Springer Ser Chem Sens Biosens (2007) 5: 237–280
DOI 10.1007/5346_029

Published online: 16 September 2006

Piezoelectric Immunosensors

Robert D. Vaughan · George G. Guilbault (✉)

Laboratory of Sensor Development, Department of Chemistry,
National University of Ireland, Cork, Ireland
g.guilbault@ucc.ie

1 General Introduction 238
1.1 Piezoelectric Immunosensors 238
1.2 Assay Formats 239
1.2.1 Capture 239
1.2.2 Displacement 239
1.2.3 Competition 241
1.3 Measurement 241
1.4 Immobilisation 243
1.4.1 Introduction to Immobilisation 243
1.4.2 Summary of Immobilisation Techniques 245
1.4.3 Comparison of Immobilisation Methods 252

2 Piezoelectric Immunosensors 255
2.1 Historical Developments 255
2.1.1 First Piezoelectric Sensor 255
2.1.2 Piezoelectric Biosensors 255
2.1.3 Piezoelectric Immunosensors 256
2.2 Piezoelectric Immunosensor Applications 262
2.2.1 Bacterial Detection 262
2.2.2 Viral Detection 266
2.2.3 Clinical Analysis 268
2.2.4 Low Molecular Weight Analytes 273
2.3 Future Trends 274

References 275

Abstract This chapter reviews the basic theory and applications of piezoelectric immunosensors. The immunosensor assay formats most often used are introduced as well as a brief explanation of the typical methods of measurement. Immobilisation is discussed, the importance of each characteristic, the basic techniques employed and a comparison of their performance as investigated by many researchers. The main historical developments of piezoelectric sensors and how these have led to early piezoelectric immunosensors are reviewed. Immunosensor applications and a comparison of sensor performance, for various analytes are summarised. The potential future of this field is also discussed.

Keywords Piezoelectric · Immunosensors · Immobilisation

1
General Introduction

1.1
Piezoelectric Immunosensors

Immunosensors are a class of biosensors that exploit the antibody–antigen interaction. They are generally more specific and sensitive than other biosensors because of the highly specific molecular recognition and affinity between an antibody and its antigen. Affinity constants are usually of the order of $10^8\ M^{-1}$ and can be as high as $10^{15}\ M^{-1}$, this being significantly higher than for other biomolecules such as enzymes, which have typical affinity constants of $10^6\ M^{-1}$. The specificity of this immuno-interaction combined with the very sensitive nature of piezoelectric (Pz) transducers makes a very powerful basis for sensor development.

Since a Pz transducer is basically a mass balance, it can be used to directly detect an immunoreaction by mass alone. This eliminates the need for labelling or the use of a secondary antibody to generate a response, as required by most other immunosensor formats.

Bulk acoustic wave (BAW) or surface acoustic wave (SAW) Pz devices are incorporated for immunosensor development. SAW devices consist of two interdigitated electrodes placed a few millimetres apart on the surface of a Pz slab. When an alternating current is passed a standing surface wave is set up on the Pz material. Addition of mass to the surface causes a change in the wave velocity and thus can be monitored by the frequency change. The quartz crystals generally incorporated are ST-cut (– 49°00′) operating between 30 and 200 MHz. Theoretically SAW devices are more sensitive than BAW devices since they oscillate at much higher frequencies. But, in comparison, they are rarely employed in immunosensor development, since they suffer from a lot of practical operational problems. BAW devices generally consist of an AT-cut (+ 35°15′) piezoelectric crystal disks, with a gold or silver electrode on each side, that typically vibrate fundamental frequencies of 5–20 MHz. This frequency will change if the surface mass of the crystal is altered. A reduction in frequency will occur if the mass is increased. For immunosensor development, crystals with a fundamental frequency of 10 MHz are most often used. Higher frequency crystals are more sensitive but can have robustness issues in solution, particularly when heavily loaded, whereas lower frequency crystals are less sensitive but less effected by liquid damping or heavy mass loading. The choice of crystal depends on its exact application and is often a compromise between sensitivity, robustness and availability.

1.2 Assay Formats

1.2.1 Capture

Direct capture assays are the most common format used in piezoelectric immunosensors. Primarily, because its simplistic format exploits the label-free advantages of piezoelectric transducers.

This format involves modification of the gold surface with an appropriate immobilisation layer followed by attachment of the specific antibody. This antibody-coated crystal can be exposed to a solution containing the analyte, which will bind specifically to the surface and cause a frequency change. In most cases, the frequency change is proportional to the mass added and thus the concentration of the analyte. A diagrammatic representation of a capture is seen in Fig. 1.

Indirect captures can also be used, but generally as a method to detect the specific antibody concentration. In this case the antigen is immobilised and used to capture the specific antibody from the solution.

Pre-coated crystals can be placed in a solution and give a direct result of the concentration of the specific analyte in a matter of minutes without any need for extra assay steps or washings. This approach is particularly useful for those developing portable sensors with a view to field analysis. Pre-prepared coated crystals can be used with a portable instrument for direct analysis in minutes without the need for a skilled operator.

The only downfall of this format is the fact that the sensitivity of the assay is heavily dependent on the mass of the analyte. Thus the analysis of light analytes such as drugs, hormones and toxins may require a different approach.

1.2.2 Displacement

The displacement assay format is often used if the analyte does not have adequate mass to cause significant frequency changes, by exploiting the large mass of the antibody. The crystal is modified with an appropriate immobilisation layer followed by attachment of the antigen. The antibody is then added, which specifically binds to the immobilised antigen. The prepared crystal is exposed to a solution containing the antigen. The immobilised antibody will have a higher affinity for the free antigen in solution and will be displaced from the immobilised antigen, thus causing a frequency increase as the mass of the crystal is reduced due to loss of the immobilised antibody. The overall change in mass will be proportional to the concentration of the free analyte in solution. Since the antibody, which has significant mass, causes the

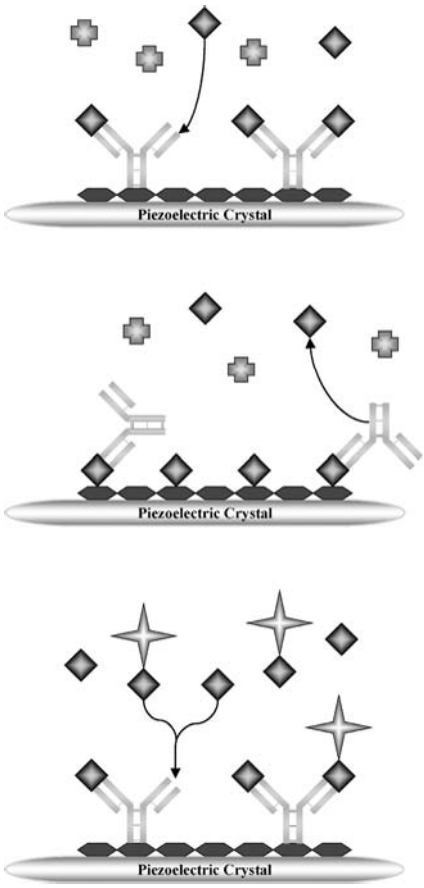

Fig. 1 A typical direct capture (*above*). The immobilised antibody captures the specific analyte from the solution, which results in a decrease in frequency. A typical displacement (*centre*). The immobilised antibody is displaced from the surface and specifically binds to the free antigen in solution resulting in a frequency increase. A typical direct competition (*below*). Free antigen and labelled antigen in solution compete for the available binding sites on the immobilised antibodies: immobilisation layer, non-specific antigen, antibody, antigen, labelled antigen

frequency change, a good sensitivity is often achieved even for very low molecular weight analytes. This assay format generally has fewer problems due to non-specific binding effects since removal from the surface is what is being measured. Thus, applications to real samples are less hindered by matrix effects.

As with the capture, prepared crystals can be applied to a real sample and give results in a few moments without the need for extra assay steps or washings. A diagrammatic representation of a displacement is seen in Fig. 1.

1.2.3 Competition

Another assay format used for the detection of low molecular weight analytes is the competition. A disadvantage of this method is the fact that there is often a need for the use of labels, the lack of which is one of the major advantages of Pz detection. But since the analyte being tested is light, conjugates would have been already prepared in the production of antibodies to make it immunogenic. Thus a ready supply is generally available. Also since the type of conjugate label used is irrelevant as long as it has sufficient mass, they can be easily prepared.

In a competitive assay the crystal is prepared as it would for a direct capture. The antibody-coated crystal is exposed to a solution containing the analyte and a fixed concentration of conjugated analyte. The conjugated analyte has sufficient mass to produce a significant frequency change upon specific binding to the immobilised antibodies. As the concentration of the free analyte is increased it will compete more with the conjugated analyte for the specific binding sites of the immobilised antibodies, thus a lower frequency change will be observed. The overall mass binding to the surface will be less. Since the conjugate label used is irrelevant, heavier ones can be incorporated to enhance the sensitivity of the assay. The major disadvantage for real sample analysis lies in the fact that a mixing step (the antigen and labelled antigen) has to be incorporated. Thus the assay will take longer to perform and potentially involves exposure to harmful analytes.

Competition assays can also be performed by immobilisation of the analyte to the crystal surface. A fixed concentration of the specific antibody is pre-incubated with the free analyte in a test solution, and this solution added to the crystal. Any free antibody will specifically bind to the immobilised analyte on the crystal surface. The lower the concentration of analyte in the test solution the more antibody will bind to the surface and thus a larger frequency change will be observed. This format eliminates the need for a label (but the immobilised analyte is often a conjugate) but involves an extra incubation step. A diagrammatic representation of a capture is seen in Fig. 1.

1.3 Measurement

Pz immunosensor analysis is mainly carried out in liquid phase. Measurements can be taken in two different ways, dip and dry or directly in solution.

Dip and dry involves reading the crystal's frequency while dry in the air. It is exposed to a sample or modified in some way. After sufficient time, in which binding or immobilisation of a species is complete, the crystal is washed and dried. The frequency is recorded again in a dry state in the air. The change in frequency, from before and after modification, is propor-

tional to the mass of the species immobilised on the surface. The dip and dry method can be very useful since both sides of the crystal can be used, with increased mass change and thus increased sensitivity. Also, solution effects due to viscosity etc. are eliminated. It is also very attractive to field analysis as the crystal can literally be dipped into the sample for a period of time, after a quick rinsing and drying step instant results can be obtained. The sensitivity of the method is part of its downfall, since it can be plagued by interferences form environmental effects, especially humidity changes. Repeatability and reproducibility are often the biggest validation obstacles to the development of dip and dry type sensors. The use of a reference crystal is often used to counteract these environmental effects.

Measurements taken directly in solution are less affected by changing environmental conditions and much more reproducible and repeatable results are generally obtained. Damping effects due to the liquid, however, can reduce the sensitivity of the sensor. Also the behaviour of a crystal in solution may not be due directly to the effect of mass on the surface. The frequency response of a layered crystal in solution is dependent on many factors, such as the viscosity and temperature of the liquid, and the thickness and rigidity of the immobilised layers.

Analysis is generally carried out in specifically designed flow cells (Fig. 2) and frequency changes can be recorded in real time yielding kinetic informa-

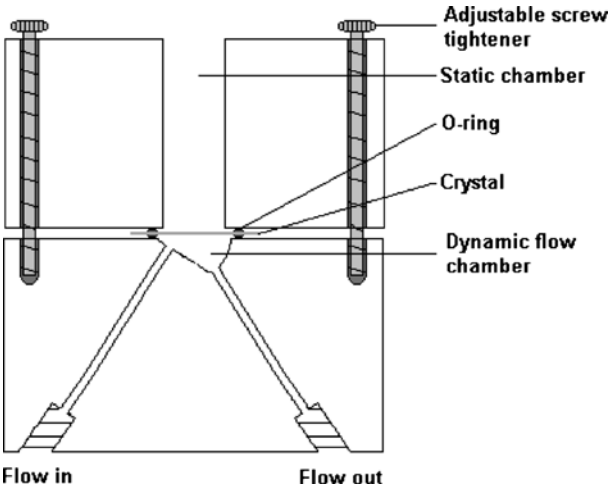

Fig. 2 Diagram of a typical flow cell used for solution analysis. The cell allows exposure of one side of the crystal to a static solution and allows exposure of the other side to a dynamic solution. The dynamic side of the flow cell typically has a specially designed chamber to help eliminate bubbles. Low pressure peristaltic pumps are generally incorporated. The flowing solution can greatly increase sensitivity since large volumes of dilute solutions can be passed over the crystal surface

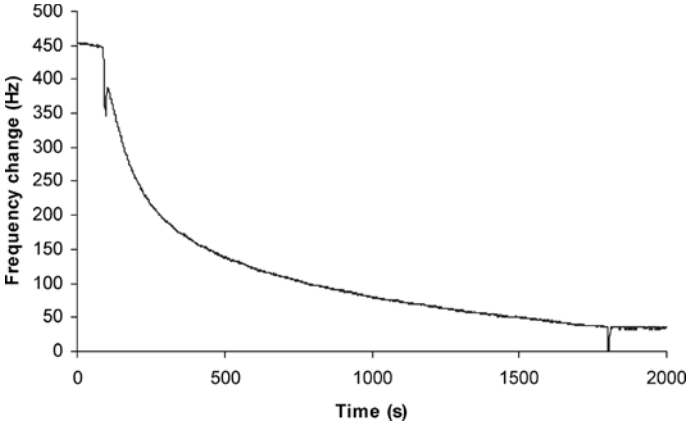

Fig. 3 Real-time Pz response of a direct capture. The antibody-coated crystal is in solution. After 100 s the specific antigen is added. A sharp decrease is initially recorded as the antigen binds to the immobilised specific antibodies on the crystal surface due to the mass increase. The frequency change eventually levels off as equilibrium at the surface is reached. The total frequency change is proportional to the concentration of the analyte added

tion if required. The flow cells allow exposure of gold surface on one side of the crystal to the solution and the other to the air. Analysis can be performed in a static or dynamic solution. Figure 3 shows the typical real time response of an antigen specifically binding to an antibody-coated crystal in solution.

Using a flowing solution can greatly increase the sensitivity of a sensor since very large sample volumes can be flowed over the crystal surface, greatly increasing the potential surface loading and thus the sensitivity of the sensor.

1.4 Immobilisation

1.4.1 Introduction to Immobilisation

Immobilisation of the antibody to the crystal surface is of critical importance to the overall performance of the sensor. Stability, orientation and reproducibility of the immobilised layer will affect the sensitivity, lifetime, reusability and potential applications of the sensor.

The type of immobilisation dictates the ability to regenerate the sensor surface, or the antibody surface, for reuse as well as the stability and storage conditions needed to extend the lifetime of the sensor. The importance of immobilisation is reflected in the fact that a significant percentage of Pz immunosensor publications deal with the development of novel or the improvement of existing immobilisation methods.

Biomolecular immobilisation remains the primary challenge to commercialisation of immunosensors. Many successful sensors have been produced for numerous analytes, incorporating many different immobilisation methods, but most never make it past a laboratory, with very few ever making it to manufacturing prototypes. The transfer of a laboratory-based sensor to a mass-produced product while still retaining stability, reliability and sensitivity requires simple and reproducible procedures. Complex multistep immobilisation procedures that require a trained operator to perform in a laboratory will not be easy to transfer to a manufacturing situation and would probably result in a poor quality product when compared to its laboratory-based prototype.

Stability. A stable coated surface with a long lifetime is essential. The immobilised protein layer must also have a reasonable lifetime. The surface should be chemically and mechanically stable and allow washings and exposure to different conditions without any detrimental effect on the protein layer. If commercialisation is desired then very robust layers are required and must be reasonably stable under simple storage conditions for at least one year.

Orientation. The orientation of the immobilised antibody is also very important to the sensitivity of the sensor. A large concentration of immobilised antibodies does not guarantee good sensitivity. If the immobilised antibodies are randomly orientated then a significant percentage of them may be unavailable for binding. If they are regularly immobilised, but through one or both of their Fab sites then they will be partially or completely deactivated. The most desirable immobilisation methods are those that bind to the antibodies Fc sites, thus orientating their binding sites towards the analyte in solution. This has been observed by many researchers while comparing different immobilisation procedures. Protein A treated surfaces, which bind antibodies through their Fc sites, may immobilise a significantly smaller amount of antibodies but show a much greater sensitivity towards the analyte. Coupling the antibody to the immobilisation layer should occur in such a manner so as not to cause conformational changes and affect its biological activity.

Reproducibility and capacity. An important requirement is to reproducibly produce an immobilisation layer on different crystals. Even if similar crystals can be produced in a batch, this batch production must be reproducible. Obviously, if each immobilisation layer is slightly different then each crystal will have a slightly different sensitivity and lead to errors. Generally the most simplistic methods that require minimal amount of human interference are the easiest to reproduce. The homogeneity or distribution of the coating on the surface is also very important due to the greater sensitivity of the centre of the gold electrode. The most sensitive crystals will be one that can bind most to its centre. A crystal that binds the same amount to the edges of its electrodes will be less sensitive and show a lower frequency change. Thus the distribution of the coating must also be reproducible, not just the amount.

The amount of immobilised antibody is also a very important factor that can affect the sensitivity of the sensor. It must have the potential to bind a sufficient amount of the antibody. Although this is usually not a problem, since it is often found that excess immobilised antibody can lead to a reduction in sensitivity due to steric hindrance.

Simplicity. If commercialisation is to be realised then the method must be mass producible and very simplistic. Multistep immobilisation procedures that may be easy to perform reproducibly in a laboratory-based situation may be very difficult to perform in a manufacturing situation. Generally, one step procedures that can be reproducibly carried out under ambient conditions by untrained workers are desirable. A method such as adsorption that may lead to reduced sensitivity may still be more viable when compared to a very complex method that leads to a more sensitive product. Reduced production costs will generally win out over performance if the performance difference is not too significant.

Importance of each characteristic. Overall, the importance of each characteristic depends on the application and specific situation. For research purposes generally the best sensitivity is required, often regardless of the complexity of the technique, but if the sensitivity is far superior to that required for a specific application then less complex procedures are often employed at the loss of performance. For commercialisation purposes stability and simplicity are the main objectives. An ultra-sensitive device that needs a highly trained operator and only lasts a number of days has very little, if any, commercial application.

1.4.2 Summary of Immobilisation Techniques

Non-covalent methods. The easiest method of antibody immobilisation is physical adsorption. It involves the simple addition of the antibody solution to the surface and allowing it to adsorb. The antibodies bind to the surface via van der Waals, hydrogen bonding and hydrophobic interactions. Thiol–gold interactions from sulfur residues on the antibodies also play a major role. This method has no effect on the conformation of the protein but some activity is lost due to random orientation. Loss of protein from the surface is also a problem and its rate can depend greatly on changes in pH and temperature. Thus chaotroping and reuse of the antibody surface is not possible, since it is difficult to reproducibly generate the antibody surface. However, reuse of the sensor surface is possible since all the protein layers can be easily removed.

Adsorption onto polymer-coated surfaces has also been attempted [1]. Polystyrene was used, presumably to mimic the binding capabilities of enzyme linked immunosorbant assay (ELISA). While the method showed good binding of the antibodies the sensor had a low sensitivity to cells. This was

due to random orientation of the immobilised antibodies, leading to deactivation of a high percentage of them due to binding via their Fab sites. The main advantages of polystyrene are the fact that it is inert, very cheap and widely available, but problems due to its hydrophobic nature can also yield poor results. When used in the production of ELISA plates the well surfaces are often pre-treated to produce hydrophobic and hydrophilic areas to enhance the adsorption of proteins. But this pre-treatment is not feasible for the production of individually coated Pz crystals. Sakti et al. [2] used crystals spin coated with polystyrene with a view to developing commercial Pz immunosensors. Various parameters were optimised and the authors showed that pre-prepared coated crystals as a basis for disposable immunosensors were commercially feasible. Insulin detection was used as an example to demonstrate an application of the system. Mass production of these types of crystals is possible with good reproducibility, but the loss in activity due to the random orientation of adsorbed antibodies is a major disadvantage.

Immobilisation of the antibody via adsorbed protein coatings is also widely used. Protein A and G are generally used. They are proteins isolated from the cell walls of certain pathogenic bacteria and show high affinities, in excess of 10^6 M^{-1}, for the Fc portions IgG molecules [3]. Their tertiary structures are very similar but there is significant difference in their amino acid composition and both have a mass of about 40 000 kDa. The specificity of the binding helps to reduce non-specific effects and binding through the Fc terminal ensures orientated immobilisation with no loss of antibody activity. It has been reported that each protein molecule can bind two IgG molecules [4]; however, others have suggested that they are pentavalent towards IgG binding [5]. Much lower values have been reported in Pz immunosensor work. Ratios of protein molecules to bound IgG molecules of around 1 : 2 [6] and 2 : 1 [7] have been reported. These lower values are probably due to steric hindrance and saturation of the crystal surface with protein molecules. Also a loss in activity is expected because of immobilisation and the formation of multi-layers that will inhibit some of the molecules.

The ease of use of these proteins is another advantage. They are simply adsorbed onto the gold surface of crystals and after a simple washing step are ready for use. The gold–protein bond has an association constant of 10^8 M^{-1} and is believed to be van der Waals in nature [6]. The gold–protein complex has been shown to be stable at pH 2–8 [7]. Enhanced binding of protein A has also been reported near its isoelectric point (pH 5.5) [8], but it is unclear from the author's work if this was just due to longer incubation times.

Regeneration and lifetime of antibody-coated crystals formed using these proteins varies considerably, but reports of 25 regenerations without loss in activity and lifetimes of 7 weeks have appeared [9]. Most authors have reported significant removal of the protein layers after a few regenerations of the antibody surface, however.

Their ease of use and the high sensitivity usually obtained means that these are the most common immobilisation method used in Pz immunosensor work, with protein A most often used.

Incorporation of other biological components such as biotin–avidin coupling has also been used. The system has an affinity constant of 10^{-15} M and is resistant to extreme pH, vigorous washings and chaotroping conditions. Generally they are used in conjunction with other immobilisation methods.

Covalent methods. Covalent attachment offers many advantages over adsorption but has longer and more complex immobilisation procedures. Better stability and extended lifetimes of the antibody surface are often observed. There is minimal or no loss of the antibody surface, even in solutions of high ionic strength. This allows easier chaotroping of the antibody/antigen complex and reproducible regeneration of the antibody surface, since generally none of the antibody is lost.

With commercialisation in mind, immobilisation methods need to be easily mass produced, cost effective and be able to form a stable biomolecule surface. Most covalent methods satisfy these requirements but their main downfall is the sometimes complex procedure. But despite this they are much more useful than the simple adsorption methods and it is ultimately the stability of the antibody-coated surface that will dictate the commercial potential of any sensor. A lifetime of several months is the absolute minimal requirement and such a stable sensor would have greater commercial appeal than a more sensitive reliable version with a short shelf life.

Generally, immobilisation is attempted through functional groups so as not to affect the activity of the antibodies. Functionalisation of the gold surface is easily achieved through the self-assembly of thiols or sulfides, the formation of organic polymer layers or the formation of thin inorganic layers. The covalent binding generally involves carbonyl-type reactions with nucleophilic groups (– SH, – NH, and – OH) on the protein to the functionalised surface layer.

The first Pz immunosensor to be used directly in solution incorporated an inorganic silane layer for immobilisation [10]. Incorporating results from chromatographic studies, which at the time had recently shown the high reactivity of silane-modified surfaces for proteins, the authors coated their ST-cut crystals with trimethoxyorganosilane.

Silanisation has many advantages over the use of organic polymers due to its high mechanical strength, resistant to solvents, stability, ease of handling and the fact that the structure is more or less constant at varying pH and temperature.

The most commonly used silane-coupling agent is 3-aminopropyltriethoxysilane (APTES). Treatment of the gold surface with chromic acid or HCl generates a hydroxylated surface. Water catalyses the polymer formation since it hydrolyses APTES ethoxy groups, creating a siloxane bond between APTES and the hydroxyl groups on the surface. Mild curing of the surface is

generally performed to ensure bonding of the first few layers of APTES and subsequent washings remove the other weakly bound layers. This curing has been shown to enhance the stability of the silane surface [11]. Coupling of the antibody to this silanised surface is generally performed using glutaraldehyde, which covalently cross-links the protein to the silanised gold surface. Increased sensitivity from the use of other aldehydes has been noted [12]. This was attributed to the fact that glutaraldehyde deteriorates very quickly to unsuitable polymeric products [13]. The method is based on the formation of a Schiffs base linkage between carbonyl groups and free amino groups on the silanised surface and protein.

An excellent study by Raman Suri et al. investigated various parameters and developed an optimised method for the use of APTES as an immobilisation method for Pz immunosensor development [12]. In terms of surface density, binding capacity and stability vapour deposited layers were most useful. Under optimised conditions the protein layers were reusable eight times, were very stable, withstood vigorous washing steps and have a lifetime of over 12 weeks. The system was also shown to be very reproducible, but problems in procedures such as the need for dry solvents and the pre-treatment of glassware were noted. APTES reacts strongly with glass, so laborious pretreatment of any glass to be used was required. The presence of water hydrolyses the APTES ethoxy groups and leads to catalysed polymerisation. This then electrostatically adsorbs to the surface instead of covalently binding, leading to a less stable layer that leaches with time.

There has been wide use of silane as Pz immobilisation layers, mainly because of their stability and the ability to produce reproducible surfaces. But because of the fact that the antibody is bound via a primary amine, deactivation from binding through Fab sites can be a problem. But for use in assays where the analyte is an antibody with an affinity for the immobilised species or when used to first immobilise protein A/G [12] they are acceptable, since deactivation of the primary antibody is not a problem.

Covalent immobilisation to organic polymer layers is also widely practised. The most commonly used polymer is polyethylenemine (PEI), but many others including polystyrene, polyacrylamide and various methacrylates have been incorporated. The main advantage of polymers is their versatility. They are easy to prepare, have a very large potential application and can be tailored for any use. Co-polymers, usually methacrylates, can be activated and modified easily to suite individual needs.

As with silane, immobilisation of the antibody is usually performed using glutaraldehyde and deactivation of the antibody is often a problem as a result, but the versatility of polymers means that various functionalised surfaces can be produced allowing antibody binding through other groups besides amines.

Practically, the production of polymer layers is very simple. Usually the polymer of choice is just dissolved in an appropriate solvent and evaporated onto the gold surface. Spin coating is also often used to increase the unifor-

mity and to improve the reproducibility of the formed layer. Stability of the layer is another advantage since most of the polymers used are relatively inert and can be stored for long periods without changes in the crystal surface coating occurring.

Reproducibility problems in the production of polymer layers as described above can sometimes occur. Since washing steps and drying procedures are often incorporated to remove loosely bound material, and changes in intensity or duration of these steps can result in different layers. Thus the amount of the immobilised protein will be different and reduce the reproducibility of the sensors response. The use of thick organic films produced by this method can also lead to frequency instability and loss of sensitivity. It is also difficult to control film thickness and homogeneity, which also effect the reproducibility of the surface produced. Weak adhesion between the polymer and the substrate can also be a problem. Methods, such as plasma polymerisation and electropolymerisation, have been developed to overcome these problems and to gain more control over the layer parameters.

The use of plasma polymerised layers for Pz immunosensor development was first described by Nakanishi et al. [14]. The method retained the many advantages of polymers but eliminated the need for washings. The procedure was automated so human error was eliminated and very reproducible flawless surfaces were made. The process produced very thin ($< 1\,\mu m$), highly branched and cross-linked structures that were mechanically and chemically stable. The plasma glow discharge treatment only took a minute to complete and numerous crystals could be prepared at a time. Then the crystals were ready for use. The direct cross-linking of antibodies to this surface showed good binding but poor sensitivity was observed, primarily due to deactivation of most of the immobilised antibodies. Cross-linking protein A initially to the surface followed by the antibodies ensured orientated immobilisation, and despite the lower amount that bound a much improved sensitivity of the sensor was observed. The authors also cross-linked the antibody to protein A using dimethyl pimelimidate dihydrochloride to avoid disassociation during the regeneration steps. While this was the first use of this technique in Pz immunosensor work, it had been previously used for Pz gas sensor development [15].

Monomers such as ethylenediamine are usually chosen since they lead to hydrophilic surfaces with plenty of amine groups. It has longer polymerisation rates when compared to many other possible monomers, but this allows easier control over the film thickness and increases reproducibility.

Problems of reproducibility between batches of polymerised films have been reported [16]. To avoid this, the authors developed a method of adsorbing their antibodies to the surface so that easy regeneration of the original plasma film was possible. But this led to many other potential problems in that the antibodies were randomly adsorbed and the antibody layer was not very stable. While a new surface was easily regenerated the usefulness is ques-

tionable. Generally the ability to regenerate the antibody surface is a much more desirable characteristic.

Electropolymerisation of layers again offers control over the growth and thickness of the film. Production of the layer is also quite simplistic. The gold surface is placed in a solution containing the polymer, which is electropolymerised onto the surface at the appropriate potential for the desired time. After this a very stable highly branched structure is formed on the surface that can be further modified to immobilise the antibodies. As with plasma polymerised methods many crystals can be easily prepared at a time.

Kurosawa et al. incorporated a method that eliminates the need for surface pre-treatment or immobilisation of any sort [17]. It was based on polymer agglutination of latex. A cleaned crystal was placed in a solution containing latex particles pre-labelled with antibodies. Addition of the specific analyte caused cross-linking of these particles as the antibodies bound to the analyte. This agglutination caused a change in viscosity of the solution, which in turn caused a change in the frequency of the oscillating crystal. The fact that polymer agglutination is used in many commercial test kits means that many antibody labelled latex particles for different analytes are commercially available. Also stability and lifetime of immobilised species is not a problem. The authors later incorporated initial rates analysis that allowed the detection of their analyte in 2–3 min and allowed the use of sample volumes as low as 20 μL [18]. It was shown that the latex particles in solution prevented adsorption of proteins to the crystal, thus eliminating non-specific effects. This approach to immunosensor development offers many advantages over the conventional methods of analysis and commercially is as viable as any method of pre-coated crystals.

Self-assembly. Self-assembled monolayers (SAMs) offer a method of orientated, covalent attachment of antibodies to the gold surface to form densely packed monolayers, which should lead to tightly bound, regular layers of proteins. This potentially has the advantage that exposure to a high or low pH, often used in regeneration, should lead to desorption of the electrostatically bound analyte. The covalently bound antibody should be unaffected, leading to the ideal reusable system [19]. They have the added advantage over other covalent methods, such as PEI and APTES, in that there is a high affinity between the gold and sulfur so loss of the layer over time is unlikely.

Various techniques have been used to study the kinetics and structures of SAMs [20]. Thiols and sulfides are of particular interest especially in the area of electrochemistry, mainly because of their spontaneous chemisorption, regular organisation and high thermal, mechanical and chemical stability on gold surfaces [21]. An additional advantage is the inertness of gold towards the chemisorption of most polar organic functionalities; thiols and sulfides bind very strongly via chemisorption bonds [22], thus offering a good method of functionalising gold surfaces. Monolayers of thiols on gold appear to be indefinitely stable at room temperature, but desorption at elevated

temperatures is evident [23]. Long chain thiols have been shown to be more thermally stable [23] and the adsorption to the surface has been shown to proceed by two methods [24], ionic dissociation and more favourably by radical formation.

The monolayers spontaneously and reproducibly form when the gold is placed in the thiol or sulfide solution. Lack of reproducibility may occur due to differences in the gold surface from crystal to crystal but this generally is not significant.

Due to the stability, orientation and ability to functionalise the terminal groups on the molecules they can offer a very versatile method for immobilisation of biomolecules to gold electrode surfaces for biosensor development [25, 26].

The functionality of the layer depends on the terminal functional groups on the molecule used. Generally compounds with carboxylic acid or amine functional groups are used and this allows covalent attachment of the antibody using a carbodiimide or glutaraldehyde, respectively. This results in a highly ordered layer with orientated covalent attachment of antibodies to the surface, without altering their biological activities [27]. While the immobilisation may not change the conformation the orientation of its binding may lead to deactivation of the antibody. The thin layers (monolayer) used ensure no loss of sensitivity or frequency instability, often observed when using thick organic polymer layers. The strength of the sulfur–gold bond and the covalent attachment of the antibody ensure reproducible regeneration of the antibody surface. Even exposure to harsh conditions should not affect the layer. Regeneration of the antibody surfaces using most methods of immobilisation is generally only possible a few times before a loss in activity is observed. Even if the antibody is covalently bound, weak adhesion between the layer and the substrate will lead to its loss from the surface.

Duan et al. used a monolayer of thioctic acid to covalently immobilise monoclonal antibodies to a gold electrode while performing amperometric analysis [28]. Frey et al. demonstrated potential biosensor application of this immobilisation method by binding polylysine to gold surface plasmon resonance (SPR) electrodes via a SAM of 11-mercaptoundecanoic acid [29].

The functionalisation of antibodies for the immobilisation onto Pz crystals has been performed by Caruso et al. [30] and Neuman et al. [31]. Both authors used similar procedures to those initially described by Leggett et al. [32]. The method introduced functionality to the protein, which had high affinity for the chosen substrate, gold scanning tunnelling microscopy (STM) electrodes in Leggetts' case and gold Pz crystals in Caruso and Neumans' case. The procedure involved the thiolisation of the antibodies using Trauts reagent (2-iminothiolane). The methods were based on the original work performed by Traut and coworkers [33–35]. They discovered that unlike most imidates, 2-aminothiolane was very stable in acidic or neutral solutions and did not cause any protein cross-linking, and thus was a very useful compound for

the thiolisation of proteins. The fact that the thiolisation occurs at an amine group means that deactivation of the antibody may occur if the Fab amino groups were thiolated. Also if all amino groups were thiolated then random orientation of the immobilised antibodies could potentially occur, although after self-assembly a regular surface is likely to have occurred. Caruso et al. also observed strange results while using the thiolated antibody solution after several days. This was attributed to the fact that the antibodies may have oxidised and formed disulfide bonds. If this was the case then polymerisation of the antibodies was a possibility and thus this method of immobilisation is only useful for antibodies that are used immediately.

Park et al. also described a method for the thiolisation of antibodies and self-assembled these onto gold electrodes [36].

Protein engineering to tag protein A molecules with cysteine residues and to self-assemble these onto gold surfaces has been performed [37]. This method would incorporate the simplicity, reproducibility and covalent advantages of self-assembly with the orientational advantages of protein immobilisation and potentially lead to the most useful immobilisation method. Cleaving of antibodies to break the disulfide bridges and self-assembling these portions onto electrodes has also been attempted by many authors.

As with most immobilisation methods that rely on antibody binding through amine groups, deactivation can be a problem. But Spangler and Tyler have described a method of covalent antibody attachment to a SAM without any loss of antibody activity [38]. It is based on the oxidation of the antibody prior to use and then binding of this to an activated monolayer on the gold surface. This type of approach was first suggested by Taylor [39]. This method was based on the oxidation of sugar residue in the Fc portion of the antibody. The oxidised antibodies could then be immobilised onto an activated transducer surface, resulting in orientated attachment without any loss in activity. This method had not been applied to any immunosensor when suggested by Taylor.

Other monolayer-forming techniques such as the use of Langmuir–Blodgett films to immobilise antibodies have been reported [40]. The method of formation of the layers is very simplistic but their practical applications are limited.

1.4.3
Comparison of Immobilisation Methods

All immobilisation methods have different advantages and disadvantages over others. Many authors have compared different methods with varying results.

Plomer et al. compared protein A, APTES and PEI as immobilisation layers while detecting *E. coli* [41]. Best results were obtained using protein A immobilisation but regeneration of the antibody surface was not possible. Atilla and

Seuluman observed similar results while detecting cortisol [42]. Better sensitivity was found using protein A-coated crystals but regeneration of the antibody surface was not possible. König and Grätzel also found crystals coated with protein A more stable, sensitive and reproducible than those coated with PEI or APTES for the detection of enterobacteria [9] and viruses [43, 44], and were able to regenerate the antibody surface 18 [43] and 10 [9] times without detectable loss in activity. Others have also found protein A most sensitive and were able to regenerate the surface numerous times without detectable loss in activity [45]. From these results the orientated immobilisation of protein A could explain the greater sensitivity of these sensors, but this cannot be generalised since many others have found different methods of immobilisation better than protein A. Carter et al. found better sensitivity from adsorption of antibodies [46]. One explanation for this could be the differences in procedures between different researchers but this is not the case. König and Grätzel have also found PEI a better choice than protein A on other occasions [47–49]. The authors found very similar results for PEI, APTES and protein A while detecting human granulocytes [47], but PEI showed better stability. They observed poor sensitivity and very large non-specific binding with protein A while detecting lymphocytes [48, 49]. Again PEI was the immobilisation method of choice.

Caruso et al. investigated the orientational aspects of different immobilisation procedures and its effect on immunological activity [30]. They compared four antibody immobilisation methods, physical adsorption, binding to a layer of protein A, covalent attachment using thiolated antibodies (Trauts reagent) and covalent attachment to a thiol monolayer. A 30% increase in binding of the initial antibody layer was observed for the physical adsorption when compared to the other methods, but due to the random orientation enhanced binding of the second layer was not observed. Increased amounts of second layer binding were observed with the protein A layer compared to the covalent methods, due to the orientation of the protein A binding. It was not possible to separate the contributions from improved antibody alignment and non-specific binding from investigations using site-specific secondary antibodies, however.

From these reports and many other investigations it seems the choice of immobilisation methods is dependent on the specific situation and cannot be predicted. The orientation of protein A-immobilised antibodies is far superior to most other methods and an improvement in sensitivity is often evident, but the formation of reproducible stable layers and the inability to regenerate the antibody surface is a problem. Despite the results reported, the protein layers are only adsorbed to the surface and will eventually leach from the surface.

The stability of the layers produced by other methods such as SAMs often makes them more useful. In this author's experience PEI and APTES generally have the best antibody binding capacity, but the loss in activity and difficulty

with reproducibility often leads to poorer detection characteristics than other methods. Of the covalent methods of immobilisation, SAMs are the easiest to form and are more reproducible than most, if not all, methods of immobilisation. They covalently attach the antibody to the surface and combined with the strong sulfur–gold bond lead to the formation of a very stable surface. In terms of simplicity, stability and reproducibility SAMs are the best choice of immobilisation, but the loss in activity of immobilised antibodies due to binding through their Fab sites can be a problem. In cases where the analyte is an antibody and the particular orientation of the primary layer is not important, then they are the obvious choice.

Combining immobilisation methods seems to be the best option, such as incorporating the stability and reproducibility of covalent methods such as SAMs with the orientated binding of protein A/G. The first example of this was in 1987 by Muramatsu et al. [4]. The authors cross-linked protein A to a layer of APTES, although it is unlikely that it was done to combine the advantages of both immobilisation methods since this was one of the earliest Pz immunosensors and more basic fundamental problems were being overcome at this stage.

The advantages of such an approach were highlighted by Nakanishi et al. [14]. Cross-linking of antibodies to an electropolymerised surface using glutaraldehyde caused a large frequency reduction, indicating immobilisation of the antibody. However, it showed little or no response when exposed to the antigen solution due to deactivation, presumably from binding through amine groups that inhibited the Fab sites. Cross-linking protein A to the layer first resulted in less antibody binding but showed a much better sensitivity to the antigen due to the orientated binding of the antibody by protein A. The authors also cross-linked the antibody to protein A to ensure further stability. This helped prevent loss of antibody during regeneration and washing steps.

These types of immobilisations combine the stability and reproducibility of covalent immobilisation with the orientational advantages of protein A/G coupling. Together they offer many advantages over each method individually, but they have longer and more complex procedures, and in some cases this may not be justified by the increase in sensitivity. Realistically the production of a SAM, activation and immobilisation of protein A/G followed by antibody immobilisation is not too difficult, and in a laboratory-based test the potential increase in sensitivity makes the procedure worthwhile. But for commercialisation and mass production the increase in sensitivity must be significant before the increase in preparation time and cost can be justified.

2
Piezoelectric Immunosensors

2.1
Historical Developments

2.1.1
First Piezoelectric Sensor

The first analytical use of Pz crystals with view to sensor or detector development was presented by King in 1963 [50], the basis of his commercialised moisture detector. In 1964 he published details of his sorption detector [51], although earlier Oberg and Ligensjo had used Pz crystals for the monitoring of film thickness [52].

Kings sorption detector could detect moisture to 0.1 ppm and hydrocarbons such as xylene to 1 ppm. The sensors consisted of Pz crystals coated with different coatings (of varying selectivities), and their interaction with different analytes were monitored. The coatings used were based on gas chromatography (GC) stationary phases. An uncoated crystal was also incorporated as a reference.

Being the first to realise the potential of this sensor type he predicted that many more similar sensors would be developed in the future. It is also quite probable that King was the first to investigate the use of Pz crystals as detectors in solution, since he mentions the impaired ability of crystals to vibrate when a solution was placed on the surface. He states the possible reason for this is the dissipation of energy from the vibrating crystal to the liquid.

In the following years many similar sensors were developed [53–57]. During this period many fundamental problems were solved and developments in instrumentation were made, many of which are still incorporated in today's Pz immunosensor design.

2.1.2
Piezoelectric Biosensors

The use of biological coatings, such as enzymes and antibodies, was a natural progression from the initial Pz sensor development.

The first reported gas phase biosensor was in fact an enzyme-based Pz sensor [58]. Crystals coated with formaldehyde dehydrogenase were used to linearly detect formaldehyde in air from 10 ppb to 100 ppm. A portable version of the sensor was also developed. The battery-operated instrument contained a digital readout and miniature sampling pump. Weighing just 3 lbs it was used in field for on site analysis.

2.1.3 Piezoelectric Immunosensors

The first Pz immunosensor was developed by Shons et al. in 1972 [59]. They incorporated a layer of Neybar C to immobilise bovine serum albumin (BSA) to the surface of a 9 MHz Pz crystal. They used this to detect BSA antibodies in a liquid sample. Their results in terms of sensitivity were comparable to the conventional antibody assay technique of passive agglutination. This method requires hours for an assay to be completed, but the Pz sensor only required minutes.

In 1980 Olivera and Silver disclosed details of a competitive microgravimetric-based Pz immunoassay that could be used for the determination of many analytes, even low molecular weight compounds [60]. They firstly immobilised the analyte on the surface of the crystal and measured the frequency. These crystals were then exposed to a solution containing the analyte and the antibody against the particular analyte. Any free antibody in solution would bind to the immobilised antigen on the crystal surface. Thus a reduction in frequency was observed, the magnitude of which was proportional to the concentration of free antibody in solution. When the amount of antibody was fixed, the amount of free antibody in solution was proportional to the amount of analyte in solution. Thus the overall frequency change was inversely proportional to the concentration of the analyte in solution. The system was applied for the detection of many analytes, including drugs, immunoglobulins, nucleic acids and polysaccharides.

In the same year, Rice also patented another Pz-based immunoassay [61]. The sandwich-based assay was used to determine the type of antibody subclass and the concentration of the antibody present. Crystals coated with the antigen were exposed to the antibody solution. A secondary antibody against the primary antibody was then added. The frequency change observed here is indicative of the amount and type of the primary antibody present. Later, Rice described a similar assay that was used to determine the amount of a particular class or species of antibody and also the amount of total immunocomplex present in the antibody layer [62]. The assay was also based on a sandwich format and proved useful for low molecular weight antigens.

Konash and Bastiaans were the first to demonstrate the use of a Pz sensor in liquid phase [63]. They used a coated crystal as a liquid chromatography detector, vibrating in a flowing solution. The authors incorporated a reference crystal to eliminate the effect of viscosity changes in the solution. The sensitivity of the detector was relatively poor but for the first time showed that a crystal could be vibrated with a stable oscillation while submerged in a liquid.

The first Pz immunosensor to be used directly in solution was developed by Roederer and Bastiaans in 1983 [10]. This novel method, termed microgravimetric immunoassay, was based on a SAW device. Incorporating results

from chromatographic studies, which at the time had recently shown the high reactivity of silane-modified surfaces for proteins, the authors coated their ST-cut crystals with trimethoxyorganosilane. This coupling agent was used to immobilise goat anti-human IgG to the crystal surface. In their detector cell it was noted that the shift in frequency was dependent on the sample volume, but that this dependence diminished as the sample volume was increased. The sensor could measure human IgG from 0.0225 to 2.25 $mg\,mL^{-1}$ with a limit of detection of 13 μg. Regeneration of the antibody surface was also demonstrated after measurement by exposing the surface to a salt solution of high ionic strength. The primary reason for the poor sensitivity and limit of detection was due to non-specific adsorption occurring both at the reference and measuring crystal. The authors also stated, when discussing the limitations of immunosensing, that Pz devices could only be applied to analytes of high molecular weight. This statement was later shown to be untrue by many groups.

Thompson et al. were the first group to study Pz immunochemistry using a flowing solution [64]. They incorporated 5 and 2.5 MHz crystals. Crystals of lower fundamental frequency will have a lower sensitivity but will have a higher *Q* factor. These would be more stable in solution since less acoustic energy is lost to the solution than crystals of higher frequencies. Different immobilisation procedures were tested and the system was applied to the detection of IgG. This work was mainly a study of interfacial interactions and a study to show that it is feasible to oscillate Pz crystals in solution with a high degree of reproducibility.

The first Pz immunosensor for microbial pathogens was developed by Muramatsu et al. in 1986 [65]. Their Pz crystals were coated with antibodies against *Candida albicans*. The antigen was detected from 1×10^6 to 5×10^8 cells mL^{-1}. The sensor proved to be specific with no detectable response observed with the other species tested.

Muramatsu et al. later described a sensor for the detection of immunoglobulins [4]. Protein A was immobilised to the surface of crystals by cross-linking to a layer of silane via glutaraldehyde. This protein layer was then used to immobilise the immunoglobulin molecules. The authors immersed the whole crystal in the solution. Apart from when in highly conducting solutions, in which the frequency would cease due to a short circuit across the crystal, a very stable oscillation was observed. This was due to the fact that no mechanical stress was applied to the crystal, as is the case when they are in a flow cell with only one side exposed to the solution. Human IgG was determined from 10^{-4} to 10^{-2} $mg\,mL^{-1}$ in a flowing solution. The authors also used the protein-coated crystal to determine different IgG subclasses in a step gradient buffer solution. The resonance frequency was also studied in different solutions and at different temperatures.

Ebersole and Ward described their amplified immunosorbent assay (AMISA), which was used for the detection of APS reductase and human

chronic gonadotropin (hCG) [66]. After the direct capture of the antigen, a secondary enzyme-labelled antibody was added. Subsequent exposure of the surface to 5-bromo-4-chloro-3-indolyl phosphate (BCIP) resulted in a blue precipitate, which caused a large frequency change. The precipitate was due to the enzymatic dimerisation of BCIP by the immobilised alkaline phosphatase. The use of this enhancement step led to limits of detection of 5 ng mL^{-1} (10^{-14} M) of APS reductase. The hCG assay was based on the enzymatic oxidation of I^- to I_2/I_3^-. While AMISA incorporates extra steps when compared to the usual direct Pz capture it demonstrated that amplification routes via enzymatic catalysis can significantly lower detection limits. The main advantage of AMISA is the fact that the measured mass change is independent of the mass of the analyte. Thus detection of low molecular weight analytes, such as drugs and hormones, would be possible.

Davis and Leary in 1989 performed probably the first thorough investigation of Pz immunosensors in solution [8]. Using a human IgG system they continually monitored the crystals resonance in solution. They used a plexiglass reaction cell, which exposed one surface of the crystal to air and the other gold surface to the solution. Protein A was used as the immobilisation method, with different incubation times and pH buffers investigated. It was noted that immobilisation times longer than 45 min in buffers of pH 5.5 (near the iso-electric point of the protein) produced more complete coverage. This was shown by the lack of significant non-specific protein binding to the crystal. The importance of diffusion was also demonstrated. Mixing of the solution over the crystal during binding caused a sharp increase in the rate of frequency change. This was expected since the liquid depth over the crystal was 50 mm. Regeneration of the protein surface was also demonstrated by the addition of a low pH buffer. The increased frequency shifts observed clearly showed dissociation of the IgG from the protein A surface. The authors mentioned further experiments to see if any irreversible effect was caused to the protein A surface upon repeated exposure to low pH buffers. The authors obtained a frequency change of 1 Hz ng^{-1} of protein immobilised, ten times less than the value predicted by the Sauerbrey equation. They suggested that the reason was due to incomplete binding of their protein and also binding to the "non-resonating" parts of the system. The non-resonating parts referred to by the authors probably mean the areas of the crystal of lower sensitivity, away from the centre of the electrode as explained by Sauerbrey's sensitivity curve [67]. Overall, they demonstrated that continuous real-time monitoring and regeneration of a Pz immunosensor in solution was easily achieved and that countless applications could be found.

Muramatsu et al. described a Pz immunosensor for the detection of *E. coli* [68]. The specific antibody was cross-linked using glutaraldehyde to a layer of silane. Under optimised reaction times the cells could be measured from 10^6 to 10^8 cells mL^{-1}. Enhancement of the response was performed by addition of polystyrene beads coated in the specific antibody. The addition of

this third layer caused a further reduction in frequency and reduced the limit of detection to 10^5 cells mL^{-1}. Different size beads were tested and 1 μm gave best results.

Later Ebersole et al. described a novel method for the immobilisation of biological species [69] based on the spontaneous formation of avidin and streptavidin monolayers onto gold. The monolayers formed irreversibly from aqueous solutions onto freshly formed gold surfaces of Pz crystals; monolayers formed on aged gold surfaces showed lower activity. They demonstrated the use of this method using a DNA hybridisation assay and incorporated their AMISA technique to enhance the measured frequency change.

At the same time Prusak-Sochaczewski et al. also described a Pz immunosensor for the detection of *Salmonella typhimurium* [70]. They also incorporated avidin/biotin chemistry, but immobilised the avidin to a layer of polyethyleneimine, not formed as Ebersole et al. [69]. While not being the first Pz immunosensor to be published for the detection of microbial pathogens, it marked the first systematic attempt to produce a practical Pz immunosensor. The authors compared various immobilisation procedures for the attachment of their monoclonal antibodies to the surface of the crystal. Immobilisation via glutaraldehyde to a thin layer of polyethyleneimine gave best results. Bacterial cells could be detected from 10^5 to 10^9 cells mL^{-1}, although extensive incubation times were needed to measure the lower concentrations. The antibody-coated crystals displayed minimal non-specific binding when exposed to *E. coli* cells. The antibody-coated crystals displayed no detectable loss in activity for up to 4 days when stored at 4 °C, and could be reused for six to eight consecutive assays. This publication marked the way for future sensor developments. All previous Pz immunosensor publications were generally investigating fundamental parameters and performed to show that a certain assay or format could be achieved. This was the first attempt to produce a practical useful sensor. Their systematic approach, i.e. optimising parameters, investigating lifetime, reusability and regeneration would be the approach incorporated by most investigators to follow.

Prusak-Sochaczewski and Luong also published details of a reusable Pz immunosensor for the detection of human albumin [71]. Of the immobilisation methods tested, protein A proved best for the attachment of anti-human albumin to the surface of a Pz crystal. The antigen could be detected from 10^{-4} to 10^{-1} mg mL^{-1}. Regeneration was achieved by saturation of the surface with the antigen followed by subsequent immobilisation of the antibody. This new antibody layer could then be reused for the detection of the antigen. This could be repeated for five assays without loss of sensitivity, after which time sensitivity decreased. This was probably due to distance from the surface, which decays exponentially and damping of the crystals oscillation due to excess mass deposited. IgG could also be removed from the protein A layer by immersion of the crystal in a low pH buffer. But the removal was incomplete, 40% at best, so this method was not a viable method for regeneration

of the surface. Earlier work using protein A layers observed complete removal of the IgG layers under similar conditions [8], the only difference was that the earlier publications used a flowing glycine solution, which obviously helped removal.

The first gas phase immunosensor was also a Pz-based device, developed in 1986 [72]. Crystals coated with anti-parathion antibodies could detect the antigen linearly from 2 to 35 ppb under optimised conditions in real time. The system was reversible and coated crystals could be used for a week without detectable loss in activity. Interferences from similar compounds were only observed when they were present in very large concentrations. To ensure the responses observed were not non-specific adsorption of the antigen to protein molecules, the authors also tested crystals coated with IgG and BSA. Only small irreversible frequency changes of a few Hertz were observed ensuring that the responses observed were specific immunoreactions. Despite this it was noted that the antibodies appeared to be relatively much less selective in this sensor than when compared to their activity in solution. Assuming that this observation was not due to non-specific binding to the crystal, it demonstrated that the activity of antibodies in solution and gas phase was very different. Despite the promising results, the authors discussed the many unknown parameters that needed to be investigated before correct understanding of the sensor response can be achieved. The activity and nature of the immunoreaction in the gas phase is not known or well understood.

A gas phase immunosensor for the detection of cocaine has also been reported [73]. The antibody-coated crystals were used to detect cocaine in air to 0.5 ppb. Responses were observed in 30 s and full recovery was observed in 30 s, indicating a full analysis time of less than 2 min, allowing for base line stabilisation between measurements. Negligible effects were seen from the interferents tested. Coated crystals lasted 3 days without detectable loss in activity.

Preliminary results for a vapour phase tuberculosis Pz sensor have also been published [74]. Polyclonal antibodies against the antigen were immobilised onto the gold of piezoelectric crystals by two methods. Oxidised antibodies were attached to a silane layer and thiolated antibodies, were self-assembled onto the surface, using Trauts reagent. The sensor was used to detect the antigen in nebulised droplets of $\cong$ 10 μm, approximately the size of respiratory droplets. It is envisaged that this sensor could also be used for the direct detection of the analyte in expired breath. The fact that the antigen is only present in the breath of infected patients and completely absent in healthy patients means that quantitative determination is not very important. The sensor could be used for a fast positive/negative response, which is required in a clinical situation.

Obvious advantages over solution-based immunosensors are the fact that real-time analysis is possible while exposing both sides of the crystal to the

sample; in solution only one side can be exposed to the sample. Damping effects due to solution are eliminated, so larger frequency shifts can be expected as well as a behaviour closer to that predicted by the Sauerbrey equation [68], depending on the coating.

Despite the previous examples very little fundamental understanding exists about the nature of biological interactions in the gas phase. More thorough investigations are needed to determine binding affinities, association and dissociation constants, and rates of the antigen/antibody interaction. These then need to be compared to the parameters in the aqueous phase. The activity of the enzyme or antibody could be affected by many factors such as accessibility and reactivity. Orientation of the bio-component, which is affected by its immobilisation method, will probably differ significantly when in gas and liquid phase.

The possibility if gas phase biosensing could even occur has been questioned and caused an amount of controversy [75]. Rajakovic et al. [76] demonstrated the adsorption of pesticides and organics to antibody-coated Pz crystals. The responses observed were completely reversible and were shown to be due to non-specific chemisorption and not selective immunochemical binding. They coated a crystal with parathion antibodies and tested its response to a number of gaseous compounds including parathion. Similar frequency responses were observed for all compounds. Also crystals coated with anti-parathion antibodies, BSA, IgG and anti-valporic acid antibodies showed similar frequency responses to parathion. Crystals coated with anti-valporic acid were equally responsive to valporic acid and parathion. These results indicated that any frequency responses observed were directly due to adsorption of the compounds to the immobilised proteins on the crystal surfaces and not due to selective immunochemical binding by the immobilised antibody. This was not evident in the parathion immunosensor developed by Ngeh-Ngwainbi et al. [72]; where the authors showed that no non-specific binding occurred to crystals coated with non-specific proteins. Despite the views of these authors [76] it is very likely that immunosensors and biosensors in gaseous phase do work. A review on gas phase biosensors has been published [77], which describes numerous examples.

It is likely that in gas phase biosensor's water of hydration is retained in the immobilised structure, since none of authors used forceful drying procedures. It is possible that this water is sufficient to allow the bio-component to act to some extent as if it were in solution. Thus the sensors respond in an expected manner as they would in solution, but with an obvious difference in magnitude of response and activity. Even dry air contains 5 ppm or more of water, which is considered sufficient for the activity of the antibody or enzyme [78]. Partial humidification of the carrier stream has also been suggested so as to retain the behaviour of the biologically active coatings [79]. This area has been specifically reviewed elsewhere [80, 81] and also discussed in general Pz biosensor reviews [82–84].

This initial work paved the way for the development of limitless numbers of Pz immunosensors. The later developments mainly focused on the improvement and optimisation of previous methods to develop sensitive, selective and robust sensors for various analytes. Others concentrated on the development of new immobilisation procedures or assay techniques in order to improve on previous methods.

2.2 Piezoelectric Immunosensor Applications

2.2.1 Bacterial Detection

Conventional bacterial detection methods generally require laborious procedures and many hours or even days for complete analysis. The relatively large mass of bacterial cells, combined with the availability of antibodies to most species means that Pz immunosensor detection offers a very attractive alternative to microbiological methods. Generally giving results in minutes, with adequate sensitivity and selectivity. The earliest Pz immunosensors for bacterial detection have already been discussed [65, 68, 70].

Plomer et al. described a sensor for the detection of enterobacteria [85]. Of the immobilisation procedures tested, protein A was found to be most useful for the immobilisation of the antibodies. Under optimised conditions *E. coli* K12 could be measured from 10^6 to 10^9 cells mL^{-1}. The antibodies used were raised against enterobacterial common antigen (ECA), chosen so as to develop a family-specific Pz immunosensor for the detection of all enterobacteria. But the sensor's response towards other members of the family tested {*Enterobacter*, *Citrobacter*, *Proteus* and *E. coli* (wild strain)} was small. The weak response was due to the smooth surface of these strains, which masked the ECA, a glycophospholipid of the outer bacterial membrane shared by all enterobacteria. Heat treatment allowed release of this ECA, and detection to different degrees of the other strains was possible. It was also discovered that drying the antibody to the surface of the crystal had no detrimental effect on its activity. Regeneration of the antibody surface was not possible since addition of low pH buffers not only removed the antigen but partially removed the antibody layer also. Cleaning of the crystal using NaOH allowed regeneration of the gold surface, which could be reused for more than 50 assays.

König and Grätzel published results of a Pz immunosensor for the detection of various diarrhoea-causing enterobacteria [9]. Common detection methods take 2 days and in about 40% of the cases the causative pathogen cannot be determined. Unlike the sensor developed by Plomer et al. [85], the authors used specific antibodies for each strain since in clinical applications the exact pathogen is often important. Protein A was chosen as the method of

antibody immobilisation since it displayed superior sensitivity, reproducibility and was most stable when compared to other methods. The bacteria tested, including *Camylobacter* and *Shigella*, could be measured from 10^6 to 10^8 cells/crystal. The sensor was also applied to real stool samples. A 5% increase in response was observed and believed to be due to non-specific binding from other bacteria present in the stool sample. The antibody-coated crystals could be stored for 6 weeks and regenerated 25 times without detectable loss in activity.

Su and Li [86] used a SAM of an alkanethiol to immobilise antibodies for the detection of *E. coli* 0157 : H7. A detection limit of 10^3 CFU (colony forming units) mL^{-1} was obtained using dip and dry detection. Poorer performance in solution, dynamic and static, was observed.

A Pz immunosensor for the detection of *Listeria monocytogenes* was published by Jacobs et al. [87]. The bacteria could be measured to 10^5 cells mL^{-1} using dip and dry methods of detection. Analysis carried out directly in solution allowed detection to 5×10^5 cells mL^{-1}. Antibody coated crystals were stored for 17 days without detectable loss in activity. Protein A was used as the immobilisation method but not compared to others. Later the group published details of a displacement assay for the detection of this species [88]. This assay could detect the antigen from 2.5×10^5 to 2.5×10^7 cells/crystal directly in solution monitoring the response in real time. The assay was also performed in milk. Assays in milk were also shown to be specific for *L. monocytogenes*. A later study incorporated SAMs to immobilise antibodies to detect, in real time, this species in solution [89]. The sensor was specific and reusable at least ten times without detectable loss in activity. The sensitivity, however, was poor with a limit of detection of 10^{-7} cells mL^{-1}. Of note, however, was the fact that an increase in frequency was observed upon exposure of the antibody-coated crystal to the specific cells, an observation also seen by Thompson et al. [64].

A displacement assay was also used for the detection of *Pseudomonas aeruginosa* by Bovenizer et al. [90]. However, a poor detection limit of 1.5×10^7 cells mL^{-1} was found and high cross reactivity with other *Pseudomanas* species was observed. The fact that the antibodies showed high selectivity in ELISA indicated that the displacement observed was possibly due to removal of species during the washing steps and not due to any immunological reaction. Kim et al. [91] used thiolated antibodies to detect this species directly in a flowing solution. This sensor had comparable sensitivity to the previous sensor [90] and was used seven times with a relative sensitivity loss of less than 10%. Issues with selectivity were attributed to the specific antibody used.

Adsorption of antibodies was used to develop a sensor for the detection of *Vibrio cholerae* 0139 [92]. A fast detection method was required to measure and differentiate this strain with the Ogawa serotype of the bacteria. The sensor could measure the bacteria to 10^5 cells mL^{-1} against a background of the

Ogawa strain. No cross-reactivity to other bacteria such as *E. coli*, *L. monicytogenes* and *S. marcescens* was observed.

Le et al. described a sensor for the detection of *Staphyococcus aureus* [93]. They used a layer of YWG-$C_{18}H_{37}$ cross-linked with glutaraldehyde to immobilise the specific antibodies to the crystal surface. YWG-$C_{18}H_{37}$ is normally used as a protein purification ligand in chromatography and has a high affinity for antibodies. Under optimised conditions the analyte could be detected from 5×10^5 to 1×10^8 cells mL^{-1} in solution. Antibody-coated crystals could be reused 15 times and stored for 1 month without detectable loss in activity. Negligible response to *P. aeruginosa*, *S. epidermidis* and *E. coli* was observed.

Si et al. used electropolymerised films as functional coatings for antibody immobilisation for the detection of *Staphyococcus aureus* [94]. Antibodies were linked to the film using glutaraldehyde. The antigen was detected from 10^5 to 10^9 cells mL^{-1}. The antibody-coated crystal could be regenerated ten times with minimal loss in activity and was stable for up to 5 weeks, after which time only an 8% loss in activity was observed. It was also found that the sensor performed best at pH 7–8.

A SAM based immobilisation procedure was incorporated by Ben-Dov et al. for the detection of *Chlamydia trachomatis* [95]. A monolayer of cysteamine was formed on the gold surface. Using a suitable cross-linker anti-IgG was immobilised to this monolayer. Anti-*C. trachomatis* was then added to form the active sensing layer. The sensing layer was subsequently exposed to the antigen. Responses were recorded in real time directly in solution. Amplification of the response was shown by the addition of a secondary antibody. The bacteria could be detected from 260 ng mL^{-1} to 7.8 μg mL^{-1}. Analysis of the analyte was also performed directly in urine and the assay was more sensitive than current ELISA tests. The fragmented $F(ab')_2$ antibody-coated crystals were stable for up to 90 days.

Salmonella species have been the most studied bacteria in Pz immunoanalysis because of their importance to the medical field and food industries. The detection of various strains has already been described [9, 69, 71] and further sensors are summarised in Table 1.

An enzymatically amplified sandwich assay was used for the detection of *Helicobacter pylori* infection by Su et al. [103]. Elevated concentrations of the antibody against the bacteria in serum were a sign of infection. Recombinant *H. pylori* protein was immobilised to the crystal by means of an activated thioctic acid monolayer. The surface was then exposed to serum or a solution containing the specific antibody. An enzyme-labelled secondary antibody was then added, followed by an appropriate substrate. As with Ebersole and Ward's AMISA [66], an enzymatically generated product precipitated on the surface of the crystal, thus enhancing and amplifying the frequency change. The assay significantly reduced interferences from non-specific antibodies or other serum components. It was used for the analysis of positive and negative serum, using both dip and dry and real-time monitoring.

Table 1 Detection of Salmonella species using piezoelectric immunosensors. The sensor's linear range and, where available, the limit of detection have been listed

Strain	Detection	Immobilisation	Refs.
S. paratyphi	10^5–10^9 cells mL^{-1}	Electropolymerised films	[96]
S. enteritidis	3×10^5–5×10^8 cells mL^{-1}	Electropolymerised films	[97]
All strains	9.9×10^5–1.8×10^8 cells mL^{-1} 10^5 cells mL^{-1}	Thiolated antibodies	[98, 99]
S. typhimurium	3.2×10^6–4.8×10^8 CFU mL^{-1}	Thiolated antibodies	[36]
S. typhimurium	5.3×10^5–1.2×10^9 CFU mL^{-1} 10^5 CFU mL^{-1}	PEI	[100]
S. typhimurium	10^2–10^7 cells mL^{-1} 350 cells mL^{-1}	Langmuir–Blodgett films	[101]
S. paratyphi	6×10^4 cells mL^{-1}	PEI	[102]
S. typhimurium	8×10^4 cells mL^{-1}		
S. enteritidis	6×10^4 cells mL^{-1}		

Wu et al. also used the elevated antibody levels in serum as an indication of pathogen infection [104, 105]. The concentration of specific antibodies against the parasite *Schistosoma japonicum* was measured. Initially the authors used polymer agglutination to determine the antibody concentration in rabbit serum [104]. It was noted after scanning electron microscopy (SEM) analysis of the crystal surface that adsorption to the crystal surface was a dominant factor in the frequency response. Various parameters were investigated and the antibody was measured linearly from 3.6 to 42.0 μg mL^{-1}. In the later publication the authors immobilised *S. japonicum* antigen to the crystal surface using a copolymer of methacrylate [105]. This was then exposed to a solution containing the specific antibody, whose concentration was proportional to the frequency change upon binding to the immobilised antigen. The antibody was linearly measured from 7.2 to 90.0 μg mL^{-1}. The gold surface could be regenerated and reused 20 times without any detrimental effect on its frequency stability. The sensor was used to measure the degree of infection in rabbit serum. The valent value of binding of the antigen and affinity constant were also estimated.

Other non-immunosensor-based Pz sensors for the detection of bacteria have been produced. He et al. described a separated electrode Pz sensor for *E. coli* [106]. Ebersole et al. used Pz detection to monitor the growth and metabolic rates of *E. coli* cells [107]. Bao et al. developed a sensor for the detection of *Staphylococcus epidermidis* [108]. A Pz-based assay for *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Klebsiella* spp. have also been described [109].

Reviews dealing to some extent with the detection of bacteria using Pz immunosensors have appeared [110, 111].

Pz immunosensors for the detection of other micro-organisms such as plankton have also appeared. The plankton *Chattonella marina*, which causes red tide, was detected directly in sea water from 10^2 to 10^4 cells mL^{-1} by Nakanishi et al. [112]. Immobilisation of the antibodies using protein A and polystyrene gave better results than when using silane or polyethyleneimine. Protein A was the method of choice since a relatively low sensitivity to the cells was observed with the polystyrene-immobilised antibodies. This was due to the fact that fewer antibodies bound to the polystyrene surface and also to random orientation of the immobilised antibodies, leading to deactivation of a high percentage of them due to binding via their Fab sites. No interferences were observed from another plankton that causes red tide, *Alexandrium catenella*. They later described a sensor for the detection of *Alexandrium affine*, another red tide causing plankton [113]. A novel method of antibody immobilisation was incorporated, the use of plasma polymerised films, the theory of which was excellently described earlier by the authors [114]. While this was the first use of this technique in Pz immunosensor work, it had been previously used for Pz gas sensor development [115]. Plasma polymerised coated crystals performed better than polyethyleneimine and silane in terms of sensitivity and reproducibility. The importance of orientated immobilisation was discussed [114]. IgM antibodies were cross-linked via glutaraldehyde to the plasma polymerised coated crystals and were used to detect the plankton directly in seawater. The cells were measured from 10^2 to 10^5 cells mL^{-1} and the sensor showed no response to *A. catenella*.

2.2.2 Viral Detection

The first Pz immunosensor developed for the detection of viruses was reported in 1992 by Kößlinger et al. [116]. They used synthetic human immunodefficiency virus (HIV) peptides adsorbed on a Pz crystal for the detection of anti-HIV antibodies, an indication of HIV infection. The authors used very sensitive 20 MHz crystals to monitor the binding of monoclonal antibodies to the immobilised peptide layer in solution. The high relative standard deviation observed in the measurements was due to insufficiently reproducible experimental conditions. Non-specific binding of other proteins was also observed. Surprisingly, the authors observed that the Sauerbrey formula gave sufficiently accurate values for the frequency observed due to adsorption and binding of proteins.

In a later publication the group incorporated their sensor in a flow system [117]. Detection of the HIV antibodies was also carried out in serum samples. Dilution of the serum in buffer ensured the suppression of non-specific effects and that negligible viscosity effects were observed. As long as the antibody concentration in the serum was not too high and didn't saturate the receptor layer on the crystal surface, several assays could be performed on

each crystal. The sensor's performance was comparable to that of a commercial ELISA HIV kit. Similar results also appeared elsewhere [118].

The group later published details of a sensor for the detection of African swine fever virus and antibodies against the virus [119, 120]. Analysis was carried out in both serum and buffer incorporating a flow injection analysis (FIA) system, using 10 MHz crystals in serum and 20 MHz crystals in buffer. In terms of cost, experimental expenditure, response, regeneration and lifetime, immobilisation of the antibody using dimethyl sulfoxide was best when compared to other methods. Detection of the virus protein was performed in buffer and pig serum; limits of detection were $0.31\,\mu g\,mL^{-1}$ and $1\,\mu g\,mL^{-1}$, respectively. Immobilisation of the virus protein for the detection of the antibodies was best using adsorption for 4 days. Detection limits in serum and buffer, were $0.2\,\mu g\,mL^{-1}$ and $0.1\,\mu g\,mL^{-1}$, respectively. The use of different blocking solutions and the addition of substances to counteract viscosity effects were also investigated.

König and Grätzel published details of a sensor for the detection of diarrhoea-causing bacteria and viruses [9]. Immunosensors were described for the detection of rotavirus and adenovirus. Both viruses were measured linearly from $10^6$to 10^{10} virions. As explained earlier, protein A was shown to be the most favourable immobilisation method but immobilisation using silane and polyethyleneimine showed a 40% increase in response when detecting rotavirus in stool samples.

They also developed a sensor for the detection of herpes viruses [43]. The sensor was used to specifically detect five human herpes viruses, herpes simplex type 1 and 2, viracella-zoster virus, cytomegalovirus and Epstein-barr virus. Of the immobilisation procedures tested, protein A was best in terms of reusability, sensitivity and stability. Each virus was measured linearly from 54 to 1×10^9 virions/crystal. The sensor was reusable 18 times and stable for 8 weeks without detectable loss in activity. When applied to complex human specimen no non-specific effects were observed and the sensor performed identically for each virus as it did in buffer.

The authors later published details of a sensor for the detection of human hepatitis viruses type A and B [44]. Immobilisation using protein A gave best results. Both types of virus could be detected linearly from 10^5 to 10^{10} virions mL^{-1} in solution. The sensor was stable for 4 weeks and could be reused ten times without detectable loss in activity. Results were comparable to those of a commercial ELISA kit.

Susmel et al. describes a competitive immunoassay for the detection of human cytomegalovirus [121]. Direct capture of the virus from solution, using a number of immobilisation procedures, yielded unsatisfactory results, contrary to what was observed before [43]. A competitive assay performed using a monolayer of poly-L-lysine (covalently attached to a monolayer of thiosalicylic acid) yielded a preliminary linear range of $2.5–5\,\mu g\,mL^{-1}$ of gB epitope and a limit of detection of $1\,\mu g\,mL^{-1}$.

Su et al. adsorbed recombinant porcine reproductive and respiratory syndrome virus (PRRSV) protein to the surface of a Pz crystal to detect infection in pigs by the presence of PRRSV antibodies [122]. As seen in earlier work [119, 120], immobilisation of the protein via self-assembly led to deactivation, thus no significant binding of the antibody was observed. Analysis in pig serum was carried out, using both dip and dry and real time analysis. A one in ten dilution in serum was used as a compromise between loss of sensitivity and suppression of non-specific effects. Screening of positive and negative sera displayed similar results to that of a commercial ELISA kit. Regeneration of the protein-coated crystal was possible four times, after which 92% of its activity was still retained. Using harsher conditions the gold surface could be easily regenerated for reuse.

Rickert et al. described a sensor for the detection of antibodies against the foot and mouth disease virus [123–125]. Synthetic peptides constituting an epitope of the virus were immobilised using activated self-assembled thiols. The antibodies were detected in a flowing solution in real time. Regeneration of the surface was also performed.

Lee and Chang [126] used a cystamine SAM to immobilise antibodies to develop a sensor for the detection of bovine ephemeral fever virus. The unoptimised sensor was used in a flowing solution and detected the antigen in real time to 5 $\mu g\,mL^{-1}$. Positive results were observed in bovine sera samples.

2.2.3 Clinical Analysis

The detection of some of the above bacteria and viruses are of major clinical importance. The development of sensors for various analytes of clinical importance, especially human blood products has been intensely investigated.

König and Grätzel first reported the Pz detection of human erythrocytes [127, 128]. The later was an improved version of the sensor. Polyethyleneimine was used to immobilise the antibody to the crystal, which was stable for 10 weeks, if stored dry at room temperature or 4 °C, without detectable loss in activity. Regeneration of the surface was improved from eight to 12 times without detectable loss in activity. This was done by the addition of a synthetic peptide, which competed for the bound antigen and allowed regeneration of the antibody surface without the use of harsh chemicals. Analysis in blood was carried out.

A sensor for the detection of human granulocytes was also reported by the authors [47]. Immobilisation was carried out using silane, protein A and polyethyleneimine. Similar results were observed for each method but the later showed better stability. The antigen was measured linearly in blood from 2×10^3 to 3×10^5 cells. The antibody-coated surface was stable for 8 weeks without detectable loss in activity. Regeneration was not possible since removal of all the bound cells even under harsh conditions did not occur.

The authors again used polyethyleneimine to immobilise antibodies for the detection of T-lymphocytes [48] and B-lymphocytes [49]. T-lymphocytes were measured linearly from 5×10^3 to 4.5×10^5 cells. The antibody-coated crystals were stable for 10 weeks without detectable loss in activity. Regeneration was not tested. A 10% increase in response was observed when tested in whole blood, due to non-specific binding. B-lyphocytes could be measured linearly from 5×10^3 to 5.6×10^5 cells, in blood and buffer. The antibody-coated crystal was stable for 6 weeks and could be regenerated eight times without detectable loss in activity.

Many authors have developed Pz sensors capable of detecting IgGs, but generally these are used as test analytes in fundamental investigations or the development of new techniques and immobilisation methods.

Raman Suri et al. described a sensor for the detection of IgM [129]. They removed the central 5 mm of the gold electrode surface to expose more Pz surface for chemical modification. This causes an increase in the resonance frequency and a reduction of the *Q* factor, but the crystal still vibrates. This surface was then refluxed in acid to produce stable hydroxyl groups. Modification with tresyl chloride gave more surface coverage than silane and showed better sensitivity when protamine was immobilised. IgM could be measured linearly from 10^{-5} to 10^{-2} mg mL^{-1}, with a limit of detection of 10 ng mL^{-1}. Tresyl chloride treated crystals were stable for 5 days without significant loss in activity whereas silane treated crystals were only stable for 3 days. Negligible interferences were observed from IgG.

Chu et al. also described a sensor capable of detecting IgM in solution [130]. The authors used an array of 5 Pz crystals coated with different ratios of antibodies against two analytes, IgM and C-reactive protein. The array was then used for the simultaneous analysis of the dual analytes in solution. Of the linear regression models tested, least terminated squares gave best results for the estimation of analyte concentrations in mixed unknown samples. The authors also carried out kinetic studies on the immunoreaction of IgM [131]. An activated methacrylate copolymer was used to immobilise the antibodies against IgM. Various immunoreaction kinetic parameters were determined under varying conditions by continuous monitoring of the reaction using the QCM.

IgA was measured in real time, directly in saliva by Tajima et al. [132]. Immobilisation of the antibody using cysteamine gave superior results to that of adsorption. The antibody-coated crystal was reusable for 15 times without detectable loss in activity. The concentration of IgA measured in saliva using the Pz sensor was confirmed using ELISA.

Su et al. incorporated SAMs in the development of a Pz sensor for IgE [133]. Thioctic acid and cysteamine, coupled with EDC and glutaraldehyde respectively, were tested. IgE was measured linearly from 5 to 300 IU mL^{-1} and the antibody-coated crystal could be regenerated five times without detectable loss in activity. The SAM coated crystals showed three

to five times less non-specific binding than crystals prepared with other methods (polyethyleneimine, silane and protein A). The sensor's performance was comparable to that of two commercially available IgE test kits.

The detection of complement proteins has also received much attention. Pei and coworkers described a Pz immunosensor for the detection of complement C4 [134, 135]. The authors first used adsorption to immobilise the specific antibody, showing better results than for the use of polyethylenieimine [134]. They measured the protein linearly from 0.1 to 10 $\mu g\,mL^{-1}$. The sensor showed no response to any materials present in serum and the gold surface could be regenerated 15 times for repeated use. Later they self-assembled the antibodies onto a layer of protein A, a method which gave superior results to the previously tested immobilisation procedures [135]. This setup gave a better linear range of 5×10^{-4}–1×10^{-2} $\mu g\,mL^{-1}$, but the crystal was only reusable about ten times.

The group later published a similar sensor for the detection of complement C6 [136]. Immobilisation of the antibody via polyethyleneimine showed superior results to physical adsorption. Under optimised conditions the protein could be determined linearly from 10^{-4} to 10^{-2} $\mu g\,mL^{-1}$. The gold surface could be regenerated 12 times for repeated use before loss of sensitivity due to damage of the gold was observed.

Chu et al. used polymer agglutination to determine the concentration of complement C3 in serum [137]. Determination of the protein concentration under optimised conditions using initial rates and end-point methods produced linear ranges of 22.0–49.1 $\mu g\,mL^{-1}$ and 22.0–43.2 $\mu g\,mL^{-1}$, respectively. Analysis of clinical samples showed that the Pz method was comparable to turbimitry analysis.

Deng et al. [138] used a novel immobilisation procedure based on a nafion membrane to detect compliment C4. The well optimised sensor was easily prepared and allowed regeneration of the nafion surface. It could detect C4 from 0.08 to 1.6 $\mu g\,mL^{-1}$.

Lepesheva et al. incorporated the use of Langmuir–Blodgett IgG films, on Pz crystals for immunosensing [139]. Ferritin was used as a model and could be detected from 10^{-10} to 10^{-7} M.

Chu et al. used an activated copolymer of methacrylate for antibody immobilisation to develop a Pz immunosensor for the detection of α-fetoprotein in serum [140]. The analyte was measured linearly from 100 to 800 $ng\,mL^{-1}$ and no interferences from other serum components were observed. Valent values and affinity constants of the immunoreaction were also determined. Comparable results to RIA were obtained.

Pei et al. used protein A immobilisation in the development of a sensor for fibrin in serum [141]. The immobilisation gave better results than those obtained with polyethyleneimine and adsorption of BSA, glutaraldehyde was used to cross-link the antibodies to the adsorbed BSA layer. Under optimised conditions the analyte could be measured from 10^{-4} to 10^{-2} $g\,L^{-1}$. The

antibody-coated surface could be stored for 6 weeks without detectable loss in activity. No interferences from other serum components were observed and the gold surface could be regenerated ten times before loss in sensitivity was observed.

Shao et al. used polyethyleneimine-coated crystals to measure haemoglobin (bovine) directly in solution from 0.001 to 0.1 mg mL^{-1} [142]. No interference was observed from BSA and the effect of different liquid depths over the crystal was also investigated.

Muratsugu et al. described a sensor for the detection of human serum albumin (HSA) [143]. Adsorption of the antibody was the immobilisation method used. The antigen binding was monitored in real time directly in a flowing solution. The system could specifically detect HSA in the presence of BSA. The antigen could be measured from 0.1 to 100 μg mL^{-1}. The frequency change observed by the authors was larger than that predicted by the Sauerbrey equation; also the binding of the HSA was larger than that of the adsorbed antibody. This was related to the hydrophobicity and hydrophilicity of proteins.

Sakai et al. also described a sensor for the detection of HSA in a flowing solution [144]. After initial capture of HSA by the immobilised monoclonal antibodies, a solution of secondary polyclonal antibodies was added, thus performing a sandwich assay. This sandwich method was effective in significantly increasing the sensitivity of the assay due to the larger mass of the polyclonal antibodies compared to HSA. The sensor showed a linear response to the antigen from 0 to 20 ppm. The antibody-coated crystal could be reused for 30 assays over 4 days without significant loss in activity.

Xia et al. also described a Pz immunosensor for the detection of HSA based on polymer agglutination [145]. The analysis was carried out in serum but had a much lower sensitivity than the previous sensors, linearly detecting the antigen from 112 to 878 μg mL^{-1}. Rubbing the crystal and washing allowed regeneration of the surface and reuse of the crystal. The authors used a carboxymethyl cellulose polymer.

Polymer agglutination has also been applied to other analytes of clinical importance. The technique was pioneered by Kurosawa and coworkers in 1990 [17]. They used it for the analysis of C-reactive protein (CRP). In a recent publication further details of its use for the detection of CRP have appeared [146]. It was successfully used in serum for the diagnosis of disease. The technique termed latex piezoelectric immunoassay (LPEIA) was also applied for the detection of antistreptolysin O antibodies in serum [18]. The technique was very rapid, requiring an assay time of only 2–3 min, incorporating the initial rates method. Only 20 μL of serum was needed to perform an assay. The antigen could be measured up to 1040 IU mL^{-1}. It was shown that the latex particles in solution prevented adsorption of proteins to the crystal, thus eliminating non-specific effects. Clinical samples were tested and LPEIA results were confirmed using turbidimetry.

Ghourchian et al. later applied LPEIA to the detection of rheumatoid factor [147]. Stable oscillation of crystals in PBS solutions generally required sealing of one side of the crystal. This was often time consuming and altered the sensitivity of each crystal, resulting in irreproducibility from crystal to crystal. The authors improved on this by designing a cell which exposed only one side of the crystal to the solution, still allowing stirring thermostatic control of the solution over the other surface. A novel surface regeneration method was also employed and allowed 38 assays to be carried out using one crystal without detectable loss in activity. Under optimised conditions the antigen could be measured from less than 5–5.5 IU mL^{-1}. The effect of HSA and BSA were also investigated.

In combined publications both groups describe the application of LPEIA in the clinical analysis of CRP, antistreptolysine O antibody and rheumatoid factor [148, 149].

Zhang et al. [150] used a novel micro-array of crystals to develop a sensor for human chronic gonadotropin. The SAM-based system retained 70% of its activity after five regenerations and had a range of 2.5–500 mIU mL^{-1}. Its performance in real urine and serum samples was comparable to radioimmunoassay.

The application of a novel immobilisation procedure, based on cystamine SAMs and chitosan/alginate multilayers, has been demonstrated in the detection of factor B [151]. A positively charged chitosan layer was immobilised via glutaraldehyde to a cystamine SAM on the crystal surface. A layer of alginate, with covalently bound antibodies, was then electrostatically bound to the charged chitosan layer. This technique allowed a fast and reproducible regeneration of the chitosan layer, with no detectable loss in activity after eight attempts. The use of this chitosan/alginate layer demonstrated increased sensitivity when compared to direct antibody immobilisation to the cystamine SAM. Analysis in serum samples was also carried out.

Michalzik et al. [152] developed an immunosensor for the detection of bone morphogenic protein-2. 20 MHz crystals were incorporated to increase the potential sensitivity of the sensor, which had a limit of detection of 0.5 $\mu g\, mL^{-1}$. Immobilisation was performed via protein A covalently bound to a SAM of cystamine and the sensor was reused six times without detectable loss in activity. More important, however, was the fact that this publication demonstrated a practical application of this group's excellent miniaturised flow system [153]. The crystals incorporated consisted of 2 mm gold electrodes and were only 3 mm diameter in total. The flow cell was just 5.5 mm in width. The possibility of applying this technology to microfluidic transducer arrays was mentioned.

2.2.4 Low Molecular Weight Analytes

The detection of low molecular weight analytes of importance including steroids, herbicides, pesticides and toxins have also appeared. Generally, because of their low molecular weight, assay formats other than direct captures are performed or their presence is measured indirectly. The direct capture of such analytes can often lead to a sensor with low sensitivity due to minor frequency changes. Table 2 summarises some of the Pz immunosensors developed for the detection of low molecular weight analytes.

Masson et al. described a bioaffinity Pz sensor for biotin [174]. The sensor was based on the displacement of avidin from the crystal surface, which caused a large frequency increases when biotin was added to the solution. The surface was easily regenerated by addition of more avidin after analysis.

Table 2 Detection of low molecular weight analytes using piezoelectric immunosensors. Capture assays were used unless otherwise stated

Analyte	Detection	Immobilisation	Assay	Refs.
Insulin	10^{-6}–10^{-1} mg mL^{-1}	Protein A	Capture	[154]
Insulin		Spin coated	Capture polystyrene	[2]
Cortisol	36–3628 ppb	Protein A	Capture	[42]
Cocaine	10–300 ppb	Protein B	Capture	[155]
Cocaine	100 pmol L^{-1}	SAM	Competitive	[156]
Metamphetimine	0.02–100 ppm	Adsorption	Competitive	[157]
2-Phenyloxazolone	10^{-5}–10^{-8} M	Adsorption	Competitive	[158, 159]
Ricin	0.5 μg	Adsorption	Capture	[160]
Staphyllococcal enterotoxin B	0.1–10 μg mL^{-1}	Adsorption	Competitive	[161]
Staphyllococcal enterotoxin C_2		Protein A	Capture	[162, 163]
Staphyllococcal enterotoxin			Capture	[164]
Atrazine	0.03–100 μg mL^{-1}	Protein A	Capture	[165, 166]
Atrazine	0.1–100 ppb	Silane	Competition	[167]
Atrazine	0.01–1 ng mL^{-1} 0.001 ng mL^{-1}	Polystyrene	Competition	[168]
Atrazine	0.1 μg L^{-1}	Silane	Competition	[169]
2,4-Dichlorophenoxyacetic acid	0.001–100 ppb	Silane	Competitive	[170, 171]
Polycyclic aromatic hydrocarbons	1–4 nM	SAM	Displacement	[172]
Polychlorinated biphenyls			Capture Competition	[173]

The assay was very versatile and would be especially useful in the detection of low molecular weight analytes. The authors demonstrated its use using a FIA system.

Horacet and Sckladal used direct competitive assays of two test analytes, 2,4-D and 4,4′-dichlorobiphenyl, to investigate the effect of organic solvents on immunoassays [175]. The effect of different concentrations of methanol on the immunoreaction was investigated. It was demonstrated that hydrophobicity of the solvent was the important factor when determining the effect of the solvent; the less soluble the solvent was in water the lower the influence it had on the immunointeraction. The authors also performed a real-time competitive assay in toluene. The first time an immunoaffinity interaction in pure solvent has been shown. The effect on affinity and kinetics were discussed.

An enzyme-based Pz biosensor for the detection of organophosphorous and carbamate pesticides has also appeared [176]. The assay was based in the inhibitory effect of the pesticides on immobilised acetylcholinesterase. After exposure of the enzyme to the pesticides, the substrate 3-indoyl acetate was added. This was enzymatically converted to an insoluble product, the concentration of which was determined by the resulting frequency change. The rate and amount of conversion was due to the concentration of the active immobilised enzyme, which was dependent on the amount of pesticide present. The system was very similar to that used by Ebersole and Ward in their AMISA [66]. The authors measured paroxon to 5×10^{-8} M and carbaryl to 1×10^{-7} M.

2.3 Future Trends

The main disadvantage of Pz immunosensors with a view to commercialisation seems to be their stability. As can be seen from the many previous examples they generally have a working lifetime of only a few days. Even unused sensors only last a number of weeks. This lack of stability is unacceptable if commercial success is to be realised.

The Pz sensor is sensitive enough on its own to theoretically measure what is required. The antibodies are quite robust and stable when stored correctly, retaining their activity for long periods. The problems arise when the two are combined. Separately, each of the components easily has the required sensitivity and specificity for the specific detection. But a lot of this is lost when they are coupled. Even with all the advances in immobilisation techniques no significant improvement in overall sensor performance has been found, rendering most Pz immunosensors useless outside the laboratory environment. Further research is needed to try and find a method of extending the lifetime of antibody-coated crystals. Only then can their advantages be fully exploited. Another solution could be to find an alternative to the antibodies, something that retains their main advantages of biologically optimised specificity and

affinity, but is more versatile and robust. The use of synthetic peptides have already been incorporated into Pz immunosensors [116–118, 123–125]. As chemical synthesis improves, the production of molecularly imprinted polymers with the selectivity of antibodies is also a real possibility. Combinatorial techniques, which are widely used in drug production, have already been applied to the development of antibody mimics. While the initial production costs would be large, mass production would generally be very cheap, once a suitable molecule has been produced. These synthetic mimics can have the desired specificity, are cheap to produce and are generally very stable.

These synthetic alternatives are much more versatile than antibodies and can be functionalised or easily tailored for individual needs without affecting their specificity. At the moment specificity is their major drawback but with developing techniques this is improving and getting closer to that of antibodies. With these technologies the production of cheap and robust, specifically coated crystals with good stability should be possible. Then the successful commercialisation of Pz sensors would be a real possibility.

The use of sensor arrays should also play an important role in the future. They could be used to simultaneously give an accurate account of many different analytes and an overall result at once. For example, an array of Pz sensors coated with specific antibodies against different freshwater cyanobacterial toxins could be used to give an overall toxicity indication and also a specific result on the concentration and type of each toxin present. Incorporation of miniaturised flow cells and crystals, such as the excellent system developed by Michalzik et al. [152, 153], could allow real-time monitoring directly in solution.

Sakti et al. have shown how a simple modification of the crystal surface can greatly improve its performance and increase the lifetime of the silver electrodes [2]. At the same time this provides a good matrix for immobilisation. This sort of modification can help improve the commercial potential of a sensor.

Theoretically Pz immunosensors are still very promising. In the short few years since the first immunosensor huge developments have been made. As more fundamental research paves the way for a better understanding of the exact mechanistics, the benefits should be conveyed in improved sensors. To overcome the stability problem a completely new approach may be required, rather than just trying to improve on already existing techniques.

References

1. Nakanishi K, Karube I, Hiroshi S, Uchida A, Ishida Y (1996) Anal Chim Acta 325:73
2. Sakti SP, Rosler S, Lucklum R, Hauptmann P, Buhling F, Ansorge S (1999) Sensor Actuator A 76:98
3. Richman DD, Cleveland PH, Oxman MN, Johnson KM (1982) J Immunol 128:2300

4. Muramatsu H, Dicks JM, Tamiya E, Karube I (1987) Anal Chem 59:2760
5. Moks T, Abrahmsen L, Nilsson B, Hellman U, Sjqöuist J, Uhlen M (1986) Eur J Biochem 156:637
6. Horisberger M, Clerk MF (1985) Histochemistry 82:219
7. Prusak-Sochaczewski E, Luong JHT (1990) Anal Lett 23:401
8. Davis KA, Leary TR (1989) Anal Chem 61:1227
9. König B, Grätzel M (1993) Anal Lett 26:1567
10. Roederer JE, Bastiaans GJ (1983) Anal Chem 55:2333
11. Weethall HH, Curley MF (1996) Sensor Actuator B 36:527
12. Raman Suri C, Mishra GC (1996) Biosens Bioelectron 11:1199
13. Barnes C, D'Silva C, Jones JP, Lewis TJ (1991) Sensor Actuator B 3:295
14. Nakanishi K, Muguruma H, Karube I (1996) Anal Chem 68:1695
15. Kurosawa S, Kamo N, Matsui D, Kobatake Y (1990) Anal Chem 62:353
16. Wu Z, Yan Y, Shen G, Yu R (2000) Anal Chim Acta 412:29
17. Kurosawa S, Tawara E, Kamo N, Ohta F, Hosokawa T (1990) Chem Pharm Bull (Tokyo) 38:1117
18. Muratsugu M, Kurosawa S, Kamo N (1992) Anal Chem 64:2483
19. Frey BL, Corn RM (1996) Anal Chem 68:3187
20. Nuzzo RG, Fusco FA, Allara DL (1987) J Am Chem Soc 109:2358
21. Ohtani M, Sunagawa T, Kuwabata S, Yoneyama H (1995) J Electroanal Chem 396:97
22. Nuzzo RG, Zegarski BR, Dubios LH (1987) J Am Chem Soc 109:733
23. Bain CD, Troughton EB, Tao YT, Evall J, Whitesides GM, Nuzzo RG (1989) J Am Chem Soc 111:321
24. Schessler HM, Karpovich DS, Blanchard GJ (1996) J Am Chem Soc 118:9645
25. Dong S, Ling J (1997) Bioelectrochem Bioenerget 42:7
26. Wink T, Zuilen SJ, Bult A, van Bennekom D (1997) Analyst 122:43R
27. Su X, Chew FT, Li SFY (1999) Anal Biochem 273:66
28. Duan C, Mayerhoff ME (1994) Anal Chem 66:1369
29. Frey BL, Jordan CE, Kornguth S, Corn RM (1995) Anal Chem 67:4452
30. Caruso F, Rodda E, Furlong N (1996) J Coll Intr Sci 178:104
31. Neuman MR, Perlaky SC, Mickalski JP, Bass JB, McCombs CC (1995) EMBC 95, paper 7.1.3.8
32. Legget GJ, Roberts CJ, Williams PM, Davies MC, Jackson DE, Tendler SJB (1993) Langmuir 9:2356
33. Jue R, Lambert JM, Pierce LR, Traut RR (1978) Biochemistry 17:5399
34. Lambert JM, Jue R, Traut RR (1978) Biochemistry 17:5406
35. Kenny JW, Fanning TG, Lambert JM, Traut RR (1979) J Mol Biol 151
36. Park IS, Kim WY, Kim N (2000) Biosens Bioelectron 15:167
37. Aizawa M, Yun K, Haruyama T, Yanagida Y, Kobatake E (1998) Supramol Sci 5:761
38. Spangler BD, Tyler BJ (1999) Anal Chim Acta 399:51
39. Taylor RF (ed) (1991) Protein immobilisation, fundamentals and applications. Dekker, New York, p 278
40. Lepesheva GI, Turko IV, Ges IA, Chashchin VL (1994) Biochem (Moscow) 59:701
41. Plomer M, Guilbault GG, Hock B (1992) Enzyme Microb Technol 14:230
42. Atilli BS, Suleman AA (1995) Anal Lett 28:2149
43. König B, Grätzel M (1994) Anal Chem 66:341
44. König B, Grätzel M (1995) Anal Chim Acta 309:19
45. Raman Suri C, Jain PK, Mishra GC (1995) J Biotechnol 39:27
46. Carter RM, Jacobs MB, Lubrano GJ, Guilbault GG (1995) Anal Lett 28:1379
47. König B, Grätzel M (1993) Anal Lett 26:2313

48. König B, Grätzel M (1993) Anal Chim Acta 281:13
49. König B, Grätzel M (1994) Bioorg Med Chem Lett 20:2429
50. King WH Jr (1963) International symposium on humidity and moisture, paper C15.11
51. King WH Jr (1964) Anal Chem 36:1735
52. Oberg P, Ligensjo J (1959) Rev Sci Instrumen 30:1053
53. Hlavay J, Guilbault GG (1977) Anal Chem 49:1890
54. Guilbault GG (1980) Ion-Selec Electrode Rev 2:3–16
55. Guilbault GG (1981) Intern J Environ Anal Chem 10:89
56. Alder JF, McCallum JJ (1983) Analyst 108:1169
57. Guilbault GG, Jordan JM (1988) Crit Rev Anal Chem 19:1
58. Guilbault GG (1983) Anal Chem 55:1682
59. Shons A, Dorman F, Najarian N (1972) L Biomed Mat Res 6:565
60. Olivera JR, Silver SP (1980) US Patent No. 4242096
61. Rice TK (1980) US Patent No. 4236893
62. Rice TK (1982) US Patent No. 4314821
63. Konash PL, Bastiaans GJ (1980) Anal Chem 52:1929
64. Thompson M, Arthur CL, Dhaliwal GK (1986) Anal Chem 58:1206
65. Muramatsu H, Kijiwara K, Tamiya E, Karube I (1986) Anal Chim Acta 188:257
66. Ebersole RC, Ward MD (1988) J Am Chem Soc 110:8623
67. Sauerbrey G (1959) Z Phys 155:206
68. Muramatsu H, Watanabe Y, Hikuma M, Ataka T, Kubo I, Tamiya E, Karube I (1989) Anal Lett 299:2155
69. Ebersole RC, Miller JA, Moran JR, Ward MD (1990) J Am Chem Soc 112:3239
70. Prusak-Sochaczewski E, Luong JHT, Guilbault GG (1990) Enzyme Microb Technol 12:173
71. Prusak-Sochaczewski E, Luong JHT (1990) Anal Lett 23:401
72. Ngeh-Ngwainbi J, Foley PH, Kuan SS, Guilbault GG (1986) J Am Chem Soc 108:5444
73. Hahn EC (1988) PhD Thesis, University of New Orleans, New Orleans LA
74. Neuman MR, Perlaky SC, Mickalski JP, Bass JB, McCombs CC (1995) EMBC, paper 7.1.3.8
75. Borman S (1987) Anal Chem 59:1161A
76. Rajakovic J, Ghaemmaghami V, Thompson M (1989) Anal Chim Acta 217:111
77. Barzana E (1995) Adv Biochem Eng Biotechnol 53:1
78. Guilbault GG, Schmid RD (1991) Electrochemical, piezoelectric and fibre optic biosensors. In: Turner APF (ed) Advances in biosensors, vol 1. JAI-Elsevier, Amsterdam, P 264
79. Luong JHT, Guilbault GG (1991) Analytical applications of piezoelectric crystal biosensors. In: Blum LJ, Coulet PR (eds) Biosensors, principles and applications. Dekker, New York
80. Guilbault GG, Luong JHT (1988) J Biotech 9:1
81. Guilbault GG (1988) 5th Symposium on ion-selective electrodes, Matrafured
82. Bunde RL, Jarvi EJ, Rosentreter JJ (1998) Talanta 46:1223
83. Ngeh-Ngwainbi J, Suleiman AA, Guilbault GG (1990) Biosens Bioelectron 5:13
84. Walton PW, Butler ME, O'Flaherty MR (1991) Biochem Soc Trans 19:44
85. Plomer M, Guilbault GG, Hock B (1992) Enzyme Microb Technol 14:230
86. Su XL, Li Y (2004) Biosens Bioelectron 19:563
87. Jacobs MB, Carter RM, Lubrano GJ, Guilbault GG (1995) Am Lab 26
88. Minunni M, Mascini N, Carter RM, Jacobs MB, Lubrano GJ, Guilbault GG (1996) Anal Chim Acta 325:169

89. Vaughan RD, O'Sullivan CK, Guilbault GG (2001) Enz Microb Technol 29:635
90. Bovenizer JS, Jacobs MB, O'Sullivan CK, Guilbault GG (1998) Anal Lett 31:1287
91. Kim N, Park IS, Kim DK (2004) Sensor Actuator B 100:432
92. Carter RM, Mekalanos JJ, Jacobs MB, Lubrano GJ, Guilbault GG (1995) J Immunol Methods 187:121
93. Le D, He FJ, Jiang TJ, Nie L, Yao S (1995) J Microbio Meth 23:229
94. Si SH, Chen JH, He FJ, Nie LH, Yao SZ (1996) Chinese J Chem 14:222
95. Ben-Dov I, Willner I, Zisman E (1997) Anal Chem 69:3506
96. Si S, Ren F, Cheng W, Yau S (1997) Fresenius J Anal Chem 357:1101
97. Fung YS, Si SH, Zhu DR (200) Talanta 51:151
98. Park IS, Kim N (1998) Biosens Bioelectron 13:1091
99. Park IS, Kim N (1998) Biosensors '98, Berlin, 3–5 June
100. Ye JM, Letcher SV, Rand AG (1997) J Food Sci 62:1067
101. Pathirana ST, Barbaree J, Chin BA, Hartell MG, Neely WC, Vodyanoy V (2000) Biosens Bioelectron 15:135
102. Wong YY, Ng SP, NG MH, Si SH, Yao SZ, Fung YS (2002) Biosens Bioelectron 17:676
103. Su X, Li SFY (2001) Anal Chim Acta 429:27
104. Wu ZY, Shen GL, Yu RQ, Wang SP, Zeng XF (1997) Chem J Chinese U 18:1774
105. Wu ZY, Shen GL, Li ZQ, Wang SP, Yu RQ (1999) Anal Chim Acta 398:57
106. He F, Geng Q, Zhu W, Nie L, Yao S, Meifeng C (1994) Anal Chim Acta 289:313
107. Ebersole RC, Foss RP, Ward MD (1991) Bio Technol 9:450
108. Bao L, Deng L, Nie L, Yao S, Wei W (1996) Anal Chim Acta 319:97
109. Ishimori Y, Karube I, Suzuki S (1981) Appl Environ Microb 42:632
110. Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E (1999) Biosens Bioelectron 14:599
111. Guilbault GG, Luong JHT (1995) Piezoelectroc immunosensors and their applications in food analysis. In: Guilbault GG, Wagner G (eds) Food biosensor analysis. Dekker, New York
112. Nakanishi K, Karube I, Hiroshi S, Uchida A, Ishida Y (1996) Anal Chim Acta 325:73
113. Nakanishi K, Masoe A, Sako Y, Ishida Y, Muguruma H, Karube I (1996) Anal Lett 29:1247
114. Nakanishi K, Muguruma H, Karube I (1996) Anal Chem 68:1695
115. Kurosawa S, Kamo N, Matsui D, Kobatake Y (1990) Anal Chem 62:353
116. Kößlinger C, Drost S, Aberl F, Wolf H, Koch S, Woias P (1992) Biosens Bioelectron 7:397
117. Kößlinger C, Drost S, Aberl F, Wolf H (1994) Fresenius J Anal Chem 349:349
118. Aberl F, Wolf H, Kößlinger C, Drost S, Woias P, Koch S (1994) Sensor Actuator B 18–19:271
119. Uttenhaler E, Kößlinger C, Drost S (1998) Anal Chim Acta 362:91
120. Uttenhaler E, Kößlinger, Drost S (1998) Biosens Bioelectron 13:1279
121. Susmel S, O'Sullivan CK, Guilbault GG (2000) Enzyme Microb Technol 27:639
122. Su XD, Li SFY, Liu W, Kwang J (2000) Analyst 125:725
123. Rickert J, Weiss T, Kraas, Jung G, Gopel W (1996) Biosens Bioelectron 11:591
124. Rickert J, Weiss T, Gopel W (1996) Sensor Actuator B 31:45
125. Rickert J, Brecht A, Gopel W (1997) Biosens Bioelectron 12:567
126. Lee YG, Chang KS (2005) Talanta 65:1335
127. König B, Grätzel M (1992) Anal Chim Acta 276:329
128. König B, Grätzel M (1993) Anal Chim Acta 280:37
129. Raman Suri C, Raje M, Mishra GC (1994) Biosens Bioelectron 9:535
130. Chu X, Jiang JH, Shen GL, Yu RQ (1996) Anal Chim Acta 336:185
131. Chu X, Lin ZH, Shen GL, Yu RQ (1996) Chem J Chinese U 17:1025

132. Tajima I, Asami O, Sugiura E (1998) Anal Chim Acat 365:147
133. Su X, Chew FT, Li SFY (1999) Anal Biochem 273:66
134. Hu JM, Pei RJ, Hu Y, Zeng YE (1998) Chinese J Chem 16:219
135. Pei RJ, Hu JM, Hu Y, Zeng Y (1998) J Chem Technol Biotech 73:59
136. Hu J, Liu L, Danielsson B, Zhou X, Wang L (2000) Anal Chim Acta 423:215
137. Chu X, Shen GL, Yu RQ (1996) Analyst 121:1689
138. Deng T, Li JS, Wang H, Shen GL, Yu RQ (2004) J Immunol Methods 299:1
139. Lepesheva GI, Turko IV, Ges IA, Chashchin VL (1994) Biochem (Moscow) 59:701
140. Chu X, Lin ZH, Shen GL, Yu RQ (1996) Chem J Chinese U 17:870
141. Pei RJ, Hu JM, Hu Y, Zeng YE (1998) Chem J Chinese U 19:363
142. Shao B, Hu Q, Hu J, Zhou X, Zhang W, Wang X, Fan X (1993) Fresenius J Anal Chem 346:1022
143. Muratsugu M, Ohta F, Miya Y, Hosokawa T, Kurosawa S, Kamo N, Ikeda H (1993) Anal Chem 65:2933
144. Sakai G, Saiki T, Uda T, Miura N, Yamazoe N (1995) Sensor Actuator B 24:134
145. Xia C, Guoli S, Feiye X, Ruqin Y (1997) Anal Lett 30:1783
146. Kurosawa S, Aizawa H, Yoshimioto M (2000) IEEE T Ultrason Ferr 47:1256
147. Ghourchian HO, Kamo N, Hosokawa T, Akiyawa T (1994) Talanta 41:401
148. Kurosawa S, Muratsugu M, Ghourchian HO, Kamo N (1990) ACS Symp Ser 657:185
149. Muratsugu M, Kurosawa S, Ghourchian HO, Kamo N (1997) Bunseki Kagaku 46:917
150. Zhang B, Mao Q, Zhang X, Jiang T, Chen M, Yu F, Fu W (2004) Biosens Bioelectron 19:71
151. Deng T, Wang H, Li JS, Hu SQ, Shen GL, Yu RQ (2004) Sensor Actuator B 99:123
152. Michalzik M, Wendler J, Rabe J, Buttgenbach S, Bilitewski U (2005) Sensor Actuator B 105:508
153. Michalzik M, Wilke R, Buttgenbach S (2005) Sensor Actuator B 111–112:410
154. Raman Suri C, Jain PK, Mishra GC (1995) J Biotechnol 39:27
155. Atilli BS, Suleman AA (1996) Microchem J 54:174
156. Halamek J, Makower A, Skladal P, Scheller FW (2005) Biosens Bioelectron 17:1045
157. Miura N, Higobashi H, Sakai G, Takeyasu A, Uda T, Yamazoe N (1993) Sensor Actuator B 13:188
158. Yun K, Kobatake E, Haruyama T, Laukkanen ML, Keinanen K, Aizawa M (1998) Anal Chem 70:260
159. Kobatake E, Yun K, Yanagida Y, Haruyama T, Keinanen K, Aizawa M (2000) Sensor Actuator B 65:42
160. Carter RM, Jacobs MB, Lubrano GJ, Guilbault GG (1995) Anal Lett 28:1379
161. Harteveld JLN, Nieuwenhuizen MS, Wils ER (1997) Biosens Bioelectron 12:661
162. Gao Z, Chao F, Chao Z, Li G (2000) Sensor Actuator B 66:193
163. Gao Z, Tao G, Li G (1998) J Hyg Res 2:122
164. Bizet K, Gabrielli C, Perrot H, Therasse J (1998) Biosensors '98, Berlin, 3–5 June
165. Guilbault GG, Hock B, Schmid R (1992) Biosens Bioelectron 7:411
166. Kindervater R, Gebbert A, Kramer P, Schmid R (1990) DECAMA Biotechnology Conference, vol 4. VCH, Weinheim, p 19–27
167. Minunni M, Skladal P, Mascini M (1994) Life Chem Rep 11:391
168. Yokoyama K, Ibekuburo K, Tamiya E, Karube I, Ichiki N, Arikawa Y (1995) Anal Chim Acta 304:139
169. Steegborn C, Skladal P (1997) Biosens Bioelectron 12:19
170. Minnuni M, Skladal P, Mascini M (1994) Anal Lett 27:1475
171. Horacek J, Skladal P (1997) Anal Chim Acta 347:43
172. Liu M, Li QX, Rechnitz GA (1999) Anal Chim Acta 387:29

173. Pribyl J, Hepal M, Skládal P (2006) Sensor Actuator B 113:900
174. Masson M, Yun K, Haruyama T, Kobatake E, Aizawa M (1995) Anal Chem 67:2212
175. Horacet J, Skladal P (2000) Anal Chim Acta 412:37
176. Abad JM, Pariente F, Hernandez L, Abruna HD, Lorenzo E (1998) Anal Chem 70:2848