

Piezoelectric quartz crystal based label-free analysis for allergy disease

Xiaodi Su ^{a,*}, Fook Tim Chew ^b, Sam F.Y. Li ^c

^a Institute of Material Research and Engineering, No.3 Research Link, Singapore 117602, Singapore

^b Paediatrics Department, Medical Faculty, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

^c Institute of Material Research and Engineering and Department of Chemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

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Abstract

This work presents a piezoelectric (Pz) quartz crystal based label-free quantification of total IgE and allergen-specific IgE in human sera for allergy testing. An evaluation of the different brands of crystals was first initiated with respect to variability in mass sensitivity, frequency measurement reliability and stability, and surface roughness. Thereafter, for total IgE quantification, a direct assay format was adopted. By means of thioctic acid (TA) and coupling reagents, anti-human IgE antibodies were immobilized on AT-cut Pz crystals (10 MHz). The modified crystals could detect serum IgE directly corresponding to a downward frequency shift. The results showed that silver-coated crystals as compared with their gold-coated counterparts provided approximately 1.5 times higher mass detection sensitivity for total IgE in the range of 5–300 IU/ml with a linear regression line, $y = 1.8957x + 1.5603$, $R^2 = 0.995$. For the detection of allergen-specific IgE, a sandwiched assay format was used. As the allergen-modified sensor surface captured various classes of associated antibodies (IgE, IgG, etc) and interfering serum proteins as well, the initial frequency shift downwards caused by sera sample incubation would not be proportional to specific IgE levels. Thus, following sample incubation, a second incubation step with secondary anti-human IgE was added to recognize IgE from other bound substances. The frequency shift after secondary antibody binding reflected the amount of allergen-specific IgE proportionally. Compared with 10 MHz crystals, the 20 MHz counterparts provided approximately four times higher mass detection sensitivity for allergen specific IgE in the range of 0.15–17.5 IU/ml with a linear regression line, $y = 50.525x + 107.777$, $R^2 = 0.954$. Total IgE and allergen specific IgE assay results of real patients' sera using the Pz sensors agreed well with those obtained by commercially available test kits with correlation coefficient 0.96–0.98. The possibility of regenerating the quartz crystals for further re-use was also dealt with. © 2000 Elsevier Science S.A. All rights reserved.

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1. Introduction

Allergy is a common disease condition affecting 10–20% of the general population worldwide (Chew et al., 1996). Raised levels of total and allergen-specific serum immunoglobulin E (IgE) are found in patients with asthma, hay-fever, atopic dermatitis (Chew et al., 1999a,b, 2000) and many parasitic diseases. High levels of total IgE in human serum reflect the presence of allergic conditions. Further diagnosis for allergy, nevertheless, requires the detection of a number of allergen-

specific antibodies (i.e. specific IgE against components such as those of grasses, animal, dust, various food, etc).

Commercially available quantitative test kits for serum IgE are based on traditional immunoassay techniques. The IgE FAST™ (Flourescence Allergo Sorbent Test) kit by BioWhittaker (Walkersville, MD, USA), for example, uses anti-human IgE (or allergen) coated microtitration wells. Following with the application of serum samples into the wells, enzyme labeled anti-human IgE is then added. During the incubation period, IgE present in human sera is sandwiched between the bound and enzyme labeled anti-IgE antibody. Thereafter, substrate is added and the bound enzyme reacts

* Corresponding author. Tel.: +65-8748420; fax: +65-8727528.
E-mail address: xd-su@imre.org.sg (X. Su).

to generate a fluorescent product. The generated fluorescent product could then be measured, via a fluorometer using appropriate excitation wave lengths, and is directly proportional to the concentration of total IgE (or specific IgE) in the test sample. The Pharmacia–CAP system (Pharmacia and Upjohn, Sweden) offers a somewhat similar assay format for IgE quantification. The reaction carrier used is however a 3-D porous matrix coated with anti-human IgE (for total IgE quantification) or different allergens (for specific IgE quantification). These assays are nevertheless subjected to the use of enzyme labeled materials and the detection of fluorescent products by specially designed spectrophotometer instrumentation. Although precise and sensitivity enough, these traditional methods suffer from drawbacks of time-consuming procedures, complicate concepts, and the use of potentially hazardous materials and expensive instrumentation. In addition, enzyme activity is rather temperature dependent, thus enzyme immunoassay results are often less comparable between laboratories and personnel.

New technologies to be developed must be able to meet diagnostic demands better than established techniques and thus compete with them. Biosensors are one such candidate. The combination of a physical transducer with the immunologically active element(s) brings about a new generation of immunoassay devices, which would be small, self-contained, specific, cheap, and robust. By means of these devices, assay results can be produced quickly and automatically. Types of biosensor include amperometric, potentiometric, optical, and piezoelectric (Pz) crystal devices. Among these formats, Pz crystal and surface plasmon resonance (SPR) devices have been known independently as label-free methods, suitable for the direct on-line detection of immunological reactions. For example, SPR sensors are based on the excitation of surface plasmons in thin metal layers. Changes in the refractive index caused by the antigen–antibody interaction at a metal–liquid interface thus change the refraction angle of the laser light. Similarly, Pz crystals are mass sensitive transducers. Direct signal output from such devices is the oscillation frequency, which corresponds to the mass of the electrode. Any mass increase generated by adsorption of analyte(s) to the immobilized ligand thus decreases the oscillation frequency of the quartz crystal. Based on the information generated from the binding of the analytes to immobilized ligands, the immunological applications possible include the detection of antigen–antibody specificity, concentration, kinetics and affinity, and epitope mapping (Malmqvist, 1996; Homola et al., 1999). Previous comparisons made by various other investigators between the principles and applications of these two sensor formats (Köblinger et al., 1995; Skáltal and Horacek, 1999), have revealed that Pz devices offer a basic advantage over SPR, the basic instrument re-

quired is far less comprehensive and thus markedly less expensive.

The earliest Pz immunoassay was demonstrated in 1972 (Shons et al., 1972). Since then numerous combinations of Pz device with biological or immunological elements have been reported (Ngeh-Ngwainbi et al., 1990; Suleiman and Guilbault, 1994; Minunni et al., 1995; Bunde et al., 1998; O'Sullivan et al., 1999). In addition to bio-specific interaction assays (BIA) based on the interaction between antigen/antibody, nucleic acids, protein/peptide and receptors/various molecules, this devices have also been used for the evaluation of cell adhesion/cell function, blood clotting time, and depolymerization of muscle protein (Cavic et al., 1999). Most recently O'Sullivan and Guilbault (1999) presented a comprehensive review of the Pz device theory, analytical applications, and possible future commercialization trends. Parallel to traditional immunoassays, alternative assay designs and formats have actively been developed for Pz immunosensors, aimed at increase sensitivity, specificity, and the range of applications (Su et al., 2000). The more popular '*one step direct assay*', involving only one sample incubation step on the antigen or antibody modified sensor surface, has been widely used. The changes in resonant signal reflect the binding of associated analyte *directly*. This format is suitable for the detection of macromolecules (e.g. antibodies or viruses) and where the highly purified sensing receptor can be immobilized on the surface of the transducer. The *indirect* or *competitive assay* is less straightforward than direct format but provides a higher signal-to-noise ratio and additional benefits in terms of the elimination or reduction of non-specific binding. This can be achieved by pre-coating the surface with derivatives of the analyte, followed by a step exposing the coated surface to a sample mixture containing predetermined quantity of antibody and sample with the analyte to be quantified. The bound and free antigens compete for the binding sites on the antibodies, thus the binding of antibodies to the surface would be inversely reflective of the amount of antigen to be quantified. A third design, the *sandwiched assay* format, typically involves two incubation procedures. Sample incubation on the biologically modified surface is followed by secondary antibody incubation. Since the secondary antibody is fully complimentary to the target analyte, its binding can be used to specify classes or subclasses of antigen specific antibodies (Rice and Lake, 1979, 1980). In addition, this assay format is helpful in eliminating or reducing non-specific binding and would even enhance the binding signal (Sakai et al., 1995).

This paper presents a Pz device based label-free immunoassay for allergic disease. The *direct* and *sandwiched* assay formats were used for total IgE quantification and allergen-specific IgE detection, respectively.

The detection of a house dust mite, *Dermatophagoides pteronyssinus* (*Der p*) specific IgE in patients' sera was used in this study as the working example for allergen specific antibody detection. Prior to the experiments proper, we evaluated various brands of quartz crystal with different fundamental frequency and metal electrode deposition. An evaluation of their mass sensitivity variability, frequency reliability, surface roughness, and thereafter, the ability for regeneration and re-use were also dealt with in this paper.

2. Experimental

2.1. Apparatus and evaluation of the different brands of Pz crystals

Different brands of AT-cut gold- or silver-coated Pz crystals (10, 20, and 25 MHz) were purchased from International Crystal Manufacturing (ICM, Oklahoma City, OK USA), Farnell Components Pte Ltd (Singapore) or Hong Kong X'tals Limited. A laboratory-made TTL oscillating circuit was used to operate the crystals up to 25 MHz. The output frequency was continually monitored by a TF830 universal counter (Thurlby–Thandar, UK) and finally transferred to a computer using the RS 232C interface. The Labview™ program was used for all measurements, data storage and processing.

Liquid phase detection was carried out using a specially designed chamber. Quartz crystal was clamped between two Plexiglas blocks with Neoprene O-ring seals. The upper surface of the crystal can be exposed to the liquid sample and the other face was exposed to air. Typically the volume of the carrier buffer was 50 μ l and the quality of sample introduced was 5 μ l.

Atomic force microscopy (AFM) studies of surface roughness of metal electrodes were carried out using a TopoMetrix TMX 2000 Explorer system (Santa Clara, CA). AFM images with $80 \times 80 \mu$ m were taken and surface roughness was calculated after horizontal leveling. The values of Ra were taken from three crystals with three measurements for each crystal.

2.2. Chemicals and biochemicals

Thioctic acid (TA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), γ -amino propyltriethoxy silane (γ -APTES), polyethyleneimine (PEI), and glutaraldehyde (GA) were obtained from Sigma. Monoclonal anti-human IgE (I-6510) and polyclonal goat anti-human IgE (I-0632) were also purchased from Sigma. Human total IgE standards were obtained from the Total IgE II FAST Test Kit from BioWhittaker. Phosphate buffered saline (PBS) was prepared in the laboratory with the following chemi-

icals, 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4 (pH 7.2). The blocking reagent used in all experiments in this paper was bovine serum albumin (BSA) from Sigma. The wash solution used was PBS buffer with low concentration of mild detergent (0.05% Tween-20). Other chemicals used were certified reagents from Fluka or Sigma.

The *Der p* dust mite crude protein was extracted from pure dust mite culture grown in the laboratory using the standard protocol (Krantz, 1978; Chew et al., 1999a). This was achieved by culturing *Der p* mites on a mixture of rodent food pellets and tetramine fish food, followed by a separation step to isolate the mites from the food medium using a modified Tullgren apparatus. The whole mite bodies were defatted for 4 h with anhydrous ether and lyophilized prior to storage at -80°C prior to use. Extraction of the protein in PBS overnight at 4°C was then carried out. The supernatant was filtered through a 0.22μ m membrane, and the protein content quantified using Bio-Rad dye-binding Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Der p-specific IgE standards were generated in-house using pooled patient sera. Strong *Der p* IgE positive serum samples from four patients with strong positive skin prick reactions to the extract were obtained and mixed thoroughly. The mixed pooled serum was quantified by IgE FAST Plus™ Test and used as the calibrators. Prior to use, it was serially diluted to the proper concentration (14.4, 7.2, 3.6, 0.6, and 0.15 IU/ml) and re-assayed via the FAST assay to confirm the concentrations (%CV less than 5%).

2.3. Total IgE quantification

Gold coated crystals were cleaned with hot Piranha solution (30% H_2O_2 : $\text{H}_2\text{SO}_4 = 1:3$) by applying 10 μ l each of Piranha solution on both sides of the gold surface for 5 min. Silver coated crystals were soaked with 1.2 N NaOH for 5 min, followed by 1.2 N HCl for 2 min. After thorough rinsing with distilled water and ethanol, these freshly cleaned crystals were dipped into 0.1% TA in ethanol overnight. This was followed by a dipping of the crystals in 2% EDC for 30 min to further activate the crystal surface. Five microliters each of monoclonal anti-human IgE at concentration of 0.5 mg/ml was added on both sides of the modified crystals for 30 min, followed by 5% BSA incubation for 30 min to block the non-occupied residues. To investigate the immobilization procedure, frequency of each reaction step was monitored at dry state. To perform the assay of human IgE, the fabricated sensors were exposed to 5 μ l of IgE calibrators at varying concentration or human serum samples for 30 min at room temperature. Frequency values before and after sample incubation was taken as F_R and F_I , respectively.

2.4. Allergen specific IgE quantification

After chemical modification as described above, quartz crystal surfaces were exposed to 5 µl of *Der p* protein at proper concentrations for indicated time. After blocking the non-specific binding residues, standard sera with known concentrations of *Der p*-specific IgE or unknown serum samples were applied for 30 min. After washing and drying those crystals, anti-human IgE were applied for further reaction with bound IgE. A calibration curve was built using frequency change caused by anti-human IgE versus the standard *Der p*-specific IgE calibrator concentration.

2.5. Total IgE II and IgE FAST Plus™ test

These procedures were performed using standard protocols (BioWhittaker, Walkersville, MD, USA).

2.6. Regeneration of used Pz crystal

Dichromatic acid or hot Piranha solution was used to treat the used gold crystals by applying 10 µl each of the said solution on both of the electrodes for certain time. After washing and drying with distilled water, the crystals were air-dried and the frequency values were measured and compared with their original fundamental values. The regeneration ratio was calculated as

$$\frac{F - F_0}{\Delta F_{\text{whole}}} \times 100\%$$

where F_0 refers to as the original fundamental frequency without any coatings, ΔF_{whole} refers to as the accumulated frequency change after usage.

3. Results and discussion

3.1. Evaluation of the different brands of quartz crystals

Pz crystal consists of an AT cut quartz wafer sand-

wiched between two metal electrodes. The electrodes provide the means of connecting the device to an external oscillator circuit that drives the quartz crystal at its resonant frequency. The famous Sauerbrey (1959) Eq. (1) describes the relationship between the frequency change and the mass accumulated on crystal in gas phase.

$$\Delta F = \frac{-2F_0^2 \Delta m}{A(\rho_q \mu_q)^{1/2}} \tag{1}$$

Mass sensitivity (S), accordingly, can be described as in Eq. (2), which is the quadratic function of the fundamental frequency F_0 :

$$S = \frac{2F_0^2}{(\rho_q \mu_q)^{1/2}} \tag{2}$$

When launching a study using Pz crystals as reaction carriers, an understanding of the properties of the quartz crystals to be used is very important. The fundamental frequency, mass sensitivity (variability in fundamental frequency and mass sensitivity), frequency measurement reliability (and stability), type of metal electrode and surface roughness, are some of the parameters that may affect the performance of the crystals as analytical devices. Preliminary assessment of these parameters on different brands of crystals is listed in Tables 1 and 2.

Firstly, fundamental frequency (F_0) is the determinant of the mass measurement sensitivity of the Pz crystal as shown in Eq. (2). F_0 , itself depends upon the thickness of the quartz wafer, its chemical structure, its shape, and its mass. For a given design, any variations in these parameters from the manufacture, in particular, the thickness of the quartz wafer and metal electrode deposited would result in variation of F_0 between individual crystals. To evaluate the mass sensitivity, and the variability of both fundamental frequency and mass sensitivity, 25 pieces of crystals were selected from each brand and were tested using the instrumentation setup described in Section 2. After stabilization of the reading, the F_0 for each crystal was taken and variations between individuals were calculated (Table 1). Theoretically, crystals from ICM would normally be calibrated within

Table 1
Mass sensitivity evaluation for branches of quartz crystals

Brand	Fundamental frequency (MHz ± kHz)	Mass sensitivity (Hz/ng per cm ²)	CV (‰)	Electrode diameter (cm)	−dm/dF (ng/Hz)
ICM gold (Cat. 131227)	9.971 ± 7.273	0.226 ± 0.324 × 10 ^{−3}	1.44	0.51	0.903
ICM gold (Cat. 131295)	20.146 ± 18.780	0.921 ± 0.700 × 10 ^{−3}	1.86	0.35	0.104
ICM gold (Cat. 131354)	25.123 ± 24.4	1.432 ± 2.720 × 10 ^{−3}	1.06	0.35	0.067
Farnell silver (103–887)	9.994 ± 0.245	0.227 ± 1.113 × 10 ^{−5}	0.05	0.48	0.794
Farnell silver (170–234)	19.993 ± 0.455	0.907 ± 4.1 × 10 ^{−5}	0.05	0.35	0.106
HK X’stal silver (HKC/10.00/24 PF)	9.998 ± 2.889	0.227 ± 1.269 × 10 ^{−4}	0.51	0.45	0.700
HK X’stal silver (HKC/20.480/24 PF)	19.991 ± 10.308	0.907 ± 9.35 × 10 ^{−4}	1.03	0.45	0.175

Table 2
Evaluation of stability of frequency measurements and surface roughness

Brands	Noise level (Hz)	Surface roughness	
		Indirect method (kHz)	Direct method Ra (nm)
ICM/gold/polished/10 MHz	–	~2	10 ± 0.8
ICM/gold/unpolished/10 MHz	1.1	6–7	185.9 ± 32.5
ICM/gold/polished/25 MHz	21.2	–	–
ICM/silver/unpolished/10 MHz	1.7	5–5.5	177.0 ± 29.3
Farnell/silver/10 MHz	1.5	3–4	124.6 ± 19.5
Farnell/silver/20 MHz	7.7	–	–
Hong Kong X'stal/silver/10 MHz	1.9	~3	114.6 ± 11.2
Hong Kong X'stal/silver/20 MHz	4.3	–	–

± 0.2% of stated frequency measured at series resonance. Farnell provided crystals (Cat. 103887 and 170234) have frequency accuracy of ± 20 ppm. Our measurement results for 25 pieces of each brand of the crystals matched well with these theoretical specifications. Thus, it can be confirmed that our instrumentation setup is reliable to operate Pz crystals at their resonant frequency. Through the use of the Sauerbrey equation, variations in F_0 were converted into percentage CV of mass sensitivity. Taking into consideration the actual working area of the electrodes, $-dm/dF$ was also calculated for each brand of crystals. Since Farnell and Hong Kong X'tals sell crystals as electronic components to provide frequency standards, it is understandable that the variations in F_0 and mass sensitivity of their crystals are pretty low, thus making them suitable as mass measurement sensors. Being the laboratory microbalance for research projects, ICM provided crystals have acceptable accuracy in frequency.

The next parameters, stability of frequency measurements and electrode surface conditions, were evaluated as shown in Table 2. Since most of the following experiments relied on the *dip and dry* measurement approach, stability of the gas phase frequency measurements was investigated. It was observed that 20 MHz crystals took ~ 2 min to achieve a stabilized reading under our experimental conditions, whereas the frequency readings of most of the 10 MHz quartz crystals stabilized in less than 1 min. At stabilized state, the frequency readings were collected over 5 min at an interval of 2s'. The levels of noise were then determined as three times the standard deviations of the frequency readings in this period. The results showed that our instrumentation setup could operate the 10 and 20 MHz crystals adequately with noise levels of less than 2 and 4–8 Hz, respectively. As for the 25 MHz crystals, the noise levels were however less acceptable.

Surface roughness is another important property of the crystals that may affect, in particular, the liquid responses of quartz crystals. Bruckenstein and Shay (1994) previously revealed that surface roughness would increase the magnitude of the liquid phase. Liquid entrapment in grooves and holes of a rough surface would result in additional frequency change (Martin et al., 1993; Zhang et al., 1998). In this study, an indirect estimation of surface roughness was carried out via the measurement of frequency change after exposing one face of the Pz crystals to water. Our data showed that with 10 MHz crystals with smooth polished surface, the frequency shift was about 2 kHz under our experimental conditions. Crystals with rough surface (unpolished crystals) produced higher frequency change of 3–7 kHz. Direct surface roughness measurements made using AFM have verified that silver coated crystals provided by Farnell and Hong Kong X'stal are less rough than unpolished crystals provided by ICM.

Finally, the type of metal electrodes deposited may also be an important consideration in the selection of quartz crystals. The metal electrode deposited is a determinative factor, which may play a role in influencing the molecular immobilization. Gold-coated crystals have always been widely used in Pz sensor development because of its long-term stability. Silver-coated crystals have also attracted people's interests because of its cost effectiveness (Barnes et al., 1991; Chen et al., 1999; Sakti et al., 1999). In the following total IgE quantification experiments, gold-coated crystals and silver-coated crystals have been used. Although they have similar mass sensitivity and noise levels, the data showed that the different surface properties of gold or silver resulted in differences in molecule binding performance and consequently different measurement sensitivity (see below).



Fig. 1. Total IgE assay scheme Υ anti-IgE antibody, $\bullet$ IgE analyte.

3.2. Total IgE quantification

To perform the total IgE quantification, the one step direct assay format was adopted as shown in Fig. 1. It was accomplished by the modification of sensor surface using monoclonal anti-human IgE followed by sample application. Once the Pz surface had been fabricated and coated with anti-IgE (F_R), a single sample incubation step was sufficient to generate the final signal (F_I). The frequency change of this interaction ($F_R - F_I$) corresponded to the concentration of IgE (analyte) directly.

In our previous study, this concept has been established with the use of 10 MHz gold-coated crystals as the sensor element (Su et al., 1999a,b). Thioctic acid-EDC or cysteamine-GA immobilization methods were adopted. The established Pz sensors has been proved to be able to quantify total IgE in the range of 5–300 IU/ml (one international unit is defined to be equivalent to 2.4 ng IgE) with $R^2 = 0.998$. In this following study, silver-coated crystals (10 MHz) provided by Farnell (Cat. 103–887) and Hong Kong X'tals (HKC/10.000/24 PF) were used. Thioctic acid-EDC immobilization method was adopted for anti-IgE immobilization. Table 3 illustrates the immobilization results observed on these silver-coated crystals (results on the gold coated crystals from the previous study are also listed for comparison). The binding capacity was converted by using $-dm/dF$ as calculated in Table 1. It was found, for this given immobilization method, silver offered a more superior binding performance. It enabled a higher TA binding capacity and higher EDC to TA reaction ratio of 0.5–0.6 nmol/nmol (compared with the gold surface, with a ratio of 0.27 nmol/nmol). Consequently, the antibody binding amount on silver

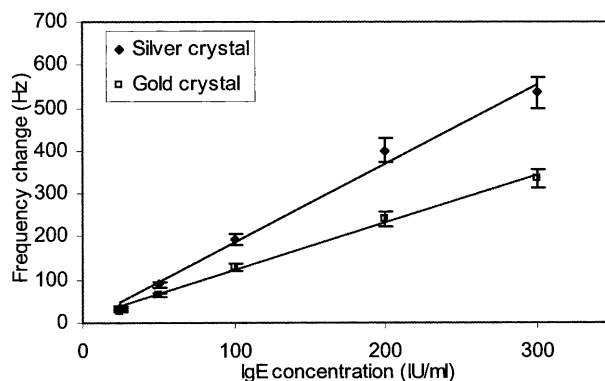


Fig. 2. Calibration curve for total IgE quantification using anti-IgE coated 10 MHz Pz crystal.

crystals was ~ 1.5 times greater than that of gold crystals. Silver crystals from Farnell were thus used for further experiments.

After blocking with 5% BSA, the anti-IgE modified Pz crystals were exposed to the IgE calibrators in the range of 25–300 IU/ml. The change in frequency as the function of IgE concentration was built as shown in Fig. 2. The linear regression results obtained with the silver and gold crystals were $y = 1.8957x + 1.5603$, $R^2 = 0.995$ and $y = 1.2378x + 4.4914$, $R^2 = 0.998$, respectively. It was observed that the detection sensitivity obtained from silver crystals was 1.53 times of that obtained from gold-coated crystals over the range evaluated in the study. The data implies that detection sensitivity was subjected to the amount of antibodies immobilized. Assay results of 28 patient serum samples obtained using the modified silver crystals indicated that the increased sensitivity brought about better agreement between Pz sensor results and those of commercial kits (FAST), particularly for samples at concentration below 100 IU/ml (Pearson correlation, 0.98; data not shown). The relative standard deviation of results obtained via Pz sensors and FAST kits for samples at this range was kept within 5–10%.

It must nevertheless be noted that the difference in molecular binding performance between gold- and sil-

Table 3
Comparison of molecular immobilization procedures different types of crystals^a

	Gold coated (ICM)		Silver coated (Farnell)		Silver coated (HK)	
	ΔF (Hz)	Binding capacity ^b (nmol/side)	ΔF (Hz)	Binding capacity ^b (nmol/side)	ΔF (Hz)	Binding capacity ^b (nmol/side)
TA	276.1 ± 27.7	1.21	744.9 ± 70.4	2.87	685.2 ± 63.6	2.32
EDC	70.2 ± 62.3	0.33	351.7 ± 40.2	1.45	371.2 ± 39.3	1.36
Anti-IgE	331.5 ± 30.1	0.0020	602.5 ± 61.3	0.0032	630.3 ± 63.3	0.00294

^a Average from at least five duplicate results.

^b Binding capacities were calculated using $-dm/dF$ of each crystal and molecular weight of the immobilized substance, e.g. TA 206.3, EDC 191.7, and anti-IgE 150 000.

ver-coated crystals observed here was not always observed for other surface treatment procedures. For example, with γ -APTES or PEI, which were conventionally used to immobilize antibodies on Pz sensor (Guilbault et al., 1992; König and Grätzel, 1993), we found that after activation with GA and immobilization of anti-IgE antibodies, there was no significant difference between the two types of crystals in terms of the binding signal after chemical treatment, antibody immobilization, and analyte detection (data not shown).

3.3. Sandwiched assay for *Der p* specific IgE

The house dust mite, *Der p*, is one of the most important indoor allergens associated with asthma and allergic rhinitis (Zhang et al., 1997; Chew et al., 1999b). Quantification of IgE against specific allergen offers further diagnosis for allergic disease. The detection of a house dust mite, *Der p*-specific IgE in patients' sera was used in this study as the working example for allergen specific antibody detection. A sandwiched format based was proposed as described in Fig. 3. Allergen protein modified quartz crystals (F_R) were exposed to human serum samples (F_1). In order to specify the class of bound antibodies, anti-human IgE was used as second antibody (F_2). As the result, *Der p*-specific IgE was sandwiched between *Der p* protein and anti-human IgE.

The crude *Der p* extract, obtained according to the procedure described in the Experimental section, had a total protein concentration of 8.9 mg/ml. Initially, 10 MHz silver-coated crystals were used to perform this assay. After treating silver electrodes with TA and EDC, *Der p* proteins in the concentration range from 0.18 to 4.4 mg/ml were applied. A pooled patient serum with high *Der p*-specific IgE was used to identify the sensitivity of the series of crystals coated with different concentration of proteins. The optimal concentration was found to be in the range of 0.5–1.0 mg/ml. At this concentration range the binding of proteins was saturated with corresponding frequency change of 550 ± 58 Hz and the coated crystals provided the highest binding signal for serum samples. Higher concentrations were not recommended because excessively high density or stacking of antigens would inhibit antibody binding or lead to less stable interactions with of subsequent reagents.

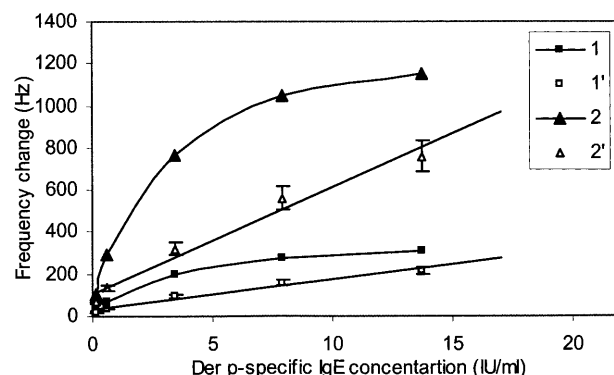


Fig. 4. Sandwiched assay results for *Der p*-specific IgE using 10 MHz of silver-coated crystals (line 1, 1') and 20 MHz silver-coated crystals (line 2, 2'). Line 1 and 2 are for the frequency changes after the first sample incubation vs. *Der p* IgE, while line 1' and 2' are for the frequency changes after the second anti-IgE binding vs. *Der p* IgE.

Experiments were also carried out to verify the function of EDC in protein immobilization process. As reported previously (Torossian et al., 1996), the presence of EDC allowed the formation of an *O*-acylurea intermediate, which promoted the formation of amide bond between carboxylic acids and amines provided by the immobilized protein. But other researcher have also reported that the $-\text{COOH}$ modified surface would adsorb proteins in the absence of coupling reagents (Yoon et al., 1996). It was believed that the electrostatic interaction between carboxylated microspheres and proteins were mainly responsible for the protein immobilization. In this study, both of the conditions with and without EDC coupling were evaluated. Results indicated that proteins immobilized through EDC coupling were more stably adsorbed. Loss of protein after multiple washing of fabricated surface was less than 5%, whereas, for protein layers immobilized without EDC, multiple washing resulted in loss of approximately 12%.

Under the optimal condition, large quantity of crystals was fabricated. After blocking non-specific binding residues, the resonant frequency was measured as F_R . Consequently, a serial of standard serum samples with *Der p* IgE concentrations of 14.4, 7.2, 3.6, 0.6, and 0.15 IU/ml were applied for 30 min. After washing and air-drying, the frequency value was taken as F_1 . The change in frequency caused by this step ($F_R - F_1$) as the function of *Der p* IgE concentration was plotted, as shown in Fig. 4 (line 1). It can be seen that this line is less linear with $R^2 = 0.79$. This may be explained as that in addition to IgE, other antibody classes, for example IgG, was also bound to the antigen coated surface. Moreover, since coated allergen was a combination of multiple crude protein(s), it is possible that some other serum components may also bind and would thus result in extra binding interfere.

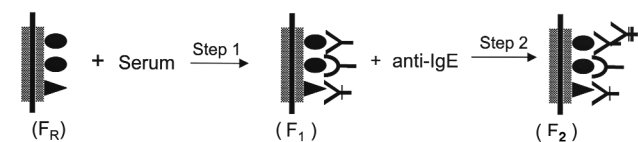


Fig. 3. Allergen specific IgE assay scheme allergen protein, crude protein component, target antibody class (IgE), interference antibody, secondary antibody specific to target, other antibody class.

To specifically indentify the bound target IgE, excess amount of goat anti-human IgE (0.1 mg/ml) was applied further for another 30 min. After washing and drying, the resonant frequency F_2 was taken. It was found that the binding of anti-human IgE ($F_1 - F_2$) was proportional well to the concentration of *Der p*-specific IgE with $R^2 = 0.978$ (Fig. 4, line 1'). Liquid phase experiments were carried out to provide further evidence for the sandwiched assay as shown in Fig. 5. *Der p* coated surface was exposed to a strong IgE positive serum at arrow 1. The frequency shift down (line 2) indicated the binding of *Der p*-specific antibodies. This was followed by the addition of a goat anti-human IgE at arrow 2. The further decrease in the frequency indicated the formation of anti-IgE/IgE complex. If *Der p* coated surface was exposed to anti-human IgE directly (line 1) without sample incubation, no significant frequency response was observed. This confirmed that the secondary antibody was attributable in sandwiched assay to specify the amount of allergen specific IgE presented in human serum.

In the clinical setting, the diagnosis of allergen specific IgE requires the classification of patients into class 0–4 corresponding to IgE concentration of <0.35, 0.35–0.75, 0.76–2.99, 3.00–17.5, and >17.5 IU/ml, respectively. Our data, nevertheless, showed that the resolution of the 10 MHz crystals was not high enough to differentiate specific IgE levels at class 0, 1, and 2.

To enhance the binding signal of the Pz sensor, there have been a variety of methods reported in the literature; for example, the use of sol particle labeled antibodies (James and Richards, 1989; Ward and Ebersole, 1996) or protein labelled antigen (Yokoyama and Ikebukuro, 1995). We ever managed to use colloidal gold conjugated anti-human IgE as the secondary antibody to possibly enhance the final binding signal in the sandwiched Pz sensor. A problem however arose, in that the huge molecules exhibited much lower response.

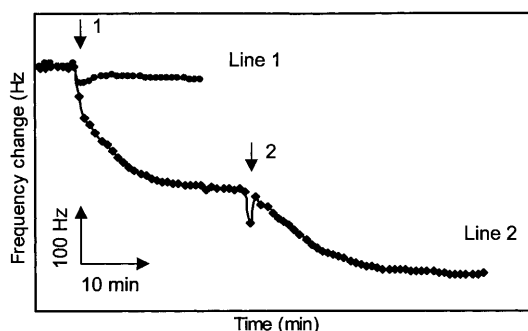


Fig. 5. Frequency responses of sandwiched assay procedures. Line 2 depicts patient serum introduction onto *Der p* protein modified Pz sensor surface at arrow 1, followed by anti-human IgE introduction at arrow 2; line 1 shows the response anti-human IgE introduction onto *Der p* protein modified surface without patient serum introduction in prior.

Pz sensor test results	Class 0	1	2	3	4
Class 4					++
Class 3				+++ +++	+
Class 2		+	+++		
Class 1		+++			
Class 0	++	+			
	Class 0	1	2	3	4
	FAST results				

Fig. 6. Cross-comparison of *Der p* IgE semi-quantification results obtained by Pz sensors and FAST test, according to the levels classified in clinical practice.

Liquid phase on-line detection additionally indicated that binding of colloidal gold conjugated anti-IgE was not completed even after a reasonably long period of time (data not shown). Similar observations were found by Horacek and Skladal (1997) in the 2,4-dichlorophenoxyacetic acid detection using BSA conjugated antibody.

Alternatively, the use of crystals with higher fundamental frequency has been proved to be successful in enhancing the resolution (Uttenthaler et al., 1998). Based on our preliminary evaluation of the different brands of crystals, the 20 MHz sliver-coated crystals from Hong Kong X'stal were used in this study. On these crystals, molecular binding signals of both the surface modification and sample detection procedures were around 4 times higher than those observed with the 10 MHz crystals. Line 2' in Fig. 4 is the calibration curve representing the linear relationship between the anti-human IgE binding signals on the 20 MHz crystals to the concentration of *Der p* IgE with $y = 50.525x + 107.77$ and $R^2 = 0.954$. With the use of well-fabricated 20 MHz Pz crystals, 19 patient serum samples were tested. Concentrations of *Der p*-specific IgE were calculated with reference to the calibration curve line 2'. Samples with $F_1 - F_2$ larger than 992 Hz, and lower than 125 Hz was identified as class 4 and 0, respectively. Samples with $F_1 - F_2$ lied in the range of 125–146, 146–259, 259–992 Hz were classified as class 1, 2, or 3, respectively. Fig. 6 shows the cross comparison of Pz sensor and FAST results, according to the levels classified in clinical practice (Spearman's rank correlation, 0.96). Typically, in the clinical setting, patients with allergen specific IgE over class 3 are confirmed as allergic. In this study, 9 of the samples recognized as over class 3 by FAST test were also tested as over class 3 by Pz sensor. In this sense, the proposed Pz sensors

were found to be reliable in diagnosis, although mismatches for samples lower than class 3 occurred more frequently. Nevertheless, since the FAST test is an enzyme-based assay, the final fluorescence signal generated by enzyme reaction is temperature dependent. Mis-classification, particularly for samples in the lower diagnosis classes, has frequently been observed with any inconsistency in incubation condition. Pz crystal-based label-free immunoassay eliminates the use of enzyme, thus provides simple assay concept and less critical assay conditions may be required.

On a separate note, it must however be acknowledged that 20 MHz crystals are thinner and more fragile than 10 MHz ones. Care must be taken to avoid broken of the crystal during the experiments, particularly during the washing and drying steps.

3.4. Regeneration possibility

The term of regeneration of used Pz crystal can be understood in two different senses. Eluting of antigen or antibody from the immune-complexes allows the modified surface to be reset for another assay. Based on the affinity of the immune-complexes and the stability of immobilized ligands, the denaturing reagents can be very mild pH 2.5 glycine–HCl buffer or more vigorous 0.001 M HCl or urea in the concentration range of 6–8 M. This concept has been established in many early studies (Guilbault et al., 1992; Steegborn and Skládal, 1997; Su et al., 1999a,b, 2000).

In this study, however, we defined regeneration as the stripping of whole coatings from the sensor surface, and then hopefully fresh metal electrodes could be rebuilt for any other molecular modification. For the case of antibody immobilized through protein A modification, antibody and protein A layers could be removed by soaking the surface in 1.2N NaOH, followed by 1.2N HCl, and finally in concentrate HCl (Guilbault et al., 1992). For hard to removed coatings, however, we found that dichromatic acid washing solution was the best choice. As shown in Fig. 7, application of 10 μ l dichromatic acid solution on used gold crystals with TA/EDC/Anti-IgE/IgE coatings for 10 min could remove all of the chemicals and proteins and eventually enabled the frequency of the crystals return to the original fundamental values. Whilst, Piranha solution could remove only 75% of those coatings within 35 min treatment. The interaction of γ -amino propyltriethoxy silane (γ -APTES) on gold is based on three gold and oxygen linkages. Regeneration of γ -APTES treated crystal could be achieved by long term treatment with hot dichromate acid solution.

The reproducibility of immobilization after regeneration was investigated. For example, used crystals with TA/EDC/anti-IgE/IgE coatings were treated using dichromate acid for 15 min. The regenerated crystals

were then re-modified by TA as described in Experimental. The resulting TA binding signal from eight duplicated crystals was 262.5 ± 29.2 Hz, which is consistent with that obtained by new crystals. The following antibody immobilization resulted in repeatable binding capacity comparable with that obtained by new crystals (data not shown). To ensure this reproducibility, the timing of dichromate acid treatment for used crystals with TA/EDC/anti-IgE/IgE coatings must be restricted to within 20 min. Any longer treatment would result in ‘over-stripping’, the frequency value would go back over its original fundamental frequency (line 1, Fig. 7), thus implying possibly irreversible ill effects to the gold electrode. The resulting gold surfaces were rougher than those of new crystals. Thus, to ensure longer life-usage of gold-coated crystals, the time required for optimal stripping of molecular coatings should be examined.

It must be pointed out that regeneration of the metal surface using such chemical treatment was only possible with gold-coated crystals, as the silver-coated crystals cannot withstand such harsh chemical treatment. It may, nevertheless, only worthwhile to regenerate the gold crystals, because of the high unite price. Silver-coated crystals are much cheaper with the unit price being less than US \$1. They can thus be used as disposables. It must, however, be acknowledged that silver-coated crystals would bring about a problem of aging and possibly shorter shelf life. It may thus be required that the fabricated sensors being sealed in nitrogen until they are used.

4. Conclusion

Pz crystal devices have shown to be an alternative method of performing immunoassay without the use of

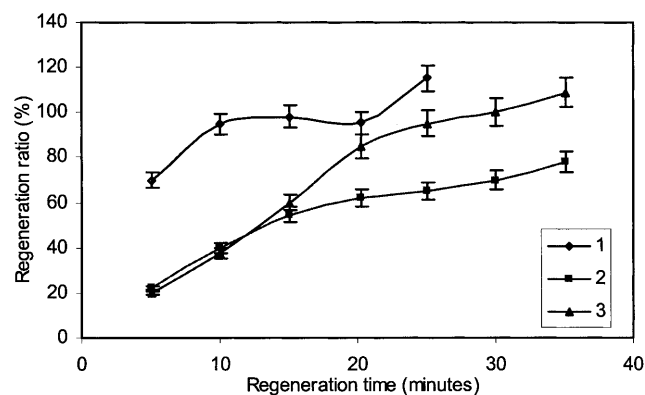


Fig. 7. Regeneration of TA/EDC/anti-IgE/IgE modified gold surface with dichromate acid solution (1) and hot Piranha solution (2); Regeneration of γ -APTES/GA/anti-IgE/IgE treated crystal with hot dichromate acid solution (3).

labeled materials and expensive instrumentation. The one step direct assay is the simplest design suitable for the detection of macromolecules where purified biosensing molecules can be immobilized. Sandwiched assay format can be used to differentiate antibody class or subclass and eliminate non-specific binding.

For any given application, the first thing need to be addressed is the selection of a proper protein immobilization approach, which permits sufficient amount of protein immobilized and yet maintaining its biological activity. At the same time, appropriate Pz crystals need to be selected, which should be suitable for said coating method and produce high detection sensitivity and stability. The most commonly used quartz crystals are the 9 or 10 MHz crystals. If the sensitivity obtained is acceptable, it is not recommended to use crystals with higher fundamental frequency since the higher F_0 would bring about the higher noise-to-signal ratios and a more demanding oscillation circuit would be required to operate them properly. In addition, as the frequency goes higher, the quartz wafer blank will become thinner and more fragile, one has to weight the handling difficulties of a higher frequency blank versus the better resolution achievable.

Gold crystals are most commonly used as reaction carriers because of their long-term stability. Silver-coated crystals have also attracted interests because of its low unit price. In this study it was found silver-coated crystals provided greater TA binding capacity than gold-coated crystals. Consequently, more antibody binding was achieved on the silver-coated crystals. This may be due to the difference in atomic size and crystallographic planes of Au and Ag. These factors would affect their atomic packing density and the binding strength with thiolated compounds. Systemically studies by AFM and XRD should be helpful to reveal insides into molecular binding performances on silver or gold and thus could direct biosensor surface modification.

Finally, this work has presented the possibility of using Pz crystal based biosensors for label-free quantification of both total IgE and allergen-specific IgE in human sera for allergy testing. For total IgE quantification, a direct assay format was adopted, while for the detection of allergen-specific IgE, a sandwiched assay format was used. The detection of a house dust mite, *Der p*-specific IgE in patients' sera was used in this study as the working example for allergen specific antibody detection. This would form the basis of further work to develop an array of Pz sensors with different allergens coated on them for simultaneous testing. In addition, the testing of different allergens from different sample sources using specific antibody modified Pz sensors is also of great interesting.

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