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Detection of two orchid viruses using quartz crystal microbalance (QCM) immunosensors

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Abstract

Quartz crystal microbalance (QCM) immunosensors are based on the principle that adsorption of substances on the surface of a quartz crystal changes its resonance oscillation frequency. A QCM immunosensor was developed for the detection of both cymbidium mosaic potexvirus (CymMV) and odontoglossum ringspot tobamovirus (ORSV) by pre-coating the QCMs with virus-specific antibodies. Upon binding of virions in either purified form or crude sap of infected orchids with the immobilised virus antibodies, the increase in mass at the QCM surface resulted in a reduction in the frequency of resonance oscillation in a manner dependent upon the amount of virus bound. The QCM was able to detect as low as 1 ng each of the two orchid viruses. This detection sensitivity is comparable to enzyme linked immunosorbent assay (ELISA) but the assay is faster. This immunoassay was shown to be specific, sensitive, rapid and economical, thus providing a viable alternative to virus detection methods. This is the first report using QCM immunosensors to detect plant viruses.

Keywords: Quartz crystal microbalance; Immunosensors; Cymbidium mosaic potexvirus; Odontoglossum ringspot tobamovirus; Plant virus detection

1. Introduction

Biosensors are devices that incorporate a biologically active sensing material into a transducer (Ngeh-Ngwainbi et al., 1990). The biological system (e.g. enzymes, antibodies, antigens) provides the specificity and selectivity to the system while the transducer (e.g. electrodes, optical sensors, piezoelectric devices) complements it with its signal processing and amplifying capacity. Biosensors utilising antibodies are termed immunosensors. In the present study, antibodies specific to the coat proteins of two orchid viruses, cymbidium mosaic potexvirus (CymMV) and odon-

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toglossum ringspot tobamovirus (ORSV), were coated onto a resonant AT-cut quartz disc which has gold electrodes on either side to create a quartz crystal microbalance (QCM) immunosensor specifically for the detection of these viruses in plant crude saps. When a voltage is applied across the antibody-coated quartz disc, it deforms slightly via a piezoelectric effect such that one face moves into a shear mode with respect to the other face (Walton et al., 1991). On removing the voltage, the crystal relaxes and oscillates with its natural frequency. Upon attraction of the target viral coat protein via its antibody, the oscillating frequency is decreased in accordance with the Sauerbrey equation (Sauerbrey, 1959).

The development of a QCM immunosensor targeted at CymMV and ORSV detection complements existing systems. CymMV and ORSV are the most prevalent and economically important orchid viruses, which have attained a worldwide distribution, infecting numerous commercially important orchid genera (Zettler et al., 1990; Wong et al., 1994). Current diagnostic methods include reverse transcription-polymerase chain reaction (RT-PCR) (Lim et al., 1993; Ryu and Park, 1995; Seoh et al., 1998), immunocapture-PCR (Barry et al., 1996), digoxigenin (DIG)-labelled cRNA probes (Hu and Wong, 1998), immuno-capillary zone electrophoresis (I-CZE) (Eun and Wong, 1999), liquid chromatography (LC)- and matrix-assisted laser desorption-ionization (MALDI)-mass spectrometry (Tan et al., 2000), TaqMan real-time RT-PCR (Eun et al., 2000) and molecular beacons (Eun and Wong, 2000). While some of these diagnostic methods are highly sensitive, QCM immunosensor method is simple and rapid in sample preparation. The coated QCMs have relatively long shelf-life of at least 1 month and the detection device is portable. It is a viable and affordable alternative for plant diagnostic clinics and field experimental stations to carry out rapid plant virus detection.

2. Materials and methods

2.1. CymMV and ORSV antisera production

CymMV and ORSV were propagated and purified as described previously (Eun and Wong, 1999). Purified CymMV virions (1 mg), suspended in 1 ml of 0.02 M sodium phosphate buffer, pH 7.0, were emulsified with an equal volume of Freud's incomplete adjuvant (Sigma-Aldrich Corp., St. Louis, USA) by vortexing for 1 min. The resulting emulsion was injected intramuscularly into the hind legs of a New Zealand White rabbit. Boost injections of 1 mg each were administered 14, 21 and 28 days after the first injection. Before each boost injection was administered, 10 ml of blood was drawn from the ear of the rabbit. The antiserum collected was aliquoted into 1.5 ml centrifuge tubes spiked with small amounts of sodium azide for long-term storage at 4 °C. The same protocol was used for the production of ORSV antiserum.

2.2. QCM and frequency counter

The circular AT-cut 10 MHz QCMs with gold electrodes on either face were purchased from International Crystal Manufacturing Co. (Oklahoma City, USA). The QCMs were 14 mm in diameter and 0.2-mm thick and the gold electrodes were 5.1 mm in diameter and 100-nm thick. A laboratory-constructed transistor–transistor logic integrated circuit (TTL-IC) based on IC 74LS504 was used to power the quartz crystals. A TF830 Universal Frequency Counter (Thurlby–Thandar, UK) was used to measure their output frequency. All frequency readings were recorded at room temperature.

2.3. Pretreatment of QCMs

Quartz crystals to be pre-treated with (γ-aminopropyl)-triethoxysilane (APTES) and poly(ethyleneimine) were immersed in 1.2 M NaOH for 25 min. After being washed with distilled water and air-dried, the quartz crystals were immersed in 1.2 M HCl for 20 min and rinsed with distilled water, followed by ethanol. The quartz crystal surface was air-dried and the initial resonance frequency (F_0) was recorded.

Hot Piranha solution (concentrated H_2SO_4: 30% H_2O_2, 3:1 v/v) (5 μl) was applied to both sides of the gold electrode surface of QCMs to be pre-treated with thioctic acid, 4-amino-thio-phenol and

3,3′-dithiopropionic acid. After an incubation period of 5–10 min, the QCM was thoroughly rinsed with distilled water, followed by ethanol. This procedure was repeated twice. The QCM surface was air-dried subsequently and the initial resonance frequency (F_0) was recorded.

2.4. Antibody immobilisation

Six different pre-antibody immobilisation treatments were carried out on separate groups of freshly cleaned QCMs. Quartz crystals were dipped in (i) 0.1 M phosphate buffered saline (PBS), pH 7.0 for 1 h at room temperature. This served as a negative control; (ii) 5% APTES (Fluka, Buchs, Switzerland) in chloroform for 2 h at room temperature. After air-drying at 70 °C, the QCMs were incubated for 2 h in 2.5% glutaraldehyde, pH 7.0 (Fluka) in 50 mM PBS, pH 7.0, rinsed with 50 mM PBS, pH 7.0 and air-dried; (iii) 4% poly(ethyleneimine) (Sigma-Aldrich Corp.) in methanol for 20 s at room temperature, air-dried and washed with methanol. After incubating for 30 min in 0.4 M glutaraldehyde, pH 7.0, the QCMs were rinsed with distilled water; (iv) 0.2% thioctic acid (Sigma-Aldrich Corp.) in ethanol overnight at room temperature and incubated for 1 h in 2.1% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Fluka) in ethanol; (v) 0.2% 4-amino-thio-phenol (Fluka) in ethanol overnight at room temperature and incubated for 3 h in 2.5% glutaraldehyde in 0.1 M PBS, pH 7.0; (vi) 0.2% 3,3′-dithiopropionic acid (DTP) (Sigma-Aldrich Corp.) in ethanol for 30 min at room temperature and incubated for 30 min in 2.1% *N*-hydroxysuccinimide in ethanol (Fluka) in 2.1% fluidic EDC.

All treated QCMs were rinsed again with ethanol and air-dried before antibody coating. Five microlitre each of 0.3 μg μl^{-1} CymMV antibody was applied on both sides of the quartz crystals for 1 h at room temperature. For QCMs pre-treated with APTES and poly(ethyleneimine), a rinsing step with 0.1 M glycine in 20 mM PBS, pH 7.0 was performed after the antibody incubation so that all unreacted aldehyde groups were blocked. All QCMs were rinsed subsequently three times with 0.1 M PBS, pH 7.0, followed by distilled water to remove salt deposits and excess antibodies. ORSV QCMs were prepared using the same procedure and concentration of ORSV antibody. The QCMs were air-dried and their resonance frequency (F_1) read and recorded, and stored at 4 °C for use in subsequent experiments.

2.5. Antibody immobilisation efficiency

Five microlitres each of purified CymMV (15 ng μl^{-1}) was applied on both sides of six separate groups of QCMs specific for CymMV coat protein. Each group consisted of five QCMs previously pre-treated as described in Section 2.4. After 1-h incubation at room temperature, the QCMs were rinsed three times with 0.1 M PBS, pH 7.0, followed by distilled water and air-dried. The resonance frequencies of the QCMs were read and recorded (F_2) and the average frequency shifts (F_2-F_1) calculated. The same method was used to prepare the ORSV QCM. Pre-treatment methods yielding the highest frequency shifts upon target binding were used for the production of QCMs for subsequent experiments.

2.6. Optimisation of incubating antibody concentrations

Five microlitres each of CymMV coat protein antibody at dilutions of 0.1, 0.2, 0.3 and 0.4 μg μl^{-1} were applied on both sides of four separate groups of DTP pre-treated QCMs. Each group consisted of five QCMs. The QCMs were treated as described in Section 2.5 and the average frequency shifts (F_2-F_1) calculated. The same procedure was repeated for ORSV coat protein antibody.

2.7. Specificity and sensitivity of QCMs

Five microlitres each of purified CymMV at dilutions of 1.0, 1.8, 4.5, 9.0, 12.0 and 15.0 ng μl^{-1} were applied on both sides of six separate groups of QCMs specific for CymMV coat protein. Each group consisted of five QCMs.

The QCMs were treated as described in Section 2.5 and the average frequency shifts (F_2-F_1) calculated. The procedure was repeated using purified ORSV to determine the specificity of the CymMV QCMs. Similar procedures were performed to determine the sensitivity and specificity of the ORSV QCMs.

2.8. Stability of QCM immunosensors

Sixty CymMV QCM immunosensors were produced and divided into six groups (with ten immunosensors per group) to determine their stability after 1, 2, 5, 10, 15 and 30 days. For each group of CymMV QCMs, five were incubated with 15 ng μl^{-1} of purified CymMV, while another five were incubated with the same concentration of purified ORSV. The QCMs were treated as described in Section 2.5 and the average frequency shifts (F_2-F_1) calculated. The procedure was reciprocated with ORSV QCMs.

2.9. QCM detection of viruses from crude sap of infected Oncidium flower

Fresh flower samples of healthy, CymMV- and ORSV-infected orchid *Oncidium* (0.5 g each) were ground in 1 ml of 0.01 M sodium borate buffer, pH 7.5. The homogenate was centrifuged at low speed to remove plant debris. The supernatant was diluted a 100-fold in 0.1 M PBS, pH 7.0 and stored at 4 °C for further analysis. Healthy crude sap samples (5 μl) were applied on both sides of five CymMV QCMs. The QCMs were treated as described in Section 2.5 and the average frequency shifts (F_2-F_1) calculated. This procedure was repeated using CymMV- and ORSV-infected crude sap samples with separate groups of five CymMV QCMs. The ORSV QCMs were tested using the same protocol. The calculated frequency shifts were computed by subtracting the measured frequency shifts values of healthy crude sap samples by that of the CymMV- or ORSV- infected crude sap samples. All tests were repeated five times.

3. Results

3.1. Efficiencies of antibody immobilisation

ORSV QCMs pre-treated with 0.1 M PBS, pH 7.0 showed no significant frequency shift upon incubation with 15 ng μl^{-1} purified ORSV suspension. The lowest significant frequency shift

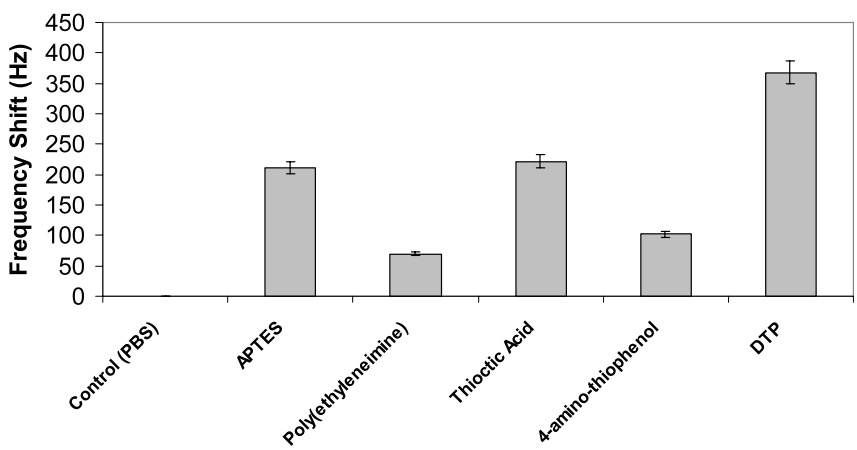

Fig. 1. Efficiencies of ORSV antibody immobilisation. Effects of PBS, APTES, poly(ethylenimine), thioctic acid, 4-amino-thiophenol and DTP on frequency shifts of QCMs coated with ORSV antibody. The error bars show S.D. obtained from five replicates of each treatment.

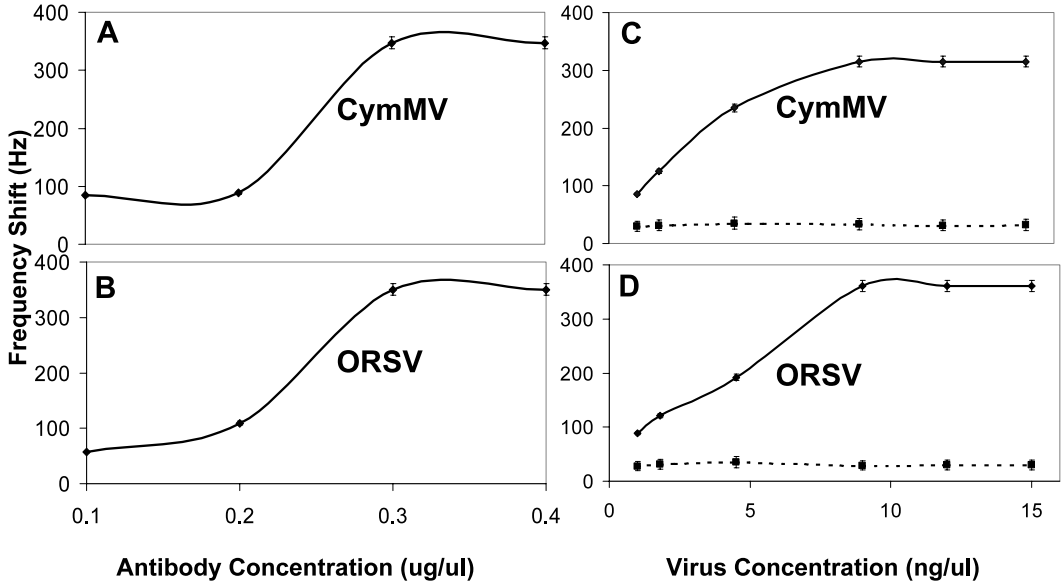

Fig. 2. Virus loading curves of (A) CymMV and (B) ORSV. Specificity and sensitivity of (C) CymMV and (D) ORSV QCM immunosensors. Solid line, target virus; dotted line, non-target virus. The error bars show the S.D. obtained from five replicates of each antibody/virus concentration combination.

observed was obtained by QCMs treated with 4% poly(ethylenimine), while those treated with 0.2% DTP yielded the highest significant frequency shifts (Fig. 1). Similar results were obtained using CymMV QCMs (data not shown). Subsequent experiments were then undertaken only with DTP pre-treated quartz crystals.

3.2. Optimal incubating antibody concentrations

The frequency shifts by the CymMV QCM increased significantly from 84 to 347 Hz, when the CymMV antibody concentration was increased from 0.1 to 0.3 μg μl^{-1}, respectively (Fig. 2A). Similar increase in the frequency shifts was observed in the ORSV QCM from 57 to 350 Hz (Fig. 2B). For both QCM immunosensors, no significant frequency shifts were observed when antibody concentrations exceeded 0.3 μg μl^{-1}.

3.3. Specificity and sensitivity of QCM immunosensors

Upon incubation of the CymMV QCMs with increasing concentrations of purified CymMV, the frequency shifts increased significantly from 85 to 315 Hz, corresponding to purified CymMV concentrations of 1.0 and 9.0 ng μl^{-1}, respectively (Fig. 2C). When the CymMV QCMs were incubated with increasing concentrations of purified ORSV, no significant changes in frequency shifts were observed (Fig. 2C). Similar results were obtained with the ORSV QCMs where the frequency shifts increased significantly from 89 to 361 Hz (Fig. 2D). The ORSV QCM did not exhibit any significant increase in frequency shifts upon incubation with increasing concentrations of purified CymMV (Fig. 2D). For both QCMs, no significant increases in frequency shifts were recorded when purified virus concentrations exceeded 9 ng μl^{-1} of their corresponding target viruses (Fig. 2C and D). Both QCM immunosensors could detect as low as 1 ng of their target purified virus. The results were highly reproducible; each CymMV and ORSV antibody/virus concentration combination was repeated five times.

3.4. Stability of QCM immunosensors

As seen in Fig. 3, there was a gradual decrease in the frequency shifts of the CymMV QCM with its target virus CymMV from 341 to 220 Hz, corresponding to storage lengths of 1–30 days, respectively. A similar trend was observed for the ORSV QCM that exhibited similar decrease in frequency shifts from 335 to 215 Hz with its target virus ORSV. Both QCMs did not show any significant frequency shifts upon incubation with the non-target virus suspensions. The results were highly reproducible. Each time point analysis was repeated five times.

3.5. QCM detection of viruses from crude sap of infected Oncidium flower

CymMV QCM immunosensors exhibited significant increase in frequency shifts upon incubation with crude saps containing the target virus when compared with the ORSV-infected crude saps. Similar results were observed with the ORSV QCM (Fig. 4). The results were highly reproducible.

4. Discussion

Since the first use of piezoelectric crystals for the immunogravimetric assay of the microorganism *Candida albicans* (Muramatsu et al., 1986), a wide repertoire of quartz crystal biosensors have been developed for the detection of human herpes viruses (Konig and Gratzel, 1994), *Vibrio cholerae* (Carter et al., 1995), African swine fever viral protein 73 (VP73) (Uttenthaler et al., 1998), *Salmonella* spp. (Park and Kim, 1998), *Staphylococcus epidermidis* (Pavey et al., 1999) and phage libraries (Hengerer et al., 1999). In the clinical setting, minute quantities of immunoglobulin E have also been quantified (Su et al., 1999). A recent review summarises the various designs and applications possible with QCM-based immunosensors, highlighting the critical issues relating to the use of such biosensors (Su et al., 2000a). There is, however, no report on the development of QCM for the detection of plant viruses.

During the last two decades, extensive work has been reported on the use of various organic and inorganic materials as quartz crystal coatings for the detection and determination of various target molecules (Guilbault and Jordan, 1988). The two most common techniques used for the immobilisation of the biologically active sensing material are (i) pre-coating the quartz crystal with a suitable material (e.g. APTES, poly(ethyleneimine)) to create a thin layer capable of forming hydrophobic and/or covalent bonds with the biomolecules (Suleiman and Guilnault, 1994); (ii) self-assembled monolayers (SAM) based on the

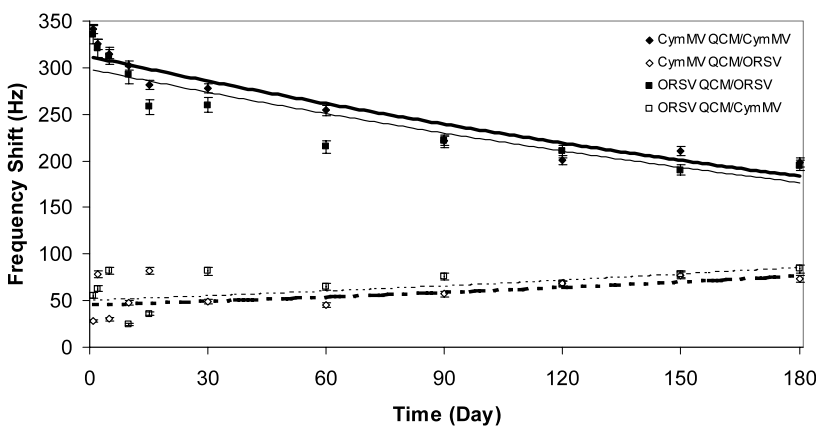

Fig. 3. Stability of CymMV (bold lines) and ORSV QCM (non-bold lines) immunosensors. Solid line, target virus; dotted line, non-target virus. The error bars show S.D. obtained from five replicates of each time point.

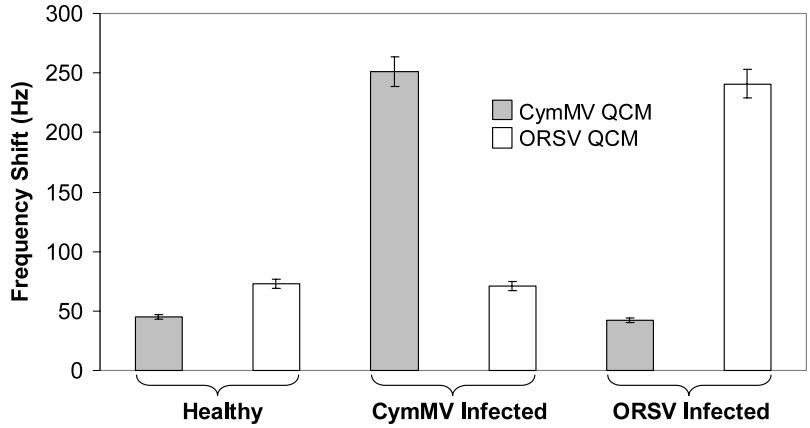

Fig. 4. Specificity of CymMV and ORSV immunosensors in the detection of target viruses from crude sap of *Oncidium* flower. The error bars show the S.D. obtained from five replicates of each sample.

strong adsorption of thiols (e.g. thioctic acid, 4-amino-thio-phenol, DTP) onto the gold electrode surfaces, coupled with linker molecules (e.g. EDC, glutaraldehyde, *N*-hydroxysuccinimide) capable of forming peptide bonds with the biomolecules (Wink et al., 1997). Although the literature provides extensive details on immobilisation techniques, the choice of an appropriate procedure for a given application has to be determined experimentally as the exact nature of intermolecular interaction between the coating and the target biomolecule is not well understood (Zhou et al., 1997). For the CymMV and ORSV QCM immunosensors, APTES yielded the highest frequency shifts observed for thin layer coating while DTP yielded the highest frequency shifts observed for SAM coatings. Factors contributing to the efficiency of the quartz crystal coating include the uniformity of the coating technique, the affinity of the coating material for the viral antibodies as well as the orientation of the coating materials (Wink et al., 1997). We believe that it is the antibody-coating and antigen-detection techniques, rather than the titre of the antiserum used, which determine the sensitivity of the detection system. Using the same CymMV and ORSV antiserum titre, the ability to detect simultaneously as little as 10 fg of both viruses in crude saps of *Oncidium* orchids was demonstrated using immuno-capillary zone electrophoresis (Eun and Wong, 1999). The development of novel antibody attachment matrices, which allows for a higher antibody monolayer density to be coated onto the quartz crystal could enhance the sensitivity of the QCM immunosensors.

There are several criteria, which all QCM-based detectors must possess, (i) the quartz crystal surface must be coated with suitable capture agents which must remain stable and well associated with the electrode during analysis; (ii) the capture agents must possess a high degree of specificity and affinity for the target analyte; (iii) the sensitivity and detection limits must be sufficiently high to be relevant biologically and the QCM detector must not respond to any other substances found in the sample (Spangler and Tyler, 1999). Both the CymMV and ORSV QCM immunosensors conform to these criteria. When coated with their respective viral antibodies, they are highly specific for their target viruses in both purified (Fig. 2C and D, and Fig. 3) as well as crude preparations (Fig. 4). Minimal frequency shifts were observed during the absence of their target viruses while significant changes in frequency shifts were observed in their presence. These traits were maintained when crude sap preparations were used. The specificity and affinity of the virus antibodies coated onto the quartz crystals for their target viruses are sufficiently high such that there was at least three-fold

difference in frequency shift between positive and negative samples (Fig. 4). In addition, both QCM immunosensors were stable for at least 30 days with minimal loss of immunochemical activity (Fig. 3), an observation common to most QCM detectors (Ngeh-Ngwainbi et al., 1990). The immobilised antibody gradually detaches from the quartz crystal surface and its sensitivity decreases with age (Suleiman and Guilnault, 1994). Our own on-going data, nevertheless, show minimal loss of immunochemical activity even up to approximately 6 months if the crystals are kept at 4 °C continuously, pre-coated and blocked with a layer of bovine serum albumin (BSA) prior to storage, and kept dry in desiccators.

To improve the detection limits and even the specificity of a mass amplified QCM-based assay, the use of specific secondary antibodies in a sandwich fashion has been suggested (Su et al., 2000b). Additionally, the use of large sol particles coated with antibodies either in a competitive or sandwich assay formats have also been suggested (Su et al., 2000a). In both of the formats, the large mass of the modified sol particle results in a large change in mass and hence a large shift in frequency. This could potentially increase the sensitivity of the assay many folds.

There are many advantages offered by QCM immunosensor detection systems. An entire detection system can be built for less than US$2000 with most of the equipment and raw materials readily available in electronic shops. QCM immunosensors, once the sensitivities and specificities have been optimised, require minimal sample preparation and very short time for data acquisition as compared with most other immunoassay techniques (Ngeh-Ngwainbi et al., 1990). Although the sensitivity and throughput of QCM immunosensors may not match up to enzyme linked immunosorbent assay (ELISA), which is currently one of the most widespread antibody-based detection system in use, the development of novel antibody attachment matrices as well as the production of quartz crystal micro-arrays could greatly enhance both the sensitivity as well as throughput. We are currently studying the feasibility of miniaturising the QCM immunosensor detection system, creating a microarray quartz crystal format similar to that of the DNA microarrays, which are currently available for nucleic acid analyses. To date, such compact and high throughput detection systems are not available for antibody-based protein detection assays. By combining the QCM immunosensor microarrays with a flow injection system, it would provide a real-time, high throughput and continuous assay. This raises the possibility of using different crystal assays in series to detect different targets in complex samples with an on-line display of the results (Suleiman and Guilnault, 1994). This could be applied in mass indexing programs such as virus-free certification, breeding, plant quarantine, and germplasm screening when speed, cost-effectiveness, specificity and sensitivity are crucial. This study demonstrates that QCM immunosensors are applicable to rapid plant virus detection using plant crude saps. This technique can be optimised to detect different plant viruses in different crops.

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