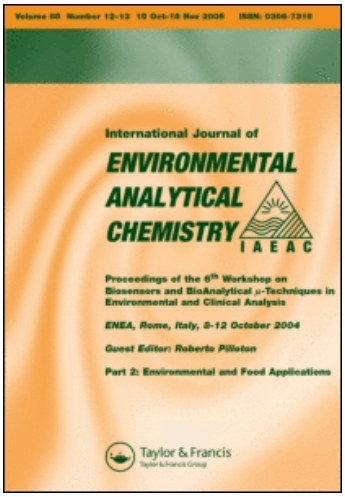

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Piezoelectric Immunosensors for Environmental Monitoring

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PIEZOELECTRIC IMMUNOSENSORS FOR ENVIRONMENTAL MONITORING

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This article is a tutorial review of piezoelectric immunosensors and their application to environmental monitoring.

The basic theory and historical developments of piezoelectricity are introduced. The development of piezoelectric sensors and eventually immunosensors for the specific detection of analytes of environmental importance are mentioned. The various assay formats and their advantages as well as examples of the different approaches taken by different analysers are also mentioned. Current developments within this laboratory on the development of piezoelectric immunosensors for the detection of okadaic acid and microcystin-LR are briefly introduced. The major drawbacks of piezoelectric immunosensors and their potential future are also discussed.

Keywords: Piezoelectricity; Piezoelectric immunosensors; Environmental analyses; Microcystin; Okadaic acid

PIEZOELECTRIC IMMUNOSENSORS

A biosensor can be defined as an analytical device incorporating a biological sensing element fixed to a suitable transducer that converts the biochemical response into a quantifiable and processible signal. Biosensors that incorporate an antibody–antigen interaction are termed immunosensors. They are based on the same principles of conventional solid-phase immunoassays, such as enzyme linked immunosorbent assay (ELISA), with the antigen, or more often the antibody, immobilised on a solid sensor surface. Conventional immunoassays require multistage processes, resulting in complex analysis procedures that require a skilled analyst. The equipment cannot be miniaturised, automation of the multistage measurements is difficult and generally need to be performed in a laboratory. Immunosensors have no such disadvantages; thus their applications are less limited. The fact that antibodies can be produced to almost any analyte means that limitless numbers of immunosensors can be produced for any area of analysis.

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They are often more specific and sensitive than other biosensors because of the highly specific molecular recognition and affinity between the 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 a typical affinity constant of $10^6\,M^{-1}$.

Piezoelectric (Pz) transducers are very robust, cheap and extremely sensitive devices that can measure mass changes in the region of 10^{-12} g, the true limit depends on the exact crystal and instrument used. This sensitivity combined with the high specificity of antibodies can lead to very useful devices for environmental monitoring. The Pz sensors used for immunosensor development are either bulk acoustic wave (BAW) or surface acoustic wave (SAW) devices, depending on the propagation of the wave.

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 waves velocity and thus can be monitored by the frequency change. The quartz crystals generally incorporated are ST-cut operating between 30 and 200 MHz. Theoretically SAW devices are more sensitive than BAW devices since they oscillate at much higher frequencies. But in reality they are rarely employed, since they suffer from a lot of practical problems, which makes BAW devices a better option.

BAW devices generally consist of Pz crystal disks that vibrate at a high frequency, typically 5–15 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. Since it is a mass balance it can be used to directly detect the immunoreaction by mass alone, eliminating the need for labelling or the use of a secondary antibody to generate the response.

The assays performed are generally direct captures, where the antibody is immobilised to the surface of the crystal. The antibody-coated surface is then exposed to the sample solution containing the analyte. As the analyte specifically binds to the immobilised antibodies it causes a change in mass, proportional to the amount bound. This can then be monitored directly, in real-time if required. BAW devices have advantages of short response times, which can be important if used in flowing solutions with fluctuating analyte concentrations. Direct conversion of the mass accumulated into a frequency shift, which represents exactly the measurable signal. Simple and cheap instrumentation means that reliable and portable sensors can be easily built. Also the crystals are relatively inexpensive and can, in some situations, be disposable, although regeneration of some sort is always possible and can be incorporated to further reduce costs. Unless stated otherwise the Pz crystals referred to are AT-cut quartz BAW devices resonating in their thickness shear mode (AT refers to the angle of the crystals cut relative to the overall crystal lattice). Pz immunosensors are often referred to as quartz crystal microbalance (QCM) immunosensors also.

The instrumentation for a Pz detector is very simple, with most users building home made devices. The oscillator circuit can be made very easily and incorporated with a frequency counter to give instant results. There are a lot of commercial instrumentation also available, individually and specifically designed for particular applications. Some incorporating pumps and specifically designed flow cells, depending on the potential use. Most instruments incorporate a second reference crystal and simultaneously record the frequency of both. The second crystal is used as a reference to compensate for frequency fluctuations caused by environmental and non-specific factors. Generally

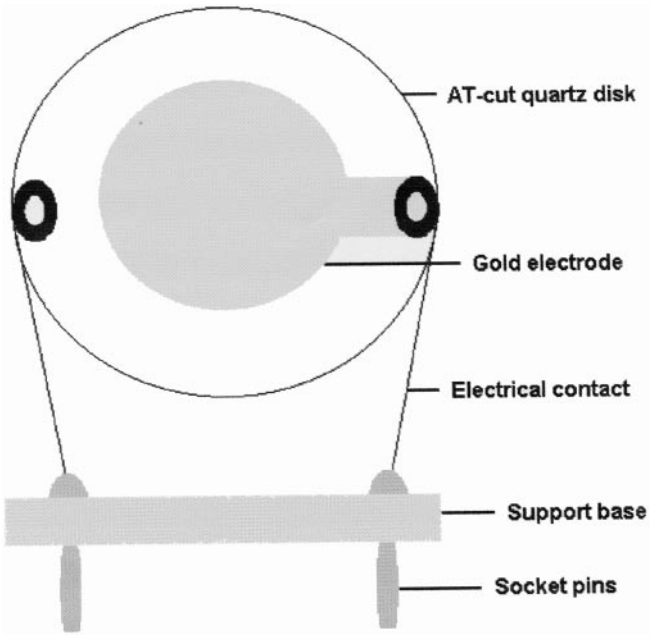

FIGURE 1 A typical piezoelectric crystal used for immunosensor development. The type shown here is a 10 MHz AT-cut crystal available from Universal Sensors Inc. The quartz disc is 0.17 mm thick, has a 14 mm diameter and is covered with a gold electrode of 7.8 mm in diameter. The crystal has an overall length (including quartz disc, support and pins) of less than 30 mm.

commercial instruments have a frequency resolution of between 0.01 and 1 Hz and can be used with crystals of different fundamental frequencies. Using the Sauerbrey equation (discussed later) the theoretical limit of detection of an instrument with a frequency resolution of 1 Hz incorporating a 10 MHz AT-cut quartz crystal is 2.1×10^{-9} g.

Crystals of many different sizes and shapes can be used, usually consisting of a circular quartz plate with an electrode on each side. The electrode is generally made of gold, but silver and other metals have also been used. Figure 1 shows a typical crystal. The sensitivity of a crystal depends on its fundamental frequency, which is effected by the crystal's electrode diameter and the thickness of the quartz. Crystals of higher fundamental frequencies (> 20 MHz) are more sensitive but are less robust and are affected more by damping from liquids, ceasing to oscillate when heavily loaded in solution. Lower frequency crystals (< 5 MHz) have a lower sensitivity but are less effected by liquid damping or heavy mass loading. For immunosensor development crystals of 9 or 10 MHz are usually incorporated. They are a good compromise between cost, sensitivity and ruggedness, capable of oscillating in most solutions under mass loading without problems, while still retaining excellent sensitivity.

Immunosensor analysis is mainly carried out in liquid phase. Measurement using Pz immunosensors can be carried out in two different ways, dip and dry or directly in solution.

Dip and dry involves reading the crystals frequency while dry in the air. It is then 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 then recorded again in a dry state in the air. The change in frequency is

proportional 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 better sensitivity. Also solution effects due to viscosity, etc., are not a problem since the reading of the frequency is done in air. 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 huge sensitivity of the method is part of its downfall, since it can be plagued by interferences from environmental effects, especially humidity.

Measurement in solution is generally carried out in specifically designed flow cells. Analysis is performed directly in solution and monitored in real-time. The flow cells allow exposure of one side of the crystal to the solution and the other to the air, to avoid short-circuiting of the crystal. Analysis can be performed in a static or flowing solution and can show real-time immobilisation or binding to the surface. Kinetic information can then be extrapolated from the recorded response if required.

THEORY AND HISTORICAL DEVELOPMENTS OF PIEZOELECTRICITY

The Pz effect can occur in any ionic crystalline solid lacking a centre of symmetry. When mechanical stress is applied to such a material the deformation of the crystal lattice induces a net dipole which results in an electrical potential across the crystal. Application of an opposite mechanical stress will induce a potential of opposite polarity.

Hypothetically, as seen in Fig. 2, (A) is a symmetrical planar molecule lacking a net dipole moment. However, if a stress is applied to the molecule parallel to one of its axes then a net dipole is induced in the molecule as seen in (B). In a crystal this induced dipole would result in a potential difference across the crystal.

If electrodes are applied to the faces of a thin stab or rod of such a material and connected to an external circuit, a current can be measured flowing through the circuit when the stress is applied to the crystal. Releasing the stress will cause a transient current to flow in the opposite direction.

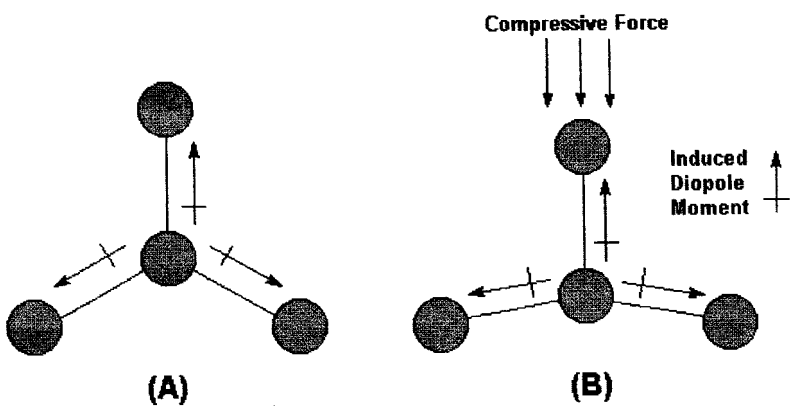

FIGURE 2 (A) is a planar symmetrical molecule possessing no net dipole. A compressive force parallel to one of its axes induces a net dipole moment in the molecule (B).

Conversely, if an alternating current is passed through the electrodes the alternating potentials induced across the crystal will result in its alternating mechanical deformation (vibration). Stable vibration will occur if the frequency of the alternating current is at that of the natural (fundamental) frequency (or natural harmonics or overtones) of the vibrating crystal. If the crystal is connected to a feedback loop in the oscillating circuit, then it becomes the frequency determining element of the circuit. In this situation, when the frequency of oscillation is close to the fundamental frequency of the crystal, the crystal offers low impedance to the exciting potential and there is efficient energy transfer between the oscillating circuit and vibrating crystal, thus the amplitude of the vibration will reach a maximum.

Of the many materials that posses Pz properties, quartz (α-quartz) plates are most commonly used. That is principally because quartz is abundant and completely oxidised so it is chemically inert, has high thermal and mechanical stability and has a high Q value (its loss of Pz effect due to mounting and acoustic radiation is low). Rochelle salt is also a commonly used Pz material because of its very high Pz effect. One drawback being it is very temperature sensitive and disintegrates above 55°C, whereas quartz can be heated to several hundred degrees without loss of its Pz effect.

A vibrating piece of quartz may exhibit several modes of vibration (longitudinal, shear and torsional in each axes). In addition to the fundamental modes, the system can vibrate at overtones of each of these and several of these can combine to form very complicated resonance modes. Depending on the plane in which the crystal is cut from and the mode in which it is vibrated, different problems can arise. Interferences from harmonics, overtones and external temperature variations can occur. The selection of one particular mode and the suppression of unwanted modes require the crystal to be cut at a very specific crystallographic orientation. Figure 3 shows a quartz crystal with the possible cuts available. Only AT ($+35°15'$) and BT ($-49°00'$) cut crystals (relative to the z-axis) are useful for sensor development because they have a temperature coefficient of zero and vibrate only in one mode, free from interferences. The quartz crystals employed are generally AT-cut thickness shear mode resonators.

Coulomb is believed to be the first to conjure up the idea of producing electricity by the application of pressure on a suitable material [1]. But it was the Curie brothers in 1880 who were the first to observe the Pz effect [2,3]. They discovered that the application of mechanical stress to the surface of some crystals (including quartz and Rochelle salts) induced an electrical potential across the crystal. The magnitude of the potential was proportional to the force of the applied pressure. This "pressure electricity" was called piezoelectricity, from the Greek word *piezein* meaning to press. Independent of the Curies, Lipmann had predicted the converse effect [4,5]. He predicted that the application of an electrical potential across the surface of certain crystals caused a deformation of the crystal structure. This effect was later verified by the Curies [6].

Sauerbrey was the first to investigate mathematically the mass-induced frequency shift of a quartz crystal with emphasis on mass measuring [7,8]. His groundbreaking work showed that the thickness of thin films could be measured by the resulting change in the frequency of the vibrating crystal. His work in 1959 "Applications of vibrating quartz for the weighing of thin layers and microweighing", presented a mathematical relationship between the mass of thin films deposited on the surface and the change in frequency observed [8].

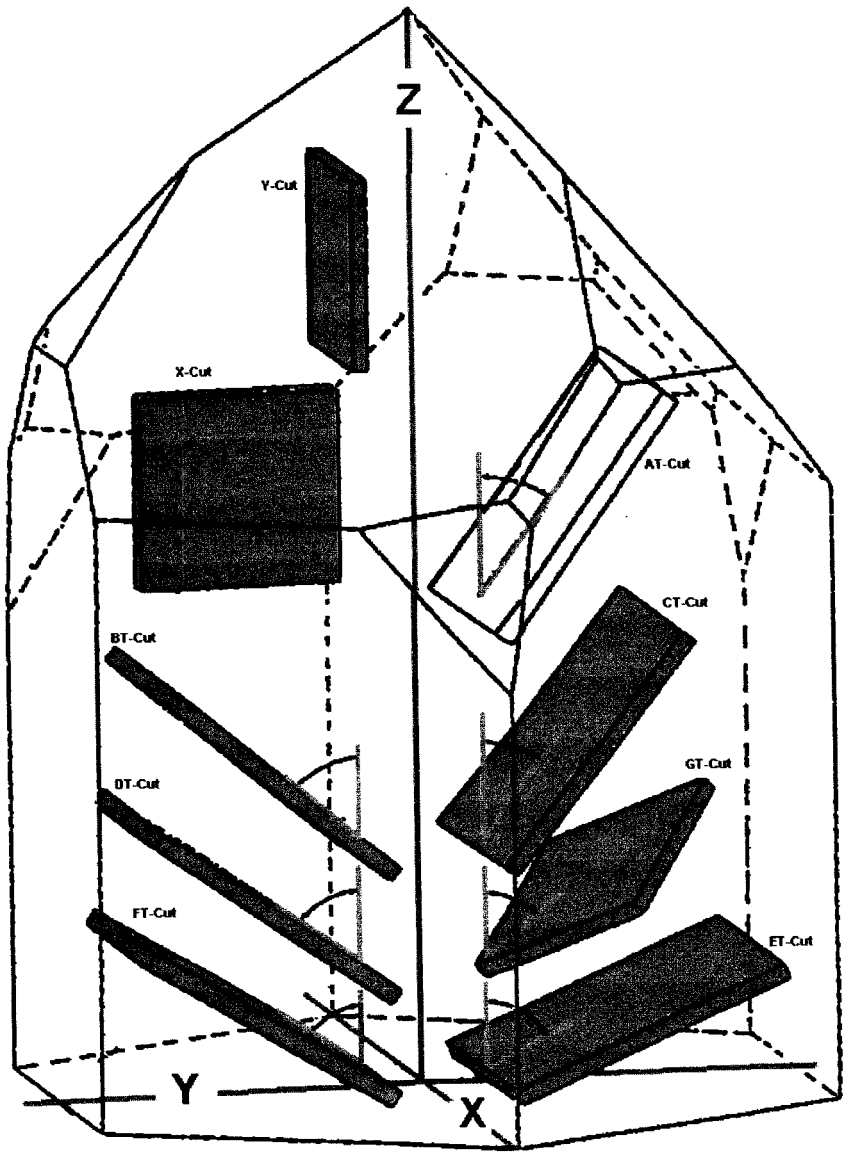

FIGURE 3 A quartz crystal, showing some of the possible orientations of cuts used to produce piezoelectric crystals.

His relationship describes the frequency change observed when a thin film is deposited on the surface of an AT-cut quartz resonator vibrating in the thickness shear mode.

$$\Delta f = -2.3 \times 10^6 f^2 \Delta m/a$$

Δf, the frequency change observed, is measured in Hz. m, the mass added to the crystal surface, is measured in g. a, the area of the electrode, is measured in cm^2. f is

the fundamental frequency of the crystal, measured in Hz. From this equation it can be seen that change in mass of a vibrating quartz crystal is inversely proportional to the mass added. Using his equation, Sauerbrey predicted that his system, consisting of a 14 MHz AT-cut quartz crystal with a 4 mm electrode, had a sensitivity of 10^{10} Hz/g for mass deposited on the centre of the crystal. With an error of frequency of 1 Hz his instrument had a precision of 10^{-10} g.

While the Sauerbrey equation is very useful, generally it cannot be applied directly to sensor situations and calibrations are generally required. The main reasons being that the equation assumes the mass added is uniformly distributed as a rigid elastic thin film. But in real practice this is never the case. For most gaseous analysis good correlation with the equation can be expected though.

PIEZOELECTRIC SENSOR DEVELOPMENT

All of the early Pz sensor work was based on the analysis of various gaseous analytes generally of environmental importance.

The first analytical use of Pz crystals with view to sensor or detector development was presented by King in 1963 [9], the basis of his commercialised moisture detector. In 1964 he published details of his sorption detector [10], although earlier Oberg and Ligensjo had used Pz crystals for the monitoring of film thickness [11].

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 was monitored. 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.

Over the following 25 years many Pz sensors for the detection of various gaseous analytes were developed. Over this time many fundamental problems were solved and developments in instrumentation were made, many of which are still incorporated in today's Pz sensor design. Reviews concerning the use of Pz crystals for gaseous analysis during this period have been published [12–16].

The use of biological coatings was a natural progression from the earlier work for gaseous analysis. Bio-recognition elements such as enzymes and antibodies can offer specificity and selectivity far superior to anything possible using the inorganic and organic coatings incorporated previously. The specificity of the biological receptor combined with the sensitivity of the QCM offered a very attractive tool for gaseous analysis.

The first reported gas phase biosensor was in fact an enzyme based Pz sensor used to determine formaldehyde in air [17]. The first gas phase immunosensor was also a Pz based device, developed in 1986 [18]. Crystals coated with anti-parathion antibodies could detect the parathion with a linear response 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 bovine serum albumin (BSA). Only small irreversible frequency changes of a few Hz were observed ensuring that the responses observed were specific immuno reactions. Despite this it was noted that relatively the antibodies appeared to be 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 immuno reaction in the gas phase is not known or well understood.

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, depending on the coating.

Despite the previous example 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 antibody could be affected by many factors such as accessibility. Orientation of the bio-component, which is affected by its immobilisation method, will probably differ significantly when in gas and liquid phase.

The possibility that gas phase biosensing can even occur has been questioned and caused an amount of controversy [19]. Rajakovic *et al.* [20] 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. 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.* [18]; where the authors showed that no non-specific binding occurred to crystals coated with non-specific proteins.

A review on gas phase biosensors has been published [21], which describes numerous examples. It is likely that in gas phase biosensors 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 [22]. Partial humidification of the carrier stream has also been suggested so as to retain the behaviour of the biologically active coatings [23]. This area has been specifically reviewed elsewhere [24,25].

The main disadvantage of detecting environmental analytes of interest using Pz immunosensors is that the sensitivity of the sensor is dependent on the mass of the analyte. Thus the detection of relatively low molecular weight analytes such as toxins, pesticides and herbicides can be problematic.

However, measurements performed in the gas phase, using dip and dry detection, have been successful. The detection of the biological warfare agent Ricin was carried out by Carter *et al*. [26]. Despite the low molecular weight of the antigen it could be detected directly to 0.5 μg. Immobilisation of the antibodies by passive adsorption proved more useful than immobilisation with protein A. Guilbault *et al*. developed a Pz immunosensor for the detection of atrazine in drinking water [27]. Despite the low molecular weight of the analyte the authors could detect it directly in solution. Protein A was used to immobilise the antibodies on the crystal. The analyte was measured from 0.03–100 μg/L. The antibody-coated crystal was reusable for 8–9 assays before a large reduction in activity was observed. The gold surface could then be regenerated and reused. Some non-specific responses were observed with other triazines, but these were due to the antibodies specificity since they were also seen in ELISA studies. It was also noted that no detrimental effect to the antibodies activity was observed upon drying on the crystal surface. The sensor was much more sensitive to the analyte than a previous attempt by the authors [28]. The low sensitivity was attributed to the antibodies used. Gao *et al*. were able to detect *Staphylococcal* enterotoxin C_2 directly [29]. Immobilisation of the antibody using protein A gave best results. Under optimised storage conditions antibody-coated crystals were stable for 12 weeks without detectable loss in activity. The antibody surface could also be regenerated and used 6 times before a sharp decrease in activity was observed. Regeneration of the gold surface was also possible. The sensor could detect the toxin in food and showed no interfering effects in the presence of other *Staphylococcal* enterotoxins [30]. However, no calibration of the sensor was reported. Bizet *et al*. were also able to detect *Staphylococcal* enterotoxins directly in a flowing solution, using highly sensitive 27 MHz crystals [31].

Generally, however when detection is performed in real-time directly in solution poor sensor sensitivity is observed because of the damping effect of the liquid. Due to the low molecular weight of these analytes even if a lot of the particular analyte was captured the small mass change would lead to very poor sensitivity. The detection of such analytes using Pz immunosensors generally incorporate assays that indirectly measure the particular analyte, i.e. a heavier compound whose concentration is dependant on the concentration of the particular analyte is often measured. The increased mass change observed due to the heavier compound increases the assay's sensitivity and allows detection of a particular analyte irrespective of its mass.

Various novel bioaffinity [32] and immunosensor [33] based approaches have been reported to successfully detect small molecular weight analytes in solution using Pz detection. These approaches were developed so they could potentially be applied to any analyte irrespective of mass since the frequency change observed was independent of the mass of the analyte.

The most popular assay format incorporated for the detection of low molecular weight analytes using Pz immunosensors is a competition. It is generally performed in one of two ways. The antibody is immobilised as for a direct capture. The free analyte then competes against a labelled analyte (Fig. 4). The conjugated label type is irrelevant since it is just added to increase the mass. The amount of the labelled conjugate that binds to the immobilised antibodies depends on the concentration of the free analyte. The second method is to initially immobilise the analyte or a conjugate of the analyte. The specific antibody is then mixed with free analyte and the resulting mixture added to the crystal surface. Any free uncomplexed antibodies will then specifically bind to the crystal surface. The amount of which will depend on the concentration

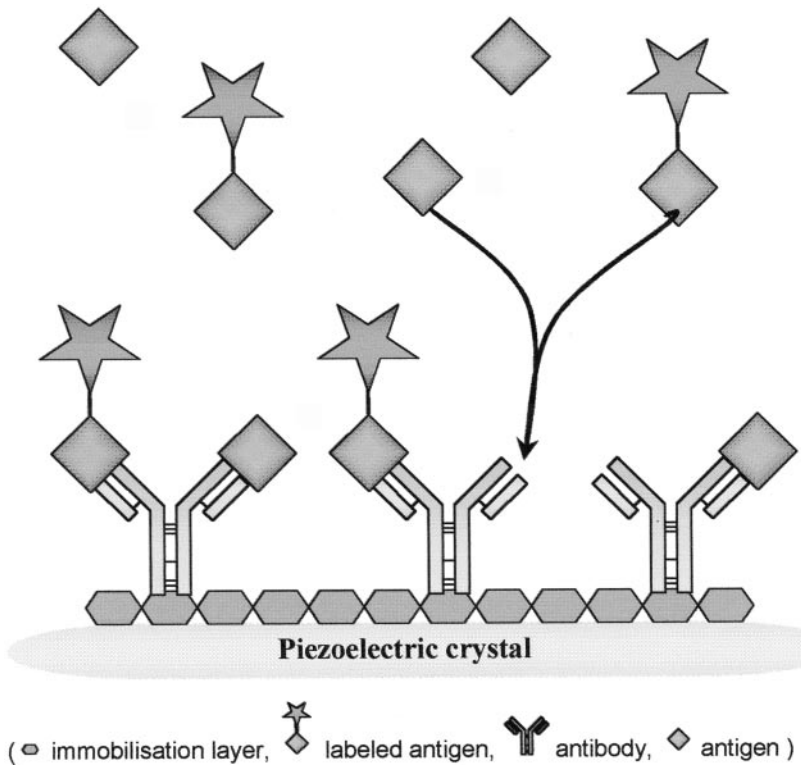

FIGURE 4 A typical direct competition assay. Free antigen competes with labelled antigen for the available binding sites on the immobilised antibodies.

of the free analyte in solution. In both cases the actual frequency change is due to the immobilisation of a conjugate or an antibody and not the analyte itself. But the extent to which they both bind depends on the concentration of the analyte. Thus the frequency change observed is dependent on the analytes concentration but independent of its mass.

The first competition Pz immunoassay was described in a patent by Olivera and Silver in 1980 [34]. Harteveld *et al.* used an indirect competitive assay to detect the biological warfare agent *Staphylococcal* enterotoxin B [35]. After adsorbing the toxin to the surface of a highly sensitive 20 MHz crystal, the antibody was pre-incubated with free toxin and added to the solution over the crystal. The change in frequency observed was due to free antibody in solution binding to the immobilised antigen, which was proportional to the concentration of the free toxin in solution. In a flowing solution the toxin could be measured from 0.1 to 10 μg/mL.

The analysis of pesticides and herbicides has also received much attention. Minunni *et al.* described a competitive assay for the detection of atrazine [36]. An atrazine derivative was immobilised to the surface of the crystal using a silane layer modified with glutaraldehyde. A solution of fixed amount of antibody and different amounts of free analyte were added. The frequency change observed due to antibody binding being proportional to the concentration of free analyte. Atrazine was measured linearly from 0.1 to 100 ng/mL in solution. Regeneration of the surface was also investigated.

A direct assay produced unsatisfactory results. Yokoyama *et al.* also described a competitive assay for atrazine [37]. They used a direct approach, immobilising antibodies using polystyrene and competing free atrazine with a protein labelled atrazine. Free atrazine could be detected linearly from 0.01 to 1 ng/mL and the sensor had a limit of detection of 0.001 ng/mL. The sensor was found to respond to other triazines tested, the lack of specificity being attributed to the antibody. Thus the sensor could be used as a general triazine detector, which is often preferable. This could be used for the total triazine determination in drinking water which would be more desirable than a specific sensor.

Steegborn and Skládal developed the first Pz immunosensor for the real-time detection of atrazine directly in solution [38]. A competitive assay similar to that of Minunni *et al.* [36] was performed but detection was carried out in a flowing solution in real-time. The lowest concentration of the analyte tested (0.1 μg/L) could be reliably detected. Higher dilutions of the antibodies gave better sensitivity and regeneration of the coated surface was possible. Association and dissociation constants were also determined. The problems involved using drying procedures for the development of Pz sensors were also discussed.

A similar set-up was earlier used by the authors for the detection of 2,4-dichlorophenoxyacetic acid (2,4-D) [39]. A BSA conjugate of the antigen and modified 2,4-D were immobilised via glutaraldehyde to a silane layer on the crystal surface. An indirect competition was then carried out. The assay was performed in a flowing solution and monitored in real-time. The analyte was measured from 0.001 to 100 ppb. Analysis in tap water was performed and no matrix effects were observed. The authors later investigated different coupling procedures based on self-assembled monolayers (SAM) to perform the same competition assay for 2,4-D [40]. Improved sensitivity of the sensor was shown over previous work [39]. From the results it was evident that sub ppb levels could be determined by the sensor. The authors later used direct competitive assays of two test analytes, 2,4-D and 4,4-dichlorobiphenyl, to investigate the effect of organic solvents on immunoassays [41]. 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 influence it had on the immunointeraction. The authors also performed a real-time competitive assay in toluene. This was the first time an immunoaffinity interaction in pure solvent had been shown. The effects on affinity and kinetics were discussed.

An enzyme based Pz biosensor for the detection of organophosphorous and carbamate pesticides has also appeared [42]. 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-indoxyl 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 similar to that used by Ebersole and Ward in their amplified mass immunosorbent assay (AMISA) [33]. The authors measured paraoxon to 5×10^{-8} M and carbaryl to 1×10^{-7} M.

Another assay format similar to a competition is a displacement. It is very similar to the second competition described earlier, in that the analyte is initially immobilised on the crystal. The specific antibodies are then bound to the immobilised antigen

molecules. Addition of free analyte results in an increase in frequency due to loss of the antibodies from the surface (Fig. 5). This occurs since the immobilised antibodies will have a higher affinity for free antigen molecules in solution than bound molecules. As with the competition the frequency change observed is dependent on the analytes concentration but independent of its mass. It has the advantage over a competition in that it eliminates the need to synthesise conjugates of the analyte. This can sometimes be problematic and introduce further errors to the assay if stability of the conjugate is a problem. Also the production of conjugates can be hazardous if toxins are being used since a lot of toxic waste can be produced. The first application of a displacement assay using Pz immunosensors was reported by Minunni *et al*. [43] for the detection of *Listeria monocytogenes*. It was later incorporated with limited success for the detection of *Pseudomonas aerugenosa* [44]. Liu *et al*. developed an immunosensor using flow injection analysis (FIA) for the detection of polycyclic aromatic hydrocarbons (PAHs) [45]. A BSA conjugate of benzo[a]pyrene was immobilised to the crystal surface using an activated SAM of thioctic acid. This was followed by binding of the specific monoclonal antibodies to the immobilised PAH layer. This was followed by addition of free benzo[a]pyrene in the flowing solution, because of the higher affinity of the antibodies for free analyte, removal of the bound antibodies was observed. Causing an increase in frequency, proportional to the concentration of the free analyte in solution. Under optimised conditions the PAH could be measured from 1 to almost

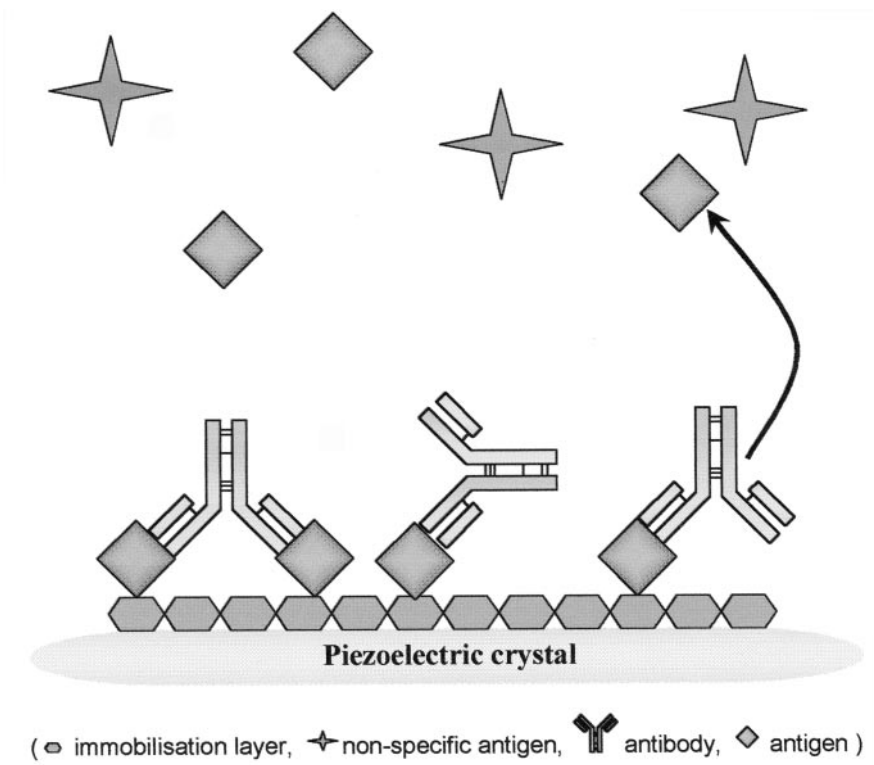

FIGURE 5 A typical displacement piezoelectric assay. The immobilised specific antibodies are displaced from the surface and bind to free antigen molecules in solution resulting in a frequency increase due to the loss in mass of the crystal surface.

4 nM. Regeneration of the BSA conjugate layer was possible but a loss in sensitivity was observed each time. The sensor's cross-reactivity to other PAHs was also investigated.

CURRENT DEVELOPMENTS WITHIN THIS LABORATORY

Investigations into the development of a Pz immunosensor for the detection of a cyanobacterial toxin, microcystin-LR, are currently being carried out in this laboratory. A competition assay was performed by first immobilising a gelatin-toxin conjugate to the crystal surface. A fixed concentration of the antibodies and different concentrations of the toxin were incubated for 15 min. This solution was then added to the crystal coated with the gelatin conjugate. Any free uncomplexed antibodies would specifically bind to the immobilised gelatin–toxin conjugate, resulting in a reduction in the crystals frequency. The amount of free antibody would depend on the concentration of the toxin in the incubated solution. A high free toxin concentration would complex a large amount of the antibodies, resulting in a small frequency change due to less uncomplexed antibodies available to bind to the immobilised conjugate. Conversely, a low free toxin concentration would complex a low concentration of the antibodies, resulting in a large frequency change due to more uncomplexed antibodies available to bind to the immobilised conjugate. Thus, the overall frequency change observed was inversely proportional to the concentration of the free toxin in solution. Various covalent and non-covalent immobilisation methods were investigated. In terms of sensitivity and reduction of non-specific binding, immobilisation of the gelatine conjugate to the crystal surface using polyethyleneimine gave best results. Preliminary results of this system can be seen in Fig. 6. The reason for the positive and negative frequency changes is due to the fact that the zero frequency was taken from the frequency before injection of the conjugate antibody solution. The consistent initial frequency increase, due to the solvent, meant that the reduction in frequency for some measurements were still positive with respect to the initial frequency. The crystal responses were

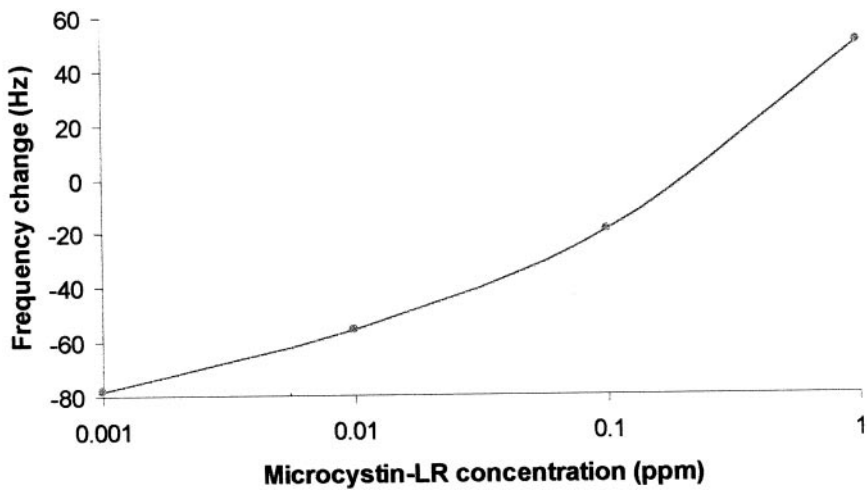

FIGURE 6 Calibration of the competition assay for the detection of microcystin-LR incorporating polyethyleneimine immobilisation.

similar to that shown in Fig. 7. Some measurements resulted in frequency reductions greater than the initial jump, resulting in negative overall frequencies. The initial increase in frequency was due to changes in viscosity of the solution over the crystal upon addition of the toxin–antibody solution, which contained a fixed amount of ethanol. Initial results seem promising and the eventual development of a sensitive portable device used for rapid or continuous on-site analyses could be a possibility. But validation, further optimisation and investigation of other experimental conditions (such as cross-reactivity with other microcystins) is required and is underway. In depth discussion on the importance of the toxin, its detection using other methods and the relevant experimental details can be found elsewhere [46].

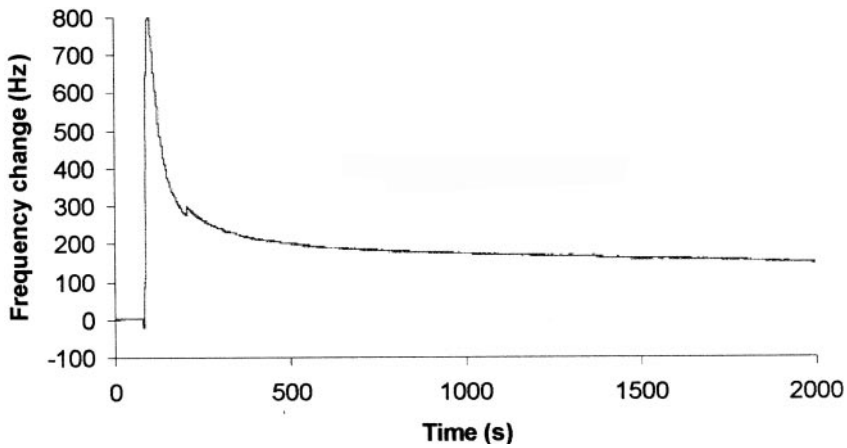

FIGURE 7 Typical crystal response upon addition of the toxin–antibody solution to a crystal with immobilised toxin conjugate. The initial jump in frequency is due to a slight change in viscosity due to ethanol contained in the solution. The subsequent frequency reduction is due to the binding of free antibodies to the immobilised toxin conjugate.

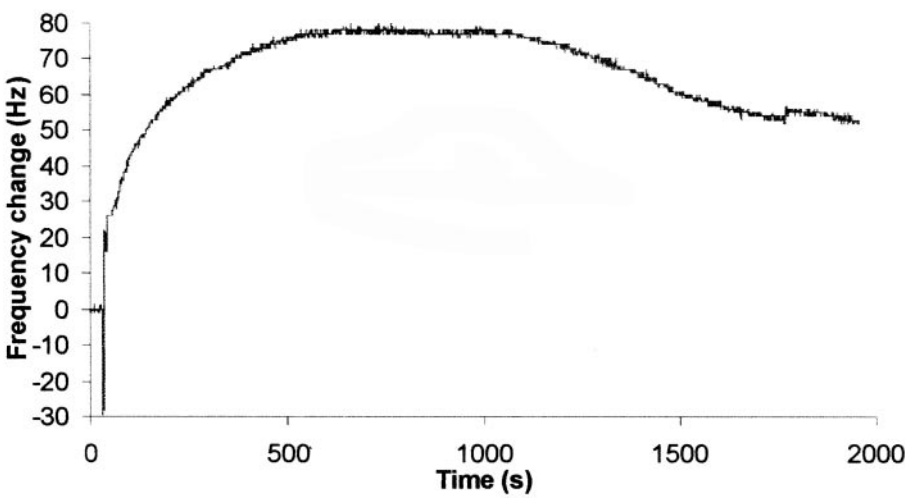

FIGURE 8 The real-time response of a displacement assay for the detection of the okadaic acid.

Development of a sensor for the detection of the sea-food toxin, okadaic acid is also underway [46]. Initial work is concentrating on the development of a displacement assay format. Okadaic acid is covalently bound directly to a SAM of cysteamine using EDC (1-ethyl-3-[3(dimethylamino)propyl]carbodiimide) and NHS (*n*-hydroxy-sulfosuccinimide). After blocking of the surface, antibodies against okadaic acid are specifically bound to the immobilised toxin. Exposure of such a crystal to a solution containing free okadaic acid results in loss of the immobilised antibodies and a frequency increase due to the reduction in mass.

The magnitude of the increase is due to the amount of antibodies lost which is dependent on the concentration of free toxin present. A typical real-time crystal response to a displacement assay can be seen in Fig. 8. Optimisation of various experimental parameters are underway. The use of okadaic acid conjugates and possibly a competition assay format may also be investigated in the future.

CONCLUSION

Pz immunosensors can be used for the rapid and sensitive detection of analytes of environmental importance, directly in solution if required and yielding instant results. Theoretically these sensors offer many advantages over other immunosensor formats. They are label free, incorporate cheap disposable electrodes, can be easily made portable and are extremely sensitive.

Despite these advantages over other sensor types many practical problems still exist, especially when the sensors are being applied to real samples. Viscosity changes in solution have an effect on the crystal's frequency. Most sensors are developed in buffer media and have to be recalibrated in a real sample matrix. Despite this, however, reproducibility problems can be encountered in different real samples with a slightly different viscosity.

Non-specific adsorption to the crystal's surface can also can also have a negative impact on the sensor's performance. Components such as proteins can non-specifically adsorb onto the crystal surface, often occurring when a sensor is used in complex media such as milk, blood and water samples. This adsorption can reduce sensitivity by blocking antibody binding sites and can result in misleading frequency changes due to the extra mass added.

The use of organic layers to immobilise antibodies on the crystal surface can cause the crystal to stop oscillating when exposed to aqueous samples, due to hydrophobic forces between the surface and the solution. Sensors affected by this can still be useful for specific detection, but not for real-time analyses directly in solution.

Lack of robustness and stability are possibly the main hindrance to commercialisation and field use of Pz immunosensors. A stable and robust protein coated crystal surface with a reasonable lifetime is often very difficult to achieve. Ideally the surface should be chemically and mechanically stable and allow washings and exposure to different conditions without any detrimental effect on the sensors performance. In reality, however, most sensors that have been produced require careful handling if their sensitivity and selectivity are to be retained.

Generally most Pz immunosensors 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.

Theoretically a Pz sensor is sensitive enough to measure most analytes of environmental interest to very low levels. 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 have the required sensitivity (provided by the Pz sensor) and specificity (provided by the antibody) for the specific detection of almost any analyte. But a lot of this is lost when they are coupled. Even with recent advances in immobilisation techniques no significant improvement 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 more versatile and robust.

The incorporation of stable synthetic recognition elements instead of antibodies, offer a real altemative [47,48]. With improving synthesis, the possibility of these recognition elements having the selectivity of biological systems in the near future is very likely. The use of crystal arrays can also help overcome the relative lack of sensitivity. These elements when immobilised result in very robust and stable sensors, the main drawbacks of Pz immunosensors. If the selectivity approaches that of immunosensors these Pz sensor should offer a real alternative to immunosensors.

Pz immunosensors still have a promising future. 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.

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