

Self-Assembled Monolayer-Based Piezoelectric Crystal Immunosensor for the Quantification of Total Human Immunoglobulin E

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Received January 20, 1999

This work presents a piezoelectric (Pz) immunosensor for the quantification of total human IgE in serum samples. The anti-human IgE is deposited on the surface of the 10 MHz AT-cut gold coated crystal resonator by self-assembled technique, and serves as a receptor layer. The highly ordered self-assembled monolayers (SAMs) ensure well-controlled surface structure and offer many advantages to the performance of the sensor. The fabricated Pz sensor can quantitatively detect human serum IgE in the range of 5–300 IU/ml with high precision (CV < 8%). A total of 28 patient serum samples are detected by the Pz sensor, and the results agree well with those given by two commercially provided test kits (Total IgE FAST Test, Pharmacia-CAP). The correlation coefficients are 0.94 between FAST and Pz sensor, and 0.90 between CAP and Pz sensor, respectively. After regeneration with urea and glycine buffer the coated crystal can be re-used five times without appreciable loss of activity. Compared with conventional cross-linking methods, nonspecific binding caused by the SAM binding method is three to five times less. © 1999 Academic Press

Key Words: piezoelectric immunosensor; self-assembled monolayer; human IgE quantification.

Immunoglobulin E (IgE)² is a four-chain molecule with a molecular weight near 190,000. It is present in

the serum of several mammalian species at extremely low concentrations. Raised levels of serum IgE are found in asthma, hay fever, atopic dermatitis, and many parasitic diseases. In clinical chemistry, IgE is usually measured in plasma or serum to diagnose immediate hypersensitivity (1). Several immunoassay methods have been reported for the measurement of total human IgE as antigen, e.g., radioimmunoassay (2), and enzyme-labeled immunosorbent assays (3). These assays use anti-IgE antibody immobilized on various solid supports, and use radiolabeled or enzyme-labeled anti-IgE antibodies to perform a sandwich immunoassay. On the basis of the traditional assay techniques, there are several commercially available allergy test kits, such as FAST-Biowhittaker (4), Pharmacia-CAP (5), and MAST-CLA system (6) being widely used. Although they are sensitive and precise enough, these conventional immunoassay methods, however, suffer from drawbacks of requiring skilled personnel and potentially hazardous and expensive chemicals. In addition, the test procedures and instrumentation are complex and time-consuming.

An alternative method to perform the assay of antigen or antibody is the use of an antigen/antibody-based biosensor. Biosensors offer the potential to use inexpensive equipment that can be taken to where the samples may be added by unskilled personnel; the result would ideally be available rapidly. Immunosensors can quantitatively or qualitatively detect antigen or antibody either by direct competitive and displacement reactions or by direct changes in transducer out-

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² Abbreviations used: IgE, immunoglobulin E; Pz, piezoelectric; SAM, self-assembled monolayer; GA, glutaraldehyde; EDC, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, γ -APTES, γ -amino

propyltriethoxy silane; PEI, polyethyleneimine; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

put (7). An example of the latter type of system is the piezoelectric (Pz) crystal devices.

The Pz crystal device consists of a quartz crystal wafer sandwiched between two metal electrodes. The electrodes provide a means of connecting the device to an external oscillator circuit that drives the quartz crystal at its resonant frequency. This frequency is dependent on the mass of the crystal, as well as the mass of any layers confined to the electrode areas of the crystal. Changes in mass on the surface of the electrode thus change the frequency of the Pz device. The relationship between the frequency change and the mass loading is described by the Sauerbrey equation [8]

$$\Delta F = -2F_0^2 \Delta M / A(\rho_q \mu_q)^{1/2}, \quad [1]$$

where ΔF is the measured frequency shift, F_0 is the resonant frequency of the Pz crystal, A is the area coated, ΔM is the mass change due to surface deposition, ρ_q is the density of the quartz crystal (2.648 g cm^{-3}), and μ_q is the shear modulus ($2.947 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2}$ for AT-cut quartz crystals). The mass sensitivity can be described as Eq. [2]:

$$S = 2F_0^2 / (\rho_q \mu_q)^{1/2} \quad [2]$$

For a 10-MHz piezoelectric crystal, the mass sensitivity of $S = 0.227 \text{ Hz ng}^{-1} \text{ cm}^2$ means that 4.4 ng/cm^2 of adsorption leads to frequency change of about 1 Hz.

In recent years, methods based on the use of piezoelectric crystal devices have been developed for immunoassay applications (9–11). The critical procedure to fabricate a Pz crystal-based immunosensor is the surface modification of the electrode, by which antigen or antibody layers are created to serve as receptors to capture the target analyte from the sample. The use of self-assembled monolayer (SAM) in various fields of research is rapidly growing. In particular, SAM has been employed in many biomedical fields as an interface layer between a metal surface and solution or vapor. Reference (12) provided a comprehensive review of the applications of self-assembled monolayers in biosensor fabrication. Browning-Kelley *et al.* (13) systematically studied the binding of antigen/antibody on thiol compound modified gold surfaces using atomic force microscopy. As they discussed, the formation of highly ordered thiolate self-assembled monolayer on the surface of the crystal will result in orientational immobilization of antibody/antigen without altering the biological activities. This feature offers numerous advantages to the sensor performance, e.g., improvement of detection limits, the ease of regeneration of the sensor, the reproducibility of the assay, as well as prevention of nonspecific adsorption.

This paper provides a method for total IgE quantification using a Pz crystal in which a novel SAM-based antibody immobilization technique has been developed. This new antibody immobilization method is able to alleviate problems encountered by other techniques, e.g., nonspecific adsorption and poor reproducibility. The fabricated IgE immunosensor has proved to be feasible for detecting human IgE in clinical diagnostics.

MATERIALS AND METHODS

Apparatus

The Pz crystals used were AT-cut 10-MHz quartz crystals with gold electrodes, purchased from International Crystal Manufacturing (Oklahoma City, OK). The quartz wafer was 14 mm in diameter and 0.2 mm in thickness, the gold electrodes were 5.1 mm in diameter and 100 nm in thickness. For those crystals, a frequency change of 1 Hz corresponds to a mass increase of $0.902 \pm 0.01 \text{ ng}$. The crystals were connected with a home-made TTL oscillating circuit based on IC 74LS04, similar to the one described in the literature (14). The output frequency was measured by using a TF830 universal frequency counter (Thurlby-Thandar, UK).

Chemicals and Biochemicals

Thioctic acid, cysteamine, glutaraldehyde (GA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), γ -amino propyltriethoxy silane (γ -APTES), and polyethyleneimine (PEI) were obtained from Sigma. Monoclonal anti-human IgE (I-6510) was also purchased from Sigma. Human IgE standards were obtained from the Total IgE II FAST Test Kit from BioWhittaker. Phosphate-buffered saline (PBS) was composed of 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4 (pH 7.2). Blocking reagent was bovine serum albumin (BSA) from Sigma. Washing solution was PBS buffer with low concentration of mild detergents. Other chemicals used were certified reagents from Fluka or Sigma.

Antibody Immobilization Procedures

Clean up of crystals. Crystals were cleaned with hot Piranha solution (30% H_2O_2 : H_2SO_4 , 1:3) by applying 10 μl each of Piranha solution on both sides of the gold surface for 5 min, followed by thorough rinse with distilled water and ethanol. This procedure was repeated twice. The surface was then blown dry with a stream of nitrogen gas and the resonance frequency (F_0) was taken.

Immobilization via thioctic acid method. The freshly cleaned crystals were dipped into 0.2% of thioc-

tic acid ethanol solution for 24 h at room temperature. The modified crystals were subsequently dipped into 2.1% EDC ethanol solution for 1 h. After washing and drying the crystals, 5 μ l each of 10 times diluted anti-human IgE was added on both sides of the crystal. After solvent evaporation, the antibody layers were immediately washed three times with PBS and distilled water to remove any residual salt deposits or loosely adsorbed protein.

Immobilization via cysteamine methods. The freshly cleaned crystals were modified using 0.1% cysteamine in water overnight. Following dipping of the treated crystals in 2.5% of GA in PBS, 5 μ l of diluted anti-human IgE was applied onto the gold surfaces. After incubation for a predetermined time the crystals were washed with PBS and distilled water and air-dried.

Blocking Procedures

A solution of 5% BSA in PBS was used to incubate with antibody-coated crystals, which was achieved by dropping a droplet of BSA solution onto each of the coated surfaces for 30 min. After washing and drying, the coated and blocked crystals were stored at 4°C.

Sample Detection Procedures

For detecting antigen, the coated and blocked crystals and all reagents were taken from the refrigerator. When coming to room temperature, the resonant frequency of the crystal was obtained as reference (I_R). Five microliters of each of the human IgE or real human serum samples at varying concentrations was applied on both sides of the crystal for 45 min. After washing and drying, the resonant frequencies were taken as F_s .

Regeneration of Used Crystal

An 8 M urea buffer was applied on the electrodes for 30 min to denature the anti-human IgE/human IgE immunocomplex. The obtained surfaces were rinsed with 0.2 M glycine-HCl buffer three times. After washing with distilled water, the regenerated surface can be reused to perform the assay.

Total IgE II FAST Test and Pharmacia-CAP

These procedures were performed using the standard protocols (4, 5).

RESULTS AND DISCUSSION

Antibody Immobilization

Gold surfaces can be easily modified by thioalkyl derivatives. The formation of SAM is due to the strong

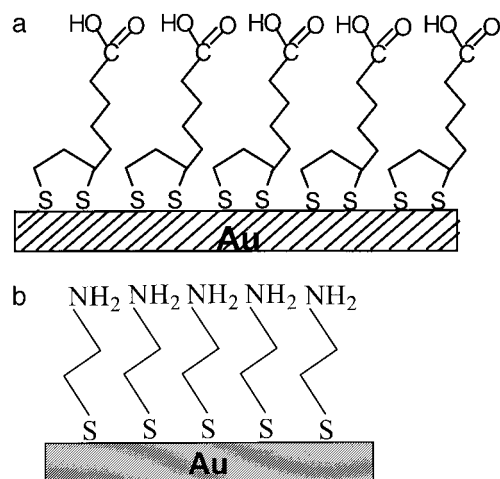


FIG. 1. Model of self-assembled monolayer: (a) thioctic acid on Au, (b) cysteamine on Au.

and spontaneous adsorption between sulfur atoms and a gold surface. Thioctic acid is a disulfide. The interaction of the disulfide group with an Au surface leads to the formation of two thiolate bonds after S-S bond cleavage. The hydrophobic alkyl chains interact with each other, and the hydrophilic -COOH head groups exposed on the surface of the monolayer are available for cross-linking as shown in Fig. 1a. Figure 1b shows the coupling of cysteamine to the gold electrode. Due to the terminal position of the thiol group on the aliphatic chain, the resultant structure is also an ordered array of a self-assembled monolayer, with the amino-reactive group toward the interface of the monolayer and available for cross-linking. To cross-link a desired protein to the SAM, a cross-linker, which can react with the appropriate functional groups, is employed. Figure 2a shows the procedure of antibody immobilization onto thioctic acid-coupled surfaces, in which the carboxyl group of immobilized thioctic acid is further activated by a carbodiimide (EDC in this case). The formation of an *O*-acylurea intermediate promotes the formation of amide bond between carboxylic acids and amines provided by the immobilized protein. Cysteamine immobilization procedures are shown in Fig. 2b. In this case GA is used as the cross-linking reagent, by which the reactive aldehyde group is obtained and subsequently used for coupling of amino groups available from proteins. To monitor the progress of the coating, the resonant frequency of each reaction step in dry state was taken. The changes in frequency caused by each step reflect the amount of material binding (Table 1). These data indicate that the two SAM methods ensure a similar amount of anti-IgE binding. One significant advantage offered by the highly ordered SAM coating method is the uniform amount of antibody binding for each individual crystal. The consistency of preparing

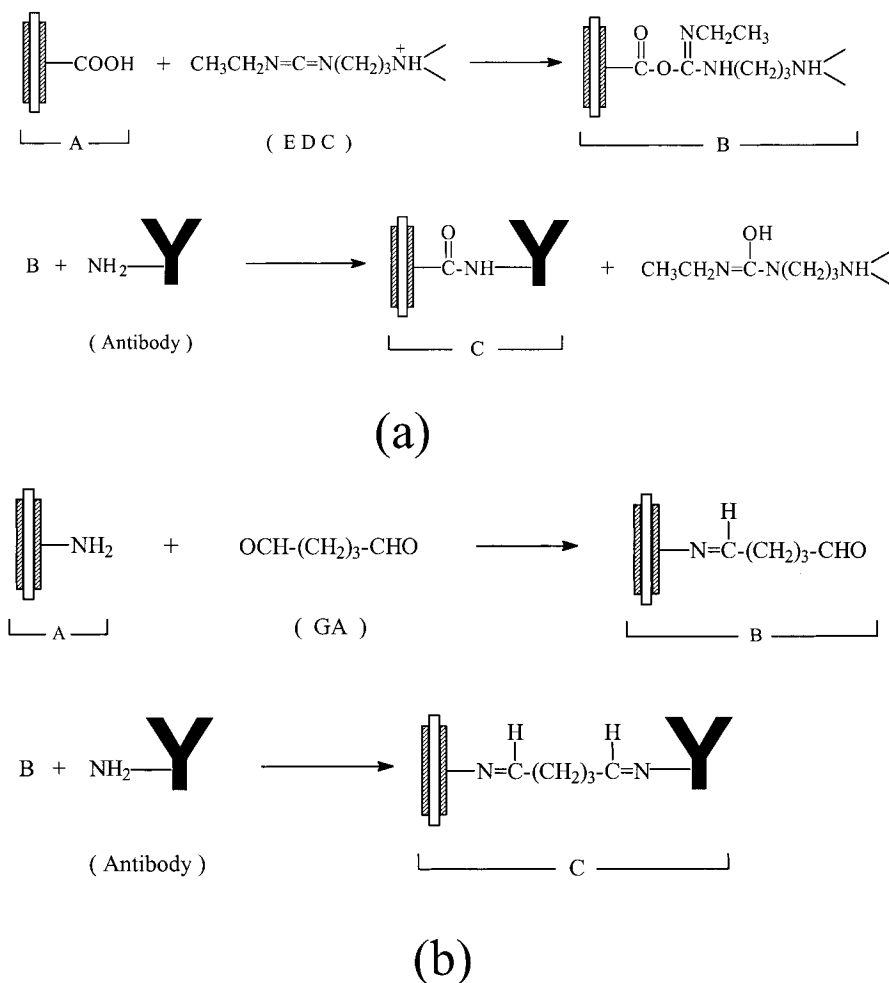


FIG. 2. Antibody immobilization procedures: (a) thioctic acid method, (b) cysteamine method.

individual Pz crystals is evaluated in Table 2. This table reports the reproducibility for nine Pz crystals prepared by either the thioctic acid method or the cysteamine method. The variability in anti-IgE binding between individual crystals is within 5%. The size of the error appears to be due to a few crystals with either

high or low coating levels. The uniform amount of antibody binding is the premise of the high reproducibility of the detection results.

Human IgE Detection

To avoid nonspecific adsorption, the antibody-coated crystals were incubated with 5% BSA in PBS before exposure to antigen. Nonactive BSA can occupy any free active residues so that adsorption of interfering proteins in the sample to be detected can be reduced. After coating and blocking, the fabricated Pz crystals can be exposed to IgE antigen. Frequency changes before and after incubation with IgE reflect the concentrations of IgE. First, 100 IU/ml of IgE antigen was selected to test the detection reproducibility of the Pz sensor. The results of five or six duplicate measurements as shown in Table 2 are in the range of 110–154 and 100–135 Hz for the thioctic acid or the cysteamine-coated method, respectively, and RSD is around 6.0%.

TABLE 1

Frequency Changes for Each Immobilization Procedure

Methods and steps	$\Delta F(\text{Hz})$ (average $\pm$ SD ^a)	RSD(%)	Binding capacity ^b (nmol/site)
Thioctic acid	276.1 $\pm$ 12.4	4.5	0.604
Anti-IgE ^c	331.5 $\pm$ 13.7	4.1	0.000997
Cysteamine	465.3 $\pm$ 24.5	5.3	2.143
GA	170.7 $\pm$ 14.5	8.5	0.888
Anti-IgE ^c	342.8 $\pm$ 15.2	4.4	0.00103

^a Standard deviations of 10 results for each method.

^b Calculated based on the sensitivity of 1 Hz $\approx$ 0.902 μg .

^c Ten times diluted antibody.

TABLE 2
Reproducibility for the SAM-Coated Pz Sensor (Frequency Change in Hz)

Crystal No.	Thioctic acid method		Cysteamine method	
	Anti-IgE binding	100 IU/ml IgE binding	Anti-IgE binding	100 IU/ml IgE binding
1	310	123	342	108
2	319	154	346	134
3	293	138	301	129
4	279	150	403	135
5	375	135	335	97
6	411	110	278	
7	341		397	
8	316		385	
9	340		302	
Average $\pm$ SD	331.5 $\pm$ 13.7	130.1 $\pm$ 7.5	342.8 $\pm$ 15.2	120.8 $\pm$ 7.7
RSD (%)	4.1	5.8	4.4	6.3

For quantification of total IgE, a standard curve was obtained by incubating various concentrations of human IgE with different antibody-coated crystals for 45 min. Human IgE standards were obtained from the Total IgE II FAST Test Kit. After dilution with PBS buffer the final IgE concentrations used were 0, 25, 50, 100, 200, and 300 IU/ml. The changes of frequency of the coated crystals before (F_R) and after (F_S) incubation with IgE correspond to the concentration of IgE. Figure 3 shows the calibration curve, in which the frequency changes versus IgE concentration plot is linear from 5 to 300 IU/ml with $r^2 = 0.998$ and a determination limit of 5 IU/ml. In this case, the detec-

tion limit is taken to be three times the standard deviation at low levels. Saturation is encountered with more than 300 IU/ml human IgE due to complete binding of all the coated antibodies. To investigate the reproducibility for the whole antigen concentration range, at least five duplicate experiments were performed on five separate coated Pz crystals for each IgE concentration. Results illustrated in Fig. 3 show that the standard deviation of frequency changes obtained for the thioctic acid-coated crystal method for the detection of human IgE is less than 8%. This results indicated that the SAM-based Pz immunosensor can provide reproducible determination in the whole con-

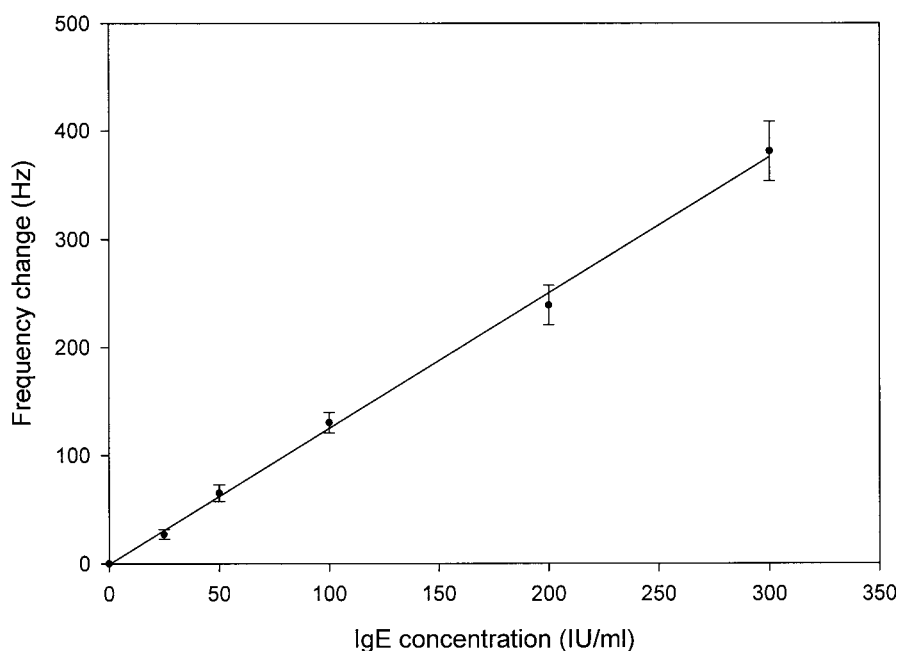


FIG. 3. Calibration curve obtained for thioctic acid-coated Pz sensor.

centration range. The cysteamine-coating method offered a similar linear range, sensitivity, and reproducibility for the detection of IgE (data not shown).

For the analysis of actual test samples, 5 μ l each of human serum was applied on both sides of the crystal for 45 min. After thorough rinsing with PBS and distilled water, the crystal was air-dried and the resonant frequency was obtained. Total IgE in the serum was calculated from $\Delta F(F_R - F_S)$ with reference to the standard curve. A total of 28 clinical samples were detected for total IgE using the SAM-based Pz immunosensor and commercially available testing methods. To investigate the correlation of Pz immunosensor with the currently used methods, cross-comparisons for the detection results using FAST, CAP, and Pz immunosensor were made. The regression lines obtained for FAST versus Pz sensor was $y = 9.6354 + 0.9725x$, and for CAP versus Pz sensor was $y = 14.1356 + 1.0298x$. The correlation coefficients correspondingly are 0.94 and 0.90, respectively. These results indicate that it is feasible to apply the proposed Pz sensor to detect human IgE in clinical diagnostics.

Estimation of Binding Ratio of Antibody and Antigen

The frequency changes caused by anti-IgE immobilization (ΔF_{Ab}) and those caused by saturated occupation of IgE antigen with antibody-coated surface (ΔF_{Ag}) can be used to estimate the interaction ratio of anti-IgE and IgE by using the Eq. [1]. Because the molecule weights of anti-IgE antibody and human IgE antigen are 150,000 and 190,000, respectively, the ratio of total amount of antibody immobilized onto the crystal (N_{Ab} , mol) to the saturated amount of antigen bound (N_{Ag} , mol) to the coated antibody can be obtained using Eq. [3]:

$$\frac{N_{Ab}}{N_{Ag}} = \frac{190,000}{150,000} \times \frac{\Delta F_{Ab}}{\Delta F_{Ag}} \quad [3]$$

The results indicated that when IgE antigen reached 300 IU/ml, the antigen binding caused by the Ab–Ag interaction reached a maximum, corresponding to a frequency change of about 395.3 Hz. Considering that the average anti-IgE binding amount is 331.5 Hz, the ratio of N_{Ab} to N_{Ag} is $(190,000/150,000) \times (331.5/395.3) \approx 1.0$, which indicates that one molecule of human IgE can bind with one molecule of coated anti-IgE antibody.

Comparison of Nonspecific Adsorption and Regeneration Ability

In addition to reproducibility, the use of SAM to immobilize antibody offers other advantages such as minimal nonspecific adsorption and ease of regenera-

TABLE 3
Comparison of Nonspecific Adsorption of BSA
on Anti-IgE-Coated Surface

Methods	Frequency change caused by BSA adsorption (Hz) ^a	BSA bound (pmol/side)
PEI	130	0.99
APTES	164	1.25
Protein A	208	1.60
Thioctic acid	48	0.37
Cysteamine	34	0.26

^a Average of at least five results.

tion of the antibody-coated crystal. To demonstrate the advantages of the SAM coating method with other methods, several anti-IgE-immobilized crystals were prepared using conventional method based on γ -APTES (15) or PEI (16), both of which form random polymer meshes adsorbed on the surface of the electrode rather than an orderly, covalently coupled SAM. In these methods, the cleaned crystals were first modified by dipping in 5% γ -APTES acetone solution or 2% PEI ethanol solution for 1 h. Anti-IgE was then cross-linked using 2.5% GA in PBS as described under Materials and Methods. This was followed by blocking of nonspecific sites with 5% BSA as described under Materials and Methods. The resonant frequencies of each crystal before and after the BSA blocking step were determined. In another experiment, 5 μ l of 1 mg/ml protein A, which has a well-known affinity for certain classes of immunoglobulins, was adsorbed onto the surface of the gold electrodes of the crystal. The protein A was dried at 37°C in an oven for 40 min before anti-IgE was added for binding with protein A. After antibody coating, 5% BSA was used to block nonspecific residues. The changes in frequency caused by BSA adsorption for different immobilization methods are listed in Table 3. These results show that conventional coating methods give approximately three to five times more nonspecific binding sites than the SAM methods. The data shown in Table 3 are mean values from at least five duplicated measurements.

After analysis had been performed, a wash with denaturing reagents to disrupt the anti-IgE/IgE immunocomplex was performed. After regeneration of the crystal, total IgE testing was then repeated. Results indicate that cross-linking via thioctic acid SAM provide superior activity after multiple regeneration cycles compared to IgE cross-linking via APTES and adsorbed protein A. Anti-IgE immobilized by the SAM method retains more than 90% of its activity after five regeneration cycles, and still retains 65% of its activity after six cycles. For the γ -APTES method and protein A method, activity of the modified surface reduce to ap-

proximately 60% and 40%, respectively, of the original after three regeneration cycles (data not shown).

CONCLUSION

The SAM-based Pz sensor coated with an anti-human IgE layer can be used to quantitatively detect total human IgE from serum samples. The well-controlled surface structure offered by SAM provides many advantages for the sensor performance, such as the reproducibility of the assay, the ease of regeneration of the sensor, as well as prevention of nonspecific adsorption. The fabricated sensor can detect total human IgE in the range of 5–300 IU/ml with CV less than 8%. The detection results for the real serum samples are in good agreement with currently used clinical methods.

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