 min of magnetic separation by application of the magnet holding block, the liquid was removed by aspiration and 200 μL of p-aminophenylphosphate (pAPP) was added to the wells and allowed to react for 3 min. Production of electroactive p-aminophenol was measured using square wave voltammetry. An Ag/AgCl electrode (0.6 cm $\times$ 7 cm) wrapped with a platinum wire that served as a counter electrode, was inserted into the test solutions during electrochemical measurements. The electrochemical response was directly proportional to the number of captured bacteria. The electrochemical detection of immunomagnetically captured bacteria is schematically represented in Fig. 14.9. This technique, with a minimum detectable level of 8×10^{-3} cells/mL of *S*. Typhimurium in buffer (achieved in ca. 80 min) proved to be very attractive for food analysis.

A rapid electrochemical ELISA coupled with an FIA system for the detection of different salmonella serotypes in meat samples has been proposed recently by Croci *et al*.[19] In this work a sandwich format was used with a microtitre plate as support for the immobilisation. Anti-mouse IgG was used as a precoating with mouse monoclonal antibodies, against boiled salmonella, as the coating. Salmonella standard solutions or treated samples reacted with rabbit polyclonal antibodies, specific for salmonella, conjugated with horseradish peroxidase. After addition of the substrate 3,3′,5,5′-tetramethylbenzidine, the activity of the label enzyme was measured by injecting the mixture from each well into an FIA system coupled with an electrochemical cell. This cell comprised a glassy carbon working electrode, a reference electrode (Ag/AgCl) and an auxiliary electrode (stainless steel). The working electrode was polarised at +100 versus Ag/AgCl. ELISA experiments carried out with different salmonella serotypes (*S*. Enteritidis, *S*. Derby and *S*. Typhimurium) and with bacteria (*E. coli*, *Klebsiella pneumoniae, Morganella morganii, Citrobacter freundii, Yersinia enterocolitica*) commonly present in food showed a good specificity for salmonella and no cross-reactivity with the other bacteria tested (Fig. 14.10). The following four-parameter logistic equation was used to fit the experimental data:

$$f(x) = \frac{a - d}{1 + (x/c)^b} + d$$

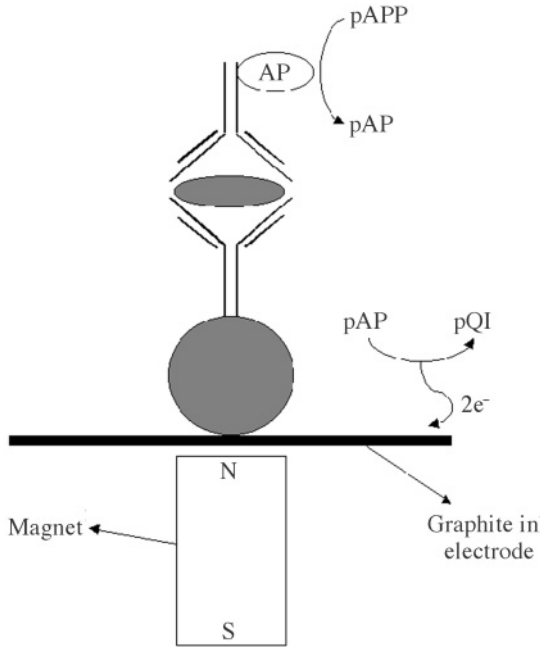


Fig. 14.9 Schematic representation of enzyme-linked immunomagnetic electrochemical assay (ELIME); pAPP = p-aminophenyl phosphate, pAP = p-amino phenol; pQI = p-quinone imine.

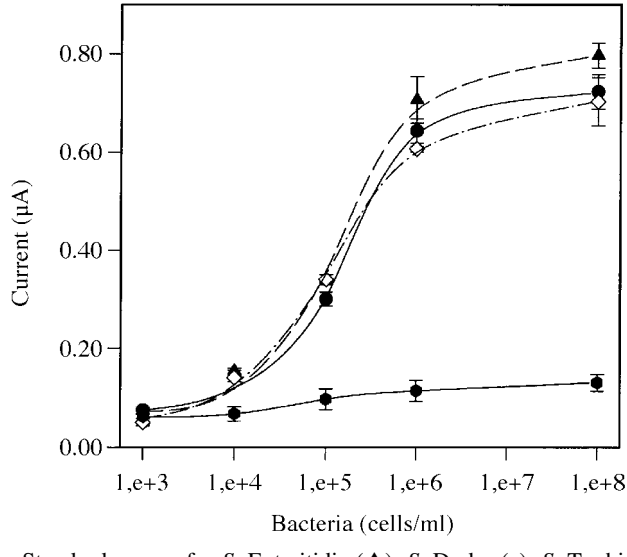
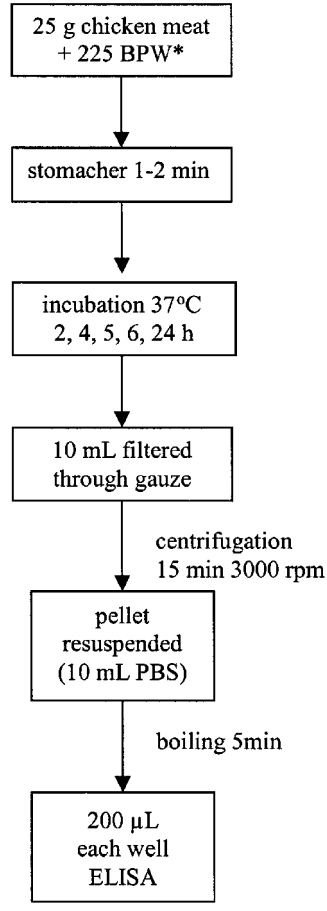


Fig. 14.10 Standard curves for *S.* Enteritidis (▲), *S.* Derby (•), *S.* Typhimurium (◇), other bacteria commonly present in foods (◇).



* BPW = buffered peptone water

Fig. 14.11 Meat sample treatment for *Salmonella* analysis.

where a and d are the asymptotic maximum and minimum values, respectively; c is the x value at the inflection point, b is the slope. Detection limits L.O.D., defined as the concentration corresponding to the $f(x)$ value obtained by adding three standard deviations of the zero point to the mean of the zero standard measurement (mean value + $3s$), was found to be 5×10^3 cells/ml.

The assay was then used to analyse samples of pork, chicken and beef experimentally contaminated, by spiking 25 g of meat with 1 ml of the salmonella standard solution (1–10 viable cells/ml), and samples not experimentally contaminated. Analysis were performed in parallel following the traditional culture method. All samples were treated according to the scheme reported in Fig. 14.11: 25 g of sample were homogenised with 225 ml of pre-enrichment broth (buffered peptone water) in a stomacher for 1–2 min and

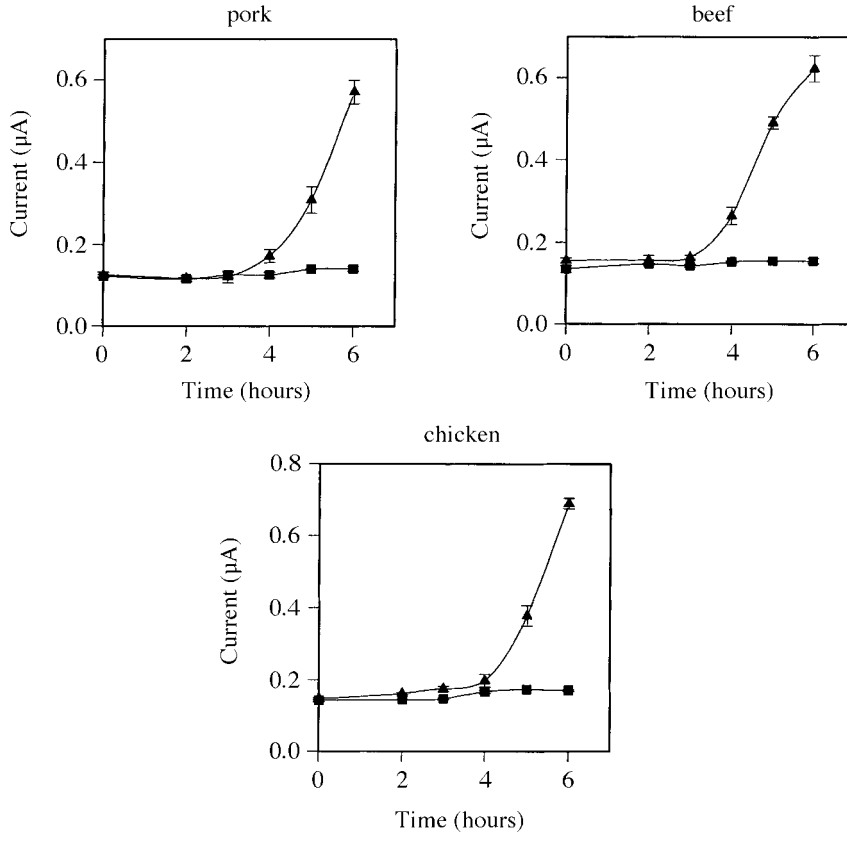


Fig. 14.12 Current measurements performed to reveal the presence of *Salmonella* on meat sample aliquots taken at different pre-enrichment times. Samples of pork, chicken and beef experimentally contaminated with *S.* Enteritidis (▲), same samples not contaminated (■).

incubated at 37ºC. Aliquots (10 ml) were taken at 2, 3, 4, 5, 6, 8 and 24 hours, filtered through gauze and centrifuged for 15 min at 3000 rpm. The supernatant was discarded and the pellet was resuspended in a final volume of 10 ml with PBS and boiled for 3 min; the volume was adjusted to the initial value (i.e. 10 ml) with PBS. All the aliquots were subjected to ELISA. The classic culture method was carried out in parallel only on the aliquots taken after 24 h of incubation, in accordance with the established procedure. The strains of salmonella isolated from the samples that were not contaminated experimentally were serologically typed in accordance with the slide-agglutination method, using commercial sera (Behring; Biogenetics). Figure 14.12 shows the results obtained when samples of meat experimentally contaminated with *S.* Enteritidis (1–10 viable cells/25 g) were analysed. As can be seen the minimum pre-enrichment time was variable, probably due to the concentration of competitor organisms naturally present in meat samples. However, five hours of pre-

enrichment were sufficient to reveal the presence of salmonella. The classic culture method confirmed the results.

Finally, only two of the ten samples of meat (pork, chicken and beef) not experimentally contaminated tested positive for salmonella with both the classic cultural method and the ELISA. Using slide-agglutination method the two strains were isolated and identified as *S.* Infantis and *S.* Anatum. The isolation of *S.* Infantis and *S.* Anatum demonstrated the efficacy of the ELISA method in revealing serotypes other than those expressly assayed.

The above features of the proposed method make it a reliable substitute for the standard procedure, particularly for testing products with a brief shelf-life or in emergency situations. The simplicity of the method, which requires no special or costly equipment and permits the simultaneous testing of numerous samples, also suggests its suitability for inclusion among the HACCP to meet food processing concerns.

14.4 Biosensors used to detect *Staphylococcus aureus*

Staphylococcus aureus is one of several bacteria causing foodborne disease outbreaks around the world. It can produce several types of enterotoxins (A, B, C, D and E) that cause gastroenteritis.[37] Therefore, the presence of the bacterium in processed food is a health hazard if the food is held at a temperature that allows growth, which permits synthesis of enterotoxins. Protein A is a cell wall constituent and it is covalently linked to the peptidoglycan structure of the bacterium. Because 99 per cent of S. *aureus* strains have this protein on the cell wall, several immunosensors have been developed using protein A as antigen for the detection of S. *aureus*. A goat-anti-human IgG modified piezoimmunosensor for S. *aureus* was reported in the literature.[18] The system consists of an oscillator, a frequency counter, a special cell and a modified piezoelectric crystal. Goat-anti-human IgG were immobilised at the surface of the crystal modified with PEG technique; the Fc portion of the IgG binds protein A with high affinity and specificity, consequently the mass of the crystal increases while the frequency of oscillation decreases proportionally. The frequency shift was correlated with a S. *aureus* concentration in the range 5×10^5–10^8 cells/mL. Because it is considered problematical to dissociate the bound analyte from the coated crystal (the antibody-antigen interaction is usually very strong), four methods of regeneration were tested in order to renew the coated crystal. The authors found that when the crystal was treated with urea (4 M) and IgG (10 mg/mL), no detectable loss of sensitivity was observed over a month when it was stored at +4ºC.

In 1996, an evanescent waves fibre-optic sensor was developed for the detection of protein A.[38] In this immunosensor, a 40 mV argon-ion laser that generated laser light at 488 nm was used together with plastic optical fibre, and antibodies to protein A were physically adsorbed onto the fibre. The principle of detection involved a sandwich immunoassay with fluorescein isothiocynate

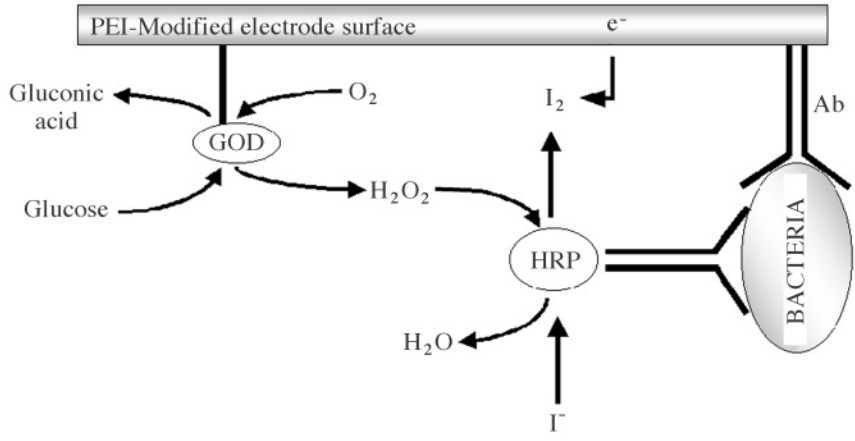


Fig. 14.13 Separation-free, amperometric enzyme-channelling immunoassay for *Staphylococcus aureus* with immobilised antibody and glucose oxidase (GOD) on the PEI-modified electrode surface.

conjugated with anti-(protein A) immunoglobulin G to produce signals of the antigen-antibody reaction. This sensor, applied only for the detection of protein A (with a detection limit of 1 ng/mL), could be suitable for food analysis.

A 'pseudo-homogeneous' amperometric immunoassay (without a washing step) was also developed for S. *aureus*, via protein A as antigen,[17,39] and its configuration is presented in Fig. 14.13. Anti-protein A IgG and glucose oxidase enzyme (GOD) are co-immobilised on a PEI (polyethylenimine) modified surface of a disposable graphite electrode in cylindrical form. The electrode was dipped into a solution containing glucose, iodide ions, and S. *aureus* cells, which in turn were bound to peroxidase (HRP) labelled anti-protein A antibodies. On the electrode surface, the GOD catalysed the oxidation of glucose to H_2O_2, and the iodine produced in the peroxidase-catalysed H_2O_2/iodide redox system was monitored amperometrically by the electrochemical reduction of iodine back to iodide. The immunosensor enabled preferential measurement of surface-bound conjugate relative to the excess enzyme-labelled reagent in the bulk solution. S. *aureus* cells were detected in pure culture at concentrations as low as 1000 cells/mL in a short assay time of 30 min.

14.5 Biosensors used to detect *Escherichia coli*

E. coli is an example of one of the most dangerous foodborne pathogens and is a typical inhabitant of the human intestinal tract which also can be a causative agent of intestinal and extra intestinal infections.[40] Certain strains of *E. coli*, such as the O157:H7, produce large quantities of a potent toxin that forms in the lining of the intestine and causes severe damage to it, resulting in haemorrhagic colitis or haemolytic uremic syndrome and may lead to death especially in

children.[41] These bacteria can easily contaminate ground beef, raw milk and chicken and water, therefore its careful control is extremely important, especially in the fields of food production and water supply. Conventional microbiological methods achieve sensitive and selective *E. coli* detection, but require days to a week to yield a result. Recently, a large number of novel techniques have been under development for a rapid assay of *E. coli*.[42–51] Only a selection of these biosensors and biosensing systems are described in this section.

Piezoelectric immunosensors based on the quartz crystal microbalance principle have been widely reported for detecting bacteria. However, antibodies are often difficult to acquire and the relation between the frequency shift and mass change does not always follow Sauerbrey's equation, and the viscosity effect cannot be neglected if the coating is not rigid enough. Further, the detection range of the method is narrow because the mass of the bacteria is usually small. Recently, a new type of PQC sensor, the series piezoelectric quartz crystal sensor (SPQC) has been introduced; it is based on the conductivity and permittivity response of the PQC and its frequency properties have been described.[52–54] This approach has greatly improved the stability and selectivity of normal piezoelectric quartz crystal sensors. It also offers the advantage of higher selectivity over classical conductivity. All these advantages make the SPQC device attractive for food tests and other applications.

A new method based on gelation of tachypleus amebocyte lysate for detection of *E. coli*, using a series piezoelectric quartz crystal sensor (SPQC), has been reported in the literature.[48] This technique depended on the change of conductivity and permittivity during the course of gelification caused by endotoxin, a constituent of the cell wall of *E. coli*. For the SPQC based on gelation of tachypleus amebocyte lysate (TAL), a well type detection cell, which consisted of two opposed Pt electrodes, was connected in series with a 9 MHz AT-cut quartz crystal and inserted in the circuit shown in Fig. 14.14(a). The cell was placed in a thermostatted water jacket at 37ºC. A diagram of the detection cell system is shown in Fig. 14.14(b). The assay was tested using series dilution of an initial *E. coli* suspension. After being heated at 60ºC for 30 min, 0.2 mL of the standard solution was incubated at 37ºC and added to dissolved TAL. When the mixture was injected into the detection cell, the conductivity and permittivity of the liquid changed and the variation of the oscillation frequency of the SPQC was recorded. In summary, the detection of *E. coli* with the SPQC-TAL method proposed in this work was based mainly on the change of conductivity and permittivity that occurs when TAL is mixed with the heated *E. coli* solution. In fact the endotoxin in the cell wall of *E. coli* is released when *E. coli* is dead, and the resonance frequency changes correspondingly. Therefore, the frequency change is related to the initial number of the bacteria. Results showed that the frequency shift was linearly related to the logarithm of the *E. coli* concentration in the range of 2.1×10^4 to 2.1×10^8 cells/mL.

In 2001, Gau *et al*.[9] reported a system for amperometric detection of *E. coli* based on the integration of microelectromechanical system (MEMS), self-

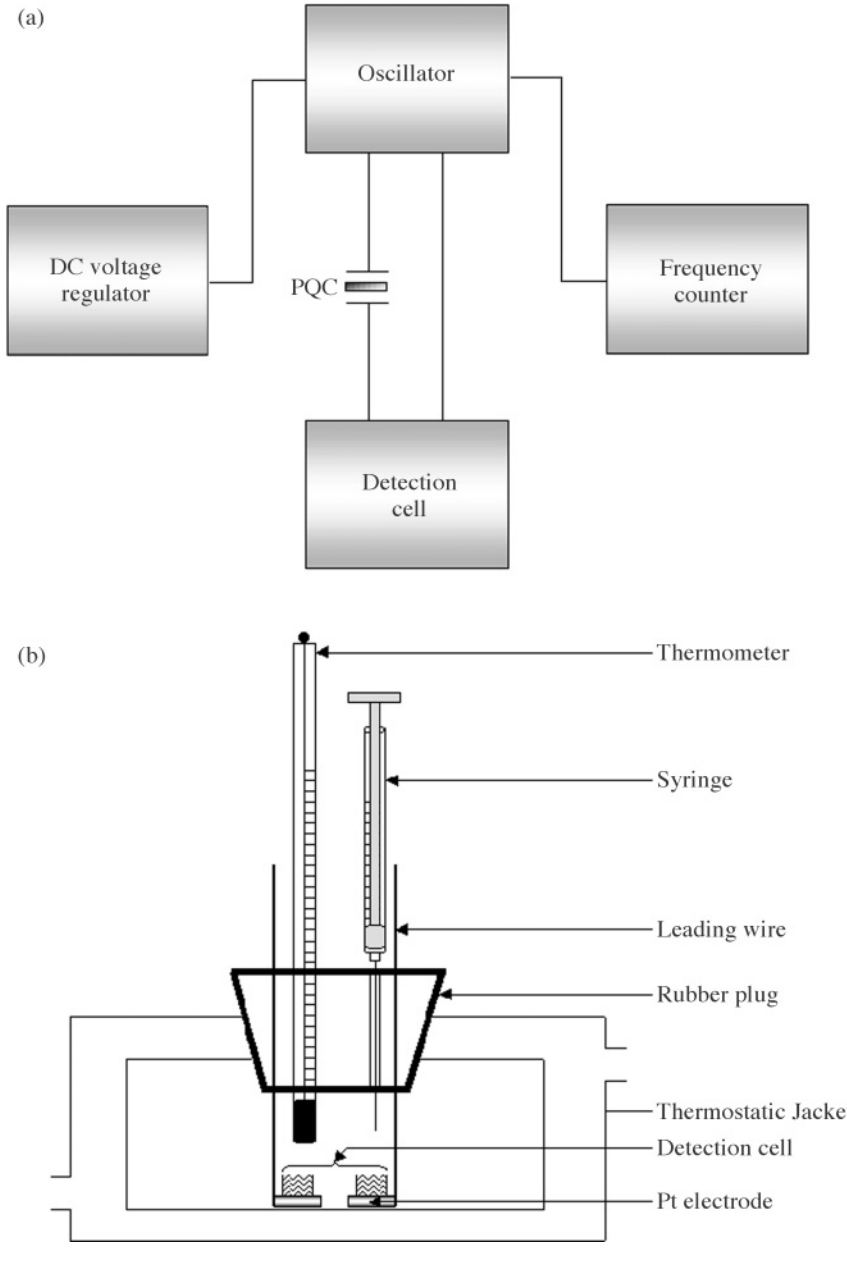


Fig. 14.14 (a) Schematic diagram of the SPQC system for detection of *E. coli*: the piezoelectric quartz crystal (PQC) is connected in series with the detection cell, the oscillator drives the crystal at its resonance frequency, and the oscillation signal was fed to a universal frequency counter. (b) Scheme of the detection cell used in the SPQC system.

assembled monolayers (SAMs), DNA hybridisation and enzyme amplification. Microelectromechanical system (MEMS) technology provides transducers to perform sensing and actuation in various engineering applications. MEMS devices are fabricated through the process of micromachining, a batch production process employing lithography. Micromachining is based on the use of lithographic methods to create three-dimensional structures using pre-designed resist patterns (or masks) and then selectively etching the undesirable parts away.[55,56] Using lithography, thin films of a wide range of materials, including metals (Au, Ag) and carbon, can be accurately patterned in μm size dimensions. SAMs is an elegant method of selectively immobilising molecules on MEMS surfaces.[57,58] Using these technologies, proteins and other biomolecules can be easily immobilised onto surfaces such as Au.[59,60] Hybridisation is one of the most effective means to detect bacteria with a high specificity. By choosing a single-stranded DNA (ssDNA) probe whose sequence is complementary only to the target bacteria's rRNA or ssDNA, the hybridisation event allows selective sensing of target cells. To maximise sensitivity, the hybridisation event can be coupled with an enzymatic reaction that leads to signal amplification. Bioassays to detect DNA hybridisation that are amplified by enzymatic reactions can still be completed within a reasonably short time.

Using MEMS technology, a detector array was fabricated. Sixteen working electrodes with their corresponding auxiliary and reference electrodes were patterned in a 2.8×2.8 cm^2 area of an Si wafer. A monolayer of the protein streptavidin was immobilised on the working electrode (Au) surface by using three different methods:

1. directly adsorbing streptavidin on Au
2. depositing a SAM of a biotinylated thiol, using biotin-DAD-C12-SH (12-mercapto(8-biotinamide-3,6-dioxaoctyl)dodecanamide) and subsequently binding streptavidin
3. depositing a SAM of biotinylated disulfide, and subsequently binding streptavidin.

The biotinylated thiol approach (Fig. 14.15(a)) yielded the best results. The assay protocol for amperometric detection of *E. coli* was conducted as follows: 50 μL of lysis reagent was added to a 250 μL sample of bacteria in culture media and incubated for 5 min at room temperature; 100 μL of probe solution (containing ssDNA complementary to one extremity of the rRNA of *E. coli* and ssDNA conjugate to fluoresceine complementary to the other extremity) was then added and the mixture was incubated for 10 min at 65ºC. 5 μL of the lysis *E. coli*/probe solution mixture was placed on the streptavidin coated working electrode of the MEMS detector array and incubated for 10 min at room temperature. After a washing step, 5 μL of anti-fluoresceine antibody conjugated with peroxidase was placed on the working electrode and incubated for 10 min (format scheme is reported in Fig. 14.15(b)). Following washing, 10 μL of a mixture containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 was placed

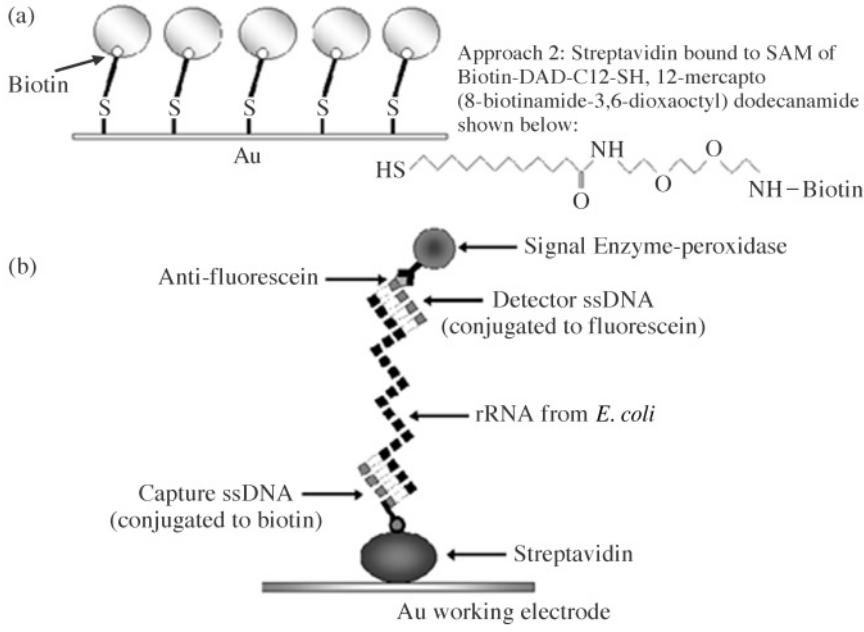


Fig. 14.15 (a) Approach used to immobilise streptavidin into Au. (b) Schematic of electrode surface upon signal detection.

on the detector array in such a way that all three electrodes (working, reference, counter) were covered by the substrate solution. The entire protocol was completed within 40 min. Amperometric current *versus* time (chrono-amperometry technique) was measured at a fixed potential (−100 mV) vs. reference electrode. At 20 s, current values reached a steady state. The detection system was capable of detecting 1000 *E. coli* cells without the use of the polymerase chain reaction for amplification. The detector array was fully reusable by cleaning the surface of the working electrodes with a mixture of H_2SO_4/H_2O_2.

Recently, Hamid *et al.* have developed a flow-injection amperometric immunofiltration assay system for the rapid detection of *E. coli*.[44,61] The method is based on the use of disposable porous filter nylon membranes which act as support for the immobilisation of anti-*E. coli* antibodies. These membranes were placed on the working electrode surface of a specially designed flow-through amperometric sensor which was inserted in a flow-injection system. As shown in Fig. 14.16, the immunosensor consisted of a disposable antibody-modified filter membrane resting on top of a hollow carbon rod, which acts as the working electrode. Another hollow carbon rod acts as a counter electrode and a hollow Ag/AgCl disk as a reference electrode. Therefore, in this design, the liquid (pumped by a peristaltic pump) flows from the inlet of the immunosensors, through the filter membrane and then through the hollow channel formed in the working, reference and counter electrodes, respectively. A sandwich

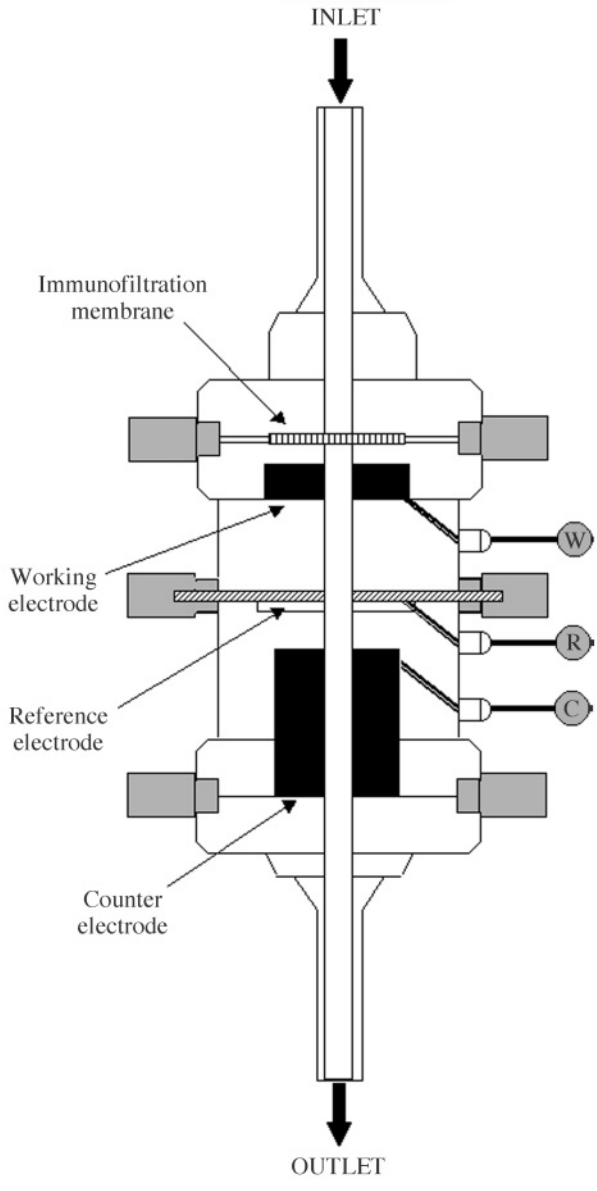


Fig. 14.16 Schematic of the flow-injection immunosensor.

immunoassay was employed with all steps performed at a fixed flow rate of 120 μL/min. An optimised volume (1 mL) of *E. coli* cells (suspension) was loaded into the injection loop and then injected into the inlet of the immunosensor. This was followed by washing with an appropriate solution. Then the same volume (1 mL) of a solution of anti-*E. coli* antibodies conjugated

to peroxidase, was loaded and injected. After washing, the activity of the peroxidase label (captured on the membrane surface) was measured using an amperometric technique with iodide-ions and H_2O_2 in a phosphate buffer. This system combined the immunofiltration technique, which eliminated diffusional limitation of the bacterial cells towards the antibodies and concentrated bacteria on the membrane (by filtering a large volume of sample), with amperometric detection delivering an impressive lower detection limit of 100 *E. coli* cells/mL.

14.6 Biosensors used to detect algal toxins and aflatoxin

Filter-feeding molluscs such as clams, oysters and mussels can become toxic to humans during the so-called 'red tides'. The phenomenon of 'red tide' is caused by the fast growth of a kind of microscopic and single-celled alga, which is usually not harmful. Unfortunately, a small number of species (HAB = harmful algae) produce potent toxins that can be transferred throughout the food chain, affecting and even killing zooplankton, shellfish, and eventually humans that feed on them either directly or indirectly.

There are four human illnesses associated with shellfish and toxic blooms: paralytic (PSP), neurotoxic (NSP), amnesic (ASP) and diarrhoeic (DSP) shellfish poisonings. Their occurrence is extremely rare. However, regulations are imposed to effectively protect the consumers from shellfish toxins. The toxins of major interest are non-protein molecules such as saxitoxin (STX) acid and domoic acid (DA). These molecules cannot be destroyed by normal cooking, freezing or smoking.

Another important phenomenon of human intoxication considers the presence of mycotoxin in food. In fact, milk is susceptible to contamination from external sources and one key analyte is aflatoxin M1.[62] This hepatocarcinogenic mycotoxin could occur in milk of cows fed with aflatoxin B1-contaminated feedstuffs. Acute effects by ingestion or inhalation of aflatoxin are primarily observed in liver damage, while chronic exposure to aflatoxin often leads to liver cancer.

The maximum content of AFM1 in milk, according with EC Directives,[63] is as low as 50 ppt, and this level of AFM1 can be controlled only by accurate and sensitive methods of analysis. The methods most widely used for the assay of naturally occurring toxins are high performance liquid chromatography (HPLC) and mouse time to death bioassay (MBA), but these methods are slow, expensive and not sufficiently rugged for routine use or for analysis in the field. The need for rapid, on-site determination of seafood poisons favours the development of biosensors in this area. In this section, new analytical procedures based on the use of electrochemical disposable immunosensors for the detection of seafood toxins are shown. Preliminary, spectrophotometric and electrochemical enzyme-linked immunosorbent assays (ELISA) have been optimised for the determination of toxins. Then, disposable immunosensors for the measurement of these toxins have been assembled using specific antibodies.

Immunoassay techniques are based on the ability of the antibodies to form complexes with corresponding antigens. The property of highly specific molecular recognition of antigens by antibodies leads to highly selective assays based on immune principles. The extreme affinity of antigen-antibody interactions results also in a great sensivity of immunoassay methods. There are different schemes of ELISA and the most popular is the competitive (direct and indirect) binding immunoassay method.[64] Direct competitive assays are based on a competition between antigens labelled (added) with enzyme and unlabelled (sample) for binding sites of antibodies, immobilised on the support, while in an indirect format the competition occurs between free and immobilised antigen versus the binding sites on the antibody labelled with enzyme. The amount of labels associated with the solid phase is inversely related to the concentration of antigen.

Electroanalytical immunosensors provide an exciting and achievable opportunity to perform food analyses away from a centralised laboratory. The most common disposable biosensors are those produced by thick-film technology. Particular attention was directed toward the screen printed electrodes (SPE), because they can combine the ease of use and portability with simple and inexpensive fabrication techniques. The modest cost of SPEs has further enhanced their desirability because it allows the device to became disposable and the development of electrochemical disposable immunosensors showed advantages in terms of sensitivity, rapidity and cost-effectiveness compared with previous analytical methods, such as MBA and HPLC, and were particularly useful for rapid screening tests. Disposable electrochemical immunosensors can be coupled with a portable instrument for the detection of seafood toxins.

14.6.1 Saxitoxin detection

Saxitoxin is one of the most lethal non protein toxins known (LD_{50} 9 μg/kg)[65] and is one of the 'Paralytic Shellfish Poisons' (PSP), produced by several marine dinoflagellates and freshwater algae. Contamination of shellfish with saxitoxin has been associated with harmful algal blooms throughout the world. According to the US Food and Drug Administration, the maximum acceptable level for paralytic poison in fresh, frozen or tinned shellfish is up to 400 mouse units (MU) or about 40–80 μg/100 g edible portion.[66] This value is equivalent to twice the minimum detection level of the mouse bioassay, the first and still most common PSP toxin testing method, which is also the official AOAC method.[67]

An electrochemical enzyme immunoassay for STX has been performed using the carbon working electrode of a disposable sensor as the solid phase for reagent immobilisation and as the signal transducer. This immunosensor was employed in a direct competitive assay involving STX labelled with antibody.[68] The used enzyme substrate was 3,3′,5,5′-tetramethylbenzidine (TMB) plus hydrogen peroxide and the product of the reaction was detected by chrono-amperometry at −100 mV for 60 s. The calibration curve for STX was carried

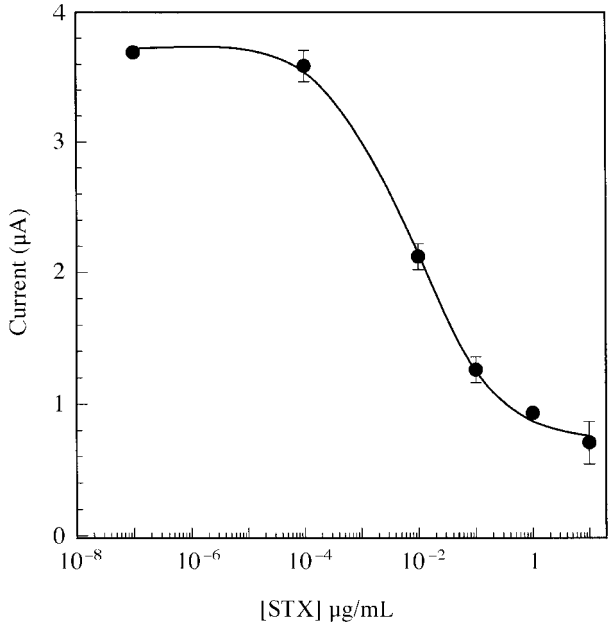


Fig. 14.17 Direct competitive curve for saxitoxin using SPEs. Antibody against STX (10 μg/mL in 50 mM carbonate buffer pH 9.6) was coated on the SPE working electrode after a pre-coating step with 2% PEI; STX-HRP (1:5000 v/v) was used as competitor.

out in the concentration range 0–10 μg/mL; results showed a working range from 10^{-4} to 1 μg/mL (Fig. 14.17).

Domoic acid

Domoic acid (DA) is a marine toxin (produced by phytoplankton species, *Nitzschia pungens*) and the main toxic agent associated with incidents of amnesic shellfish poisoning (ASP) on the east and west coasts of North America. Reliable methods for the analysis of DA and its isomers in seafood products are vitally important for the protection of the public.

A disposable immunosensor,[69,70] based on a SPE coated with BSA-DA, has been prepared for measuring DA in mussel tissue according to Kreuzer,[10] using PVA 1% (polyvinyl chloride) as blocking solution. Several dilutions of DA and fixed concentration of polyclonal antibodies against DA were prepared, followed by an excess of α-sheep IgG-AP and SPEs' measurement using DPV (differential pulse voltammetry). Once these conditions were optimised, indirect competitive analysis on SPEs was developed. A typical assay can be seen in Fig. 14.18, with an approach to the maximum signal seen at low DA concentrations. The insert of this figure shows a linear range between 5×10^{-3} and 0.1 μg/mL DA with an accompanying regression coefficient of 0.997. Errors associated with each standard were below 5 per cent ($n = 3$).

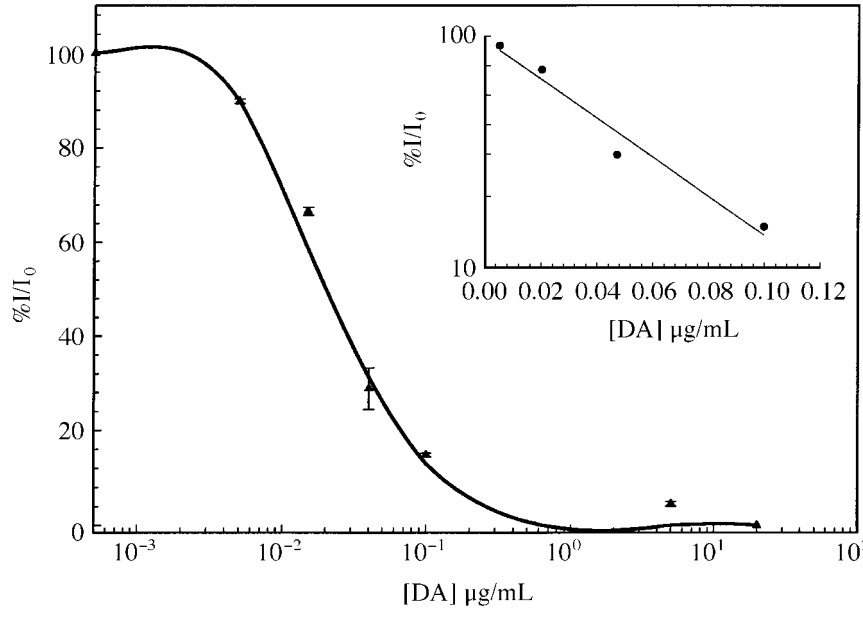


Fig. 14.18 Indirect competitive curve for domoic acid using SPEs. BSA-DA (30 μg/mL in 50 mM carbonate buffer pH 9.6) was coated on the SPE working electrode; dilutions at 1:500 v/v and 1:100 v/v were used for the primary antibody (specific for STX) and the secondary antibody (Ab_2-AP) respectively. Insert shows a linear range between 5×10^{-3} and 0.1 μg ml^{-1} of domoic acid.

The immunosensors were then applied to mussels. Mussel samples were collected and the extraction procedure performed according to Garthwaite.[71] The extraction efficiency was evaluated by the comparison of calibration curves constructed by spiking blank mussels with known amounts of DA before and after the extraction. Results of the recovery percentage ([DA] found/[DA] added $\times$ 100) for the artificial contamination mussel samples ranged from 85 to 101 per cent (Table 14.2). Repeatability and accuracy of the immunosensors were

Table 14.2 Recovery percentage obtained by spiking mussel blank with different amounts of DA

	Competitive ELISA		
DA added μg/g	DA found μg/g	RSD ($n = 6$)	% Recovery
40	34	8	85
20	16	9	80
50	9	6	90
5	5	4	100
2.5	2.8	7	111

evaluated by means of six replicates of tissue from mussels bought in different days and stores. A small portable instrument (calculator size) microprocessor controlled with LCD display and easy to use, was constructed for toxin measurement with disposable strips. This small instrument was able to perform DPV and chronoamperometric measurements in an interval range between −1000 and 1000 mV, useful for the determination of 1-naphthol which is the product of the reaction of AP with 1-naphthyl phosphate. The same experiments reported above for DA (using conventional instrument Autolab) were also carried out with the portable instrument obtaining similar results.[72]

Okadaic acid

Okadaic acid (OA) and its structural homologous are the toxins responsible for most human diarrhetic shellfish poisoning (DSP)-related illnesses. The acid was first isolated from two sponges, namely *Halichondria okadai* and *H. melanodocia* and subsequently found in the dinoflagellates *Prorocentrum lima*[73] and *Dinophysis spp.*[74] OA is a cyclic fatty acid (C_{38}) whose structure was first discovered by Tachibana.[75] Acute toxicity of various toxins from the OA group after intraperitoneal injection in mice[76] was 200 μg/kg of mouse.

An immunosensor for the detection of okadaic acid using a quartz crystal microbalance (QCM) was developed and optimised in standard solution.[77] Several coupling techniques, protein A, protein G and polyethylenimine (PEI) with glutaraldehyde (GA) cross-linking, were investigated for the determination of okadaic acid and a very good result was obtained with PEI coupling. The crystal surface was modified with polymer PEI and the free amino groups obtained were activated by GA. OA-BSA conjugate was then bound to the activated derivatives to form a cross-linked complex, which strongly attached to the gold surface of the crystal, resulting in an excellent storage lifetime of 38 days. The sensor, based on the competition between OA-BSA and free OA for the specific antibodies, showed an unsatisfactory detection limit and sensitivity. For this reason, an antibody-BSA hydrogel was adopted to improve the performance of the piezoelectric immunosensor.

The difficulty of raising antibodies against toxins and the continuing trend to reduce the use of animals for antibody production has stimulated research and development of synthetic receptors for toxins. In the past few years, molecular imprinting polymer (MIP) has been considered as one of the simplest, most straightforward, and cost-effective methods to develop artificial receptors for toxic organic compounds such as microcystins, toxins produced by aquatic microorganisms.[78]

14.6.2 *Aflatoxin M1*

A disposable electrochemical immunosensor for AFM1, which can combine the high selectivity of the immunoanalysis with the simplicity of electrochemical probes, has been developed. Immunoassay parameters, such as amount of antibodies and labelled antigen, buffer and pH, length of time and temperature

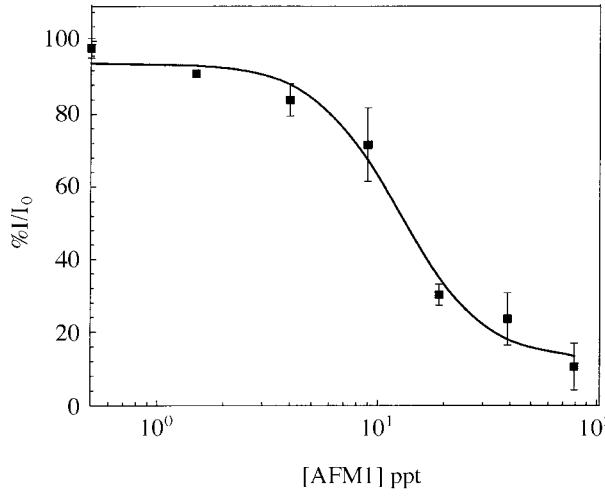


Fig. 14.19 Direct competition curve for AFM1 using SPEs. Antibody against AFM1 (10 μg/mL in PBS pH 7.4) was coated on the SPE working electrode after a pre-coating step with anti-IgG (10 μg/mL in 50 mM carbonate buffer pH 9.6); AFM1-HRP (1:20 v/v) was used as competitor.

of each pre-coating, coating, binding and competition step were evaluated and optimised. A spectrophotometric ELISA procedure, a powerful tool in biochemical trace analysis was optimised for this toxin and a working range between 30 and 480 ppt was obtained in a direct competitive format (data not shown). Electrochemical immunosensors were then fabricated by immobilising AFM1 antibodies directly on the surface of a working electrode of an SPE, and allowing the competition between AFM1 free and conjugated with *Peroxidase* (HRP). Chronoamperometric measurements were carried out at −100 mV. Results showed a working range between 30 and 250 ppt (Fig. 14.19). A preliminary study of interference and matrix effects has been performed to evaluate the suitability of these immunosensors for the analysis of aflatoxin M1 directly in milk.[79]

14.7 DNA biosensors

In recent years, various kinds of biosensors based on identification of the bacterial nucleic acid have been developed.[80–85] DNA biosensors are analytical devices that contain immobilised DNA probes that specifically hybridise to their complementary sequences in a DNA sample. The basic principle of a DNA biosensor is to detect the molecular recognition provided by the DNA probe and to transform it into a signal using a transducer. Applications of gene probes are associated with ultrasensitive detection of microorganisms, viruses and trace

amounts of special chemicals in various environments. Bacterial and viral pathogens responsible for disease states are detectable because of their unique nucleic acid sequences. Through application of molecular 'probes', labelled DNA sequences that are complementary to unique portions of bacterial DNA can be detected and identified.[81] The term nucleic acid (gene) probe describes a segment of nucleic acid which specifically recognises and binds to a nucleic acid target. The recognition is dependent on the formation of stable hydrogen bonds between the two nucleic acid strands. Samples containing bacterial nucleic acid are treated (usually by heating) to cause the double strands of nucleic acids to separate and thus become open to hybridisation with the nucleic acid probe. Because bacterial nucleic acid may be present in very small quantities, the probing process has to be preceded by an amplification technique such as the polymerase chain reaction (PCR), which amplifies the amount of nucleic acid present in the samples.[82] The gene amplification method enhances the sensitivity of DNA probes by at least three orders of magnitude. This technique uses the heat-stable DNA polymerase of *Thermus aquaticus* and allows short lengths of a double-stranded target DNA (template) to be copied in vitro thousands or millions of times, very quickly. Using PCR, bacteria can be detected directly without cultivation, by extraction and isolation of nucleic acid from real samples, followed by amplification of the DNA target and hybridisation with specific probes.

Different electrochemical approaches to detect the DNA hybridisation event have been reported in the literature. Marrazza *et al.*[86] have used an intercalating agent, daunomicine hydrochloride (a planar aromatic molecule) as an electrochemical indicator of the hybridisation reaction. Wang[87] and Lucarelli *et al.*[88] have exploited the oxidation of the guanine base for detecting the formation of the surface duplex. A more attractive approach is the use of an inosine-substitute (guanine-free) probe, immobilised onto the transducer surface, and a direct detection of the duplex formation by the appearance of an oxidation signal due to the guanine of the target DNA. Recently a DNA hybridisation electrochemical biosensor for the waterborne pathogen *Cryptosporidium parvum* has been developed.[84] This sensor relies on the immobilisation of an oligonucleotide, unique to *Cryptosporidium parvum* DNA (taken from the gene which codes for the small subunit of rRNA), onto the carbon-paste transducer and employs a cationic metal complex ($Co(phen)_3^{3+}$) to monitor by chronopotenziometry the hybridisation event. An increased electrochemical response is due to association of the indicator with the surface duplex. A very short (3 min) hybridisation period allowed μg/mL concentrations of the *Cryptosporidium* DNA sequences to be detected. Significantly lower levels of 100 ng/mL and 50 ng/mL were readily detected using 15 min and 30 min hybridisation periods. Similar hybridisation/chronopotenziometric schemes have been developed for other pathogens, such as *Escherichia coli*, *Giardia, Micobacterium tuberculosis.*[85]

14.8 Detecting microbial spoilage

The bacterial content of a sample can be determined indirectly by monitoring the microbial metabolism. Some new biosensors based on microbial metabolism are reported in this section.

In 1998 Casimiri *et al*.[89] reported a biosensor for lactate determination as an index of *E. coli* number in crude culture medium. Lactate, a major metabolite of bacterial energy metabolism, is produced in high amounts during both anaerobic fermentation of glucose and other carbohydrates, and by aerobic pathways. In this work, L-lactate excreted by *E. coli* was measured with an enzymatic L-lactate biosensor, monitoring amperometrically the oxygen consumed in the reaction catalysed by L-lactate oxidase (LOD) enzyme immobilised on the electrode surface. The L-lactate concentration measured in the growth medium was used as a parameter of cell density. The sensor gave a linear response from 5 to 300 μM of L-lactate. To improve the sensitivity of the biosensor, the enzyme L-lactate oxidase was co-immobilised with L-lactate dehydrogenase (LDH). In this way L-lactate is recycled between the two enzymes according to the coupled reaction:

$$\text{L-lactate} + O_2 \xrightarrow{\text{LOD}} \text{pyruvate} + H_2O_2$$

$$\text{pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+$$

The sensitivity provided by substrate regeneration is revealed via the oxidase reaction. The bi-enzyme electrode allowed measurement of an L-lactate concentration as low as 30 μM, which corresponds to 5×10^5 cells/mL. A lower detection limit could be obtained if glucose was added to the culture medium; in comparison, the standard spectrophotometer cell estimation ($\lambda =$ 600 nm) detected concentrations from 10^7 to 10^9 cells/mL of *E. coli* cultured under similar conditions.

Another electrochemical biosensor based on the Clark-type oxygen electrode was developed by Endo *et al*. for the rapid determination of *Enterococcus serolicida*.[90] The cell suspension was filtered through a cellulose nitrate membrane (pore size, 0.45 μm). The membrane along with the captured cells was set on the platinum working cathode of a Clark oxygen electrode and covered with a dialysis membrane. The microbial electrode was immersed in 0.05 M phosphate buffer until the output current became stable. The electrode was then taken out and placed in a solution containing sodium azide, which suppressed the growth of most microorganisms except *E. serolicida*. This biosensor showed a poor sensitivity with a linear response in the range 1.4×10^7 to 7.2×10^7 cells/mL and an assay time of 2 h.

The main drawbacks of the biosensors based only on the monitoring of microbial metabolism are their poor selectivity and slow response time. To improve the selectivity, the monitoring of microbial metabolism has to be preceded by an immunological recognition of the target bacterium. An interesting approach has been developed for rapid determination of *E. coli*

using an immunomagnetic separation coupled with flow-injection system/amperometric detection.[20] Electrochemical measurements, carried out using redox mediators (potassium hexacyanoferrate (III) and 2,6-dichlorophenolindophenol (DCPIP)) reduced by microbial metabolism, allowed the quantitative detection of viable *E. coli* cells without the use of enzyme labels.

14.9 Future trends

The development of biosensors for food analysis has lagged behind other application areas. The clearly defined and well financed needs in medicine have driven some of the most successful biosensor developments to date such as home blood glucose monitors. Public money has stimulated exploration of similar technology for environmental applications. The food industry, however, has been characterised by low profit margins and a somewhat conservative approach to new technology. Public concern for food safety and increased demands for food labelling, including the requirement for information about the presence of material derived from genetically manipulated organisms, is likely to provide more impetus for innovative approaches to food analysis in the future. Considerable research is now being sponsored by government organisations into biosensors for the detection of biological warfare agents. Demands to develop portable and simple instruments suitable for the battlefield and for wide application in combating bioterrorism are likely to provide a rich source of technology that can readily be transferred to microbiological analysis in the food industry. Hence, while the principles elucidated in this chapter could only be illustrated by a limited number of directly relevant examples to date, biosensor technology is likely to be a far greater contributor to safety in the food industry in the future.[91–93]

14.10 References

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