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Detection of cancer biomarkers by piezoelectric biosensor using PZT ceramic resonator as the transducer

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ABSTRACT

A novel piezoelectric biosensor using lead titanate zirconate (PZT) ceramic resonator as transducer was developed for label-free, cost-effective, and direct detection of cancer biomarkers. We designed a dual sensing scheme where two ceramic resonators were connected in parallel, in which one resonator was used as the sensing unit and the other as the control unit, in order to minimize environment influences including temperature fluctuation and to achieve the required frequency stability for biosensing applications. Detection of selected cancer biomarkers, such as prostate specific antigen (PSA) and α-fetoprotein (AFP) was carried out to evaluate the performance of the biosensor. The device showed high sensitivity (0.25 ng/ml) and fast detection (within 30 min) with small amount of sample (1 μl), which is compatible to that required by clinical measurements. The results also showed that the ceramic resonator-based piezoelectric biosensor platform could be utilized with different chemical interfaces, and the miniaturized size of the ceramic resonators makes it suitable for fabricating sensor arrays for multiplex detection.

1. Introduction

Biosensing is achieved based on interfacial changes at transducer surface due to binding or reactions between target molecules and recognition elements on the transducer surface (Yang, Gong, 2010; Li, et al., 2012). Piezoelectric biosensors are mass sensitive detectors using an oscillating piezoelectric resonator as the transducer coated with biological materials such as antibodies or enzymes that have high selectivity and affinity for target molecules. The sensor signals are usually detected by measuring the fundamental frequency variations of the piezoelectric resonator (Babacan et al., 2000).

Quartz crystals are the most commonly used piezoelectric trasducers in biosensing applications such as clinical diagnosis and environmental monitoring (Marx, 2003; Percival et al., 2001; Su et al., 2000; Yao et al., 2009; Zhang et al., 2011). Specifically, numerous combinations of piezoelectric quartz crystal resonators with immunological elements have been reported (Deng et al., 2005; Soumetz et al., 2009; Su et al., 2000; Yuan et al., 2002). Quartz crystals are usually manufactured for frequencies from a few tens of kilohertz to tens of megahertz, which is related to the thickness of the resonator. It is difficult and costly to produce very thin and small quartz resonators with higher frequency. As the sensitivity of piezoelectric biosensors depends on the oscillating frequency as well as the area of the electrodes on the resonator, these physical barriers place a limit on the application of quartz crystal biosensors with regard to sensitivity and cost.

Ceramic resonators are another type of popular piezoelectric transducers which are made of high-stability piezoelectric ceramics, generally lead zirconium titanate (PZT) which is an inorganic compound with the chemical formula $Pb[Zr_xTi_{1-x}]O_3$ $0 \le x \le 1$). Similar to quartz crystal, the thickness of the ceramic substrate determines the resonance frequency of the resonator. Ceramic resonators are widely used for oscillation frequency stabilization in many electronic and ultrasonic applications (De Cicco and Morten, 2008; Lee and Kim, 2005; Li et al., 2007; Zhu et al., 2011). For example, ceramic material has been mostly used as a substrate to fix other sensing materials (Akdogan et al., 2005; Kielczynski et al., 1999; Setter, 2002). However, its piezoelectricity

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property was rarely explored as a sensing element. Limited attempts on the use of ceramic resonators in biosensor development were reported where the piezoelectric ceramic resonators employed had very low resonant frequency (less than 200 kHz) and narrow detection range (100–700 Hz) (Eklund et al., 2000; Lee et al., 2005; Verissimo et al., 2003). Although the precision of frequency control in ceramic resonators is reduced when compared to quartz crystal resonators, ceramic resonators are easily mass produced with low cost, mechanical ruggedness, and small size, which provide an attractive alternative to quartz crystals as transducers for developing biosensors, rere particularly miniaturized sensor arrays.

In the present study, we developed a piezoelectric biosensor using PZT ceramic resonator as the transducer for immobilization of recognition elements and detection of target analytes. A sensing scheme with two (dual) ceramic resonators connected in parallel was designed, in which one resonator was used as the sensing unit and the other as the control unit. This approach may greatly reduce environment influences including temperature fluctuation to achieve good frequency stability for the sensor system. Two cancer biomarkers, prostate specific antigen (PSA) and α-fetoprotein (AFP) were used as the target analytes to demonstrate the feasibility of using ceramic resonators as the core component of a piezoelectric biosensor. The biosensor measurement is based on the relative frequency change of the dual ceramic resonators due to specific antibody–antigen interaction. Our results showed that ceramic resonators could be an effective alternative to quartz crystal resonators as a transducer for piezoelectric biosensor with the potential to construct sensor arrays for simple, label-free, cost-effective, and multiplex detection of various analytes.

2. Material and methods

2.1. Apparatus and Reagents

PZT ceramic resonators with different frequencies (40 MHz, 50 MHz, and 70 MHz) were fabricated by Murata Manufacturing Co., Ltd. (Kyoto, Japan). The ceramic wafer had gold plated electrodes with a diameter of 0.71 mm on both sides and the dimension of the ceramic wafer is 2 mm × 1.6 mm × 0.55 mm. The frequency of the ceramic resonator was recorded by an impendence analyzer (HP4195A, Hewlett Packard) with a detection range of 10 Hz–500 MHz.

Protein A was obtained from Sigma Chemical Co. (St Louis, MO). Nafion (perfluorinated ion-exchange resin, 5%, w/v solution) was obtained from Aldrich. Bovine serum albumin (BSA) was purchased from Bio-Rad Laboratories (Rockville Center, NY). Prostate-specific antigen (PSA) and α-fetoprotein (AFP) were provided by Calbiochem (La Jolla, CA). PSA and AFP antibodies were from GeneTex, Inc. (San Antonio, Texas). Potassium phosphate/ sodium phosphate were from Sigma for preparation of phosphate buffered saline buffer (PBS, 50 mM, pH 7.4). All chemicals used were of analytical grade unless otherwise stated. Deionized water (resistivity $> 18\ M\Omega\ cm$) was used throughout the experiments.

2.2. Selection and measurement of PZT ceramic resonators

PZT ceramic resonators with different frequencies (40 MHz, 50 MHz, and 70 MHz) were connected to an impedance analyzer to record frequency variation for 30 min at room temperature. The integrity of the electrode surface was inspected under optical microscope and morphology analyzed by scanning electron microscope (XL30 ESEM FEG, Philips Electron Optics, the Netherlands). For the dual sensor scheme, two 40 MHz ceramic resonators (separated by at least 100 K Hz) were connected in parallel to an Impedance Analyzer and placed in a shielding metal box for frequency measurement at the same time. As two resonators were under the same environment, one of them was used for sample measurement and the other one as reference to eliminate environmental interferences.

2.3. Immobilization of antibody to ceramic resonator surface

The ceramic resonator surface was first cleaned by a plasma cleaner. Protein A and Nafion were used as the linker groups to immobilize antibodies on the resonator surface, respectively. The linker solution (1 μl of 2 mg/ml protein A or 0.5 ul of 1% Nafion solution, respectively) was applied onto the cleaned gold surface and incubated for 24 h. After incubation, the surface was washed with deionized water and dried under N_2. Then 1 μl of PSA antibody or AFP antibody solution (1 mg/ml) was added onto the modified ceramic resonator surface and incubated for 1 h at room temperature, followed by incubation with 3% of BSA for 2 h to block non-specific binding sites. After washing and drying, the biosensor was ready for antigen detection.

2.4. Immunoassay procedure

One microliter of PSA or AFP in PBS solution at different concentrations was applied onto the biosensor surface and incubated for 30–60 min to allow antibody–antigen interaction. After incubation, the surface was washed by deionized water and dried under N_2. Detection signal was recorded as relative frequency change before and after applying antigen.

To investigate the utility of this ceramic biosensor for clinical sample analysis, various concentrations of PSA were spiked into the normal human serum, e.g. 2.5, 10 and 50 ng/ml and the frequency change were analyzed upon incubation and detection.

3. Results and discussion

3.1. Sensitivity of piezoelectric ceramic resonators

The resonance frequency is related to thickness of the piezoelectric ceramic wafer:

$$f = \frac{N}{t} = \frac{\rho}{m}N \tag{1}$$

where f (Hz) is the fundamental resonance frequency, N (cm · Hz) is the frequency constant, t (cm) is the wafer thickness, ρ (gcm^{-3}) is the density, of the piezoelectric material and m ($g\ cm^{-2}$) is the area density of mass.

Assuming the deposited mass Δm (g) on the wafer surface is even and the frequency will change accordingly:

$$f + \Delta f = \frac{\rho N}{m + \Delta m/A} \tag{2}$$

by integrating Eqs. (1) and (2):

$$\Delta f = -\frac{f^2}{\rho N} \cdot \frac{\Delta m/A}{1 + \Delta m/A/m} \tag{3}$$

when $\Delta m \ll m$:

$$\Delta f = -\frac{f^2}{\rho N} \cdot \frac{\Delta m}{A} = -\frac{f^2}{AN\rho} \cdot \Delta m \tag{4}$$

For ceramic resonators, the density (ρ) and frequency constant (N) are $7.54\ g\ cm^{-3}$ and 7.85×10^5 cm Hz respectively. By substituting these values into Eq. (4), the calculation equation for

Table 1
Comparison of ceramic resonators with quartz crystals.

Name	Oscillation frequency	Vibration mode	Temperature coefficient	L1/ mH	C1/ pF	Co/ pF	R1/ Ω	Ω^α	Price/ $USD	Wafer area/ mm^2	Electrode area/mm^2
Ceramic resonator	40 MHz	Thickness extension (TE)	$\leq 10^{-5}$/°C	0.21	0.077	6.4	20	2600	~0.5	3.2	0.39
Quartz crystal	10 MHz	Thickness shear (TS)	$\leq 10^{-6}$/°C	7.5	0.34	10	10	47,000	~10	153.8	50.2

ceramics should be:

$$\Delta f = -1.69 \times 10^{-7} \cdot f^2 \cdot \frac{\Delta m}{A} \quad (5)$$

The sensing surface area (A) of 40 MHz ceramic resonator used in this study is 0.39 mm^2. Therefore, the sensitivity of this device is 69.3 Hz/ng. Based on theoretical calculation, the sensitivity of a 10 MHz quartz crystal microbalance with an electrode area of 50.2 mm^2 is 0.45 Hz/ng (Tang et al., 2002), which indicates that the ceramic resonator may theoretically be more sensitive than the quartz crystal resonator because of its higher resonant frequency and smaller size.

3.2. Optimization of piezoelectric ceramic resonators

A comparison between the most commonly used piezoelectric quartz crystal resonators (10 MHz) and piezoelectric ceramic resonators (40 MHz) used in this study was carried out. We compared the size of the resonator and its electrodes, temperature coefficient, equivalent circuit parameters and cost of these two different resonators (Table 1). Firstly, ceramic resonators, which are usually operated under thickness extension mode, exhibit much higher oscillation frequency (1–100 MHz) than quartz crystal resonators which are often operated under thickness shear mode (1–10 MHz). Secondly, the temperature coefficient of ceramic resonators is much greater than quartz crystal resonators, as PZT is a polycrystalline material while quartz is a crystalline material. This indicates that ceramic resonators are more sensitive to temperature fluctuations in the surrounding environment and thus have less stable resonant frequency than quartz crystal resonators. Thermal stability is a key parameter required for practical purposes. The temperature coefficient of piezoelectric resonator is dependent on material properties as well as the dimension and vibration mode of the resonator (Erhart et al., 2007). Changing the composition of the piezoelectric ceramics may also improve the thermal stability (Lee et al., 2004; Lente et al., 2004). Thirdly, ceramic resonators are much lower-cost than quartz crystal resonators. Furthermore, it is much easier to produce thinner and smaller ceramic resonators with smaller size and electrode area than quartz resonators.

The biosensor development involved the utilization of ceramic resonator as the core component for the detection of biomarkers in clinical samples. The piezoelectric ceramic resonator chip is similar to a 'sandwich' structure, which is composed of a layer of ceramic wafer in the midst and a layer of gold electrode on both sides of the ceramic layer, respectively (Fig. 1a). These gold electrodes are used to connect the ceramic wafer with an external oscillation circuit or an impendence analyzer, which makes the ceramic resonator generate vibration under resonance frequency. The gold surface serve as support for antibody immobilization for biosensor development.

In order to compensate the large frequency shifts of the ceramic resonators induced by environmental influence, a parallelly connected (dual) ceramic resonator scheme was developed in our study (Fig. 1b and c). In the dual design scheme, two 40 MHz ceramic resonators (so-called two channels) were

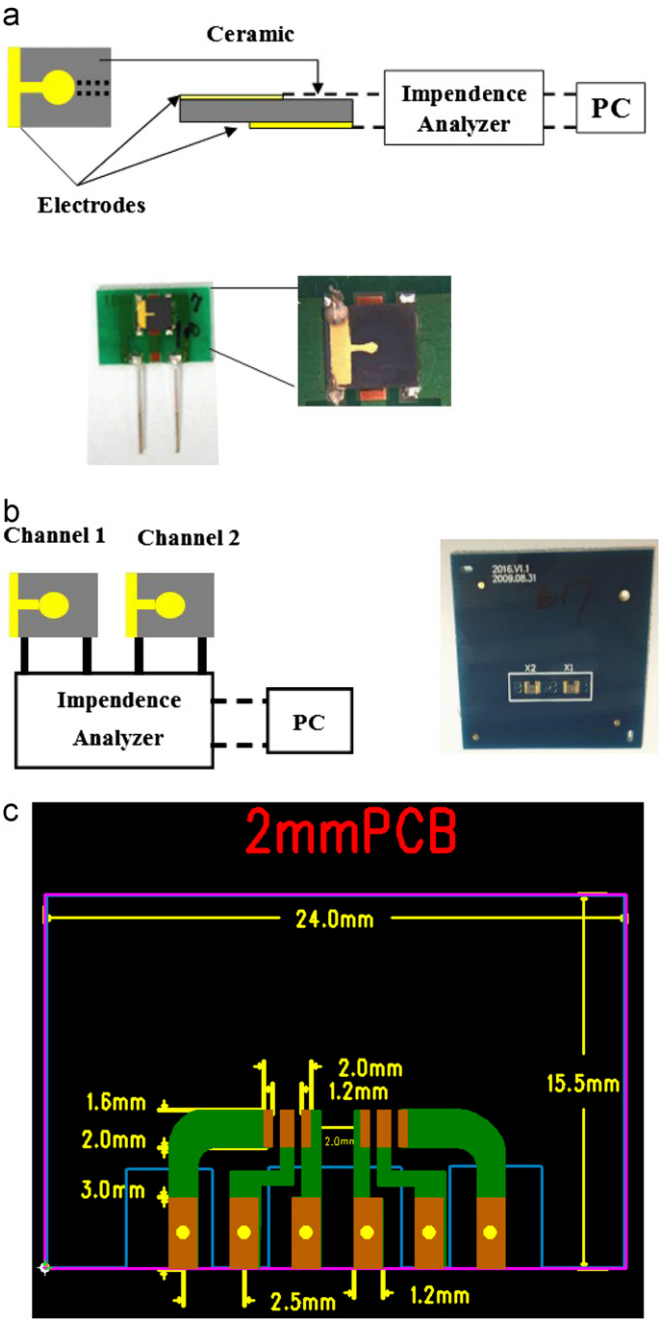

Fig. 1. (a) Schematic layout of ceramic resonator and the measurement system (up) and photo of a ceramic resonator (below); (b) Schematic layout of dual ceramic resonators and the measurement system (left) and photo of a ceramic resonator (right); (c) Dimensions of the electrode leads and connections on the printed circuit board for dual ceramic resonators.

connected in parallel to an Impedance Analyzer and placed in a shielding metal box for frequency measurement at the same time. The fundamental frequencies of the two resonators were selected

such that they differ by at least 100 KHz in order to minimize cross-talk when connected on the same printed circuit board (discussed below). As the two resonators were placed under the same environment, one of them was used for sample measurement and the other as reference to reduce environmental interferences.

Ceramic resonators with different frequencies and electrodes were evaluated with regard to the frequency stability, temperature effect and electrode surface in order to find the suitable resonator for biosensor development. The stability of PZT ceramic resonators with different frequencies (40 MHz, 50 MHz, and 70 MHz) was evaluated under fixed environmental temperature within a fixed period of time. As showed in Fig. 2a, the 40 MHz ceramic resonator showed about 3.75 ppm frequency shift within 30 min at 25 °C, which is smaller than those of 50 MHz (5 ppm) and 70 MHz (5.7 ppm) ceramic resonators, respectively. This result indicated that the 40 MHz ceramic resonator is more stable than 50 MHz and 70 MHz resonators from the same manufacturer. In addition, the 40 MHz ceramic resonator showed smooth gold electrode surface with regular round shape compared to those of 50 MHz and 70 MHz resonators (Fig. s1). Therefore, the 40 MHz ceramic resonators were selected for biosensor development in this study.

3.3. Dual ceramic resonators design

As mentioned above, the resonance frequency of the ceramic resonator is more sensitive to environment influences such as radio waves and temperature than the quartz crystal resonator. Fig. 2b showed the frequency change of a 40 MHz ceramic resonator as a function of the surrounding temperature. The resonator frequency was recorded for 60 min and the room temperature was made to shift between 20 °C and 22 °C. A shielding metal box was placed outside the resonator to eliminate other possible environment influences. As shown in Fig. 2b, the frequency change was in parallel with the room temperature change with a delay time (~2 mins), which was due to the delayed heat transfer from outside to the resonator by the metal box. A large frequency shift (~450 Hz/°C) was detected which corresponds to a temperature coefficient of 10^{-5} Hz/°C. Therefore, a parallelly connected (dual) ceramic resonator scheme was developed.

One of the issues encountered by the dual resonator design was how to avoid the interference between the parallelly connected resonators due to their close proximity. Although the coupling factor is quite weak due to the energy trapping effect for a well-detuned resonators pair on one plate even though the interval distance is small (Lu et al., 2005), high frequency

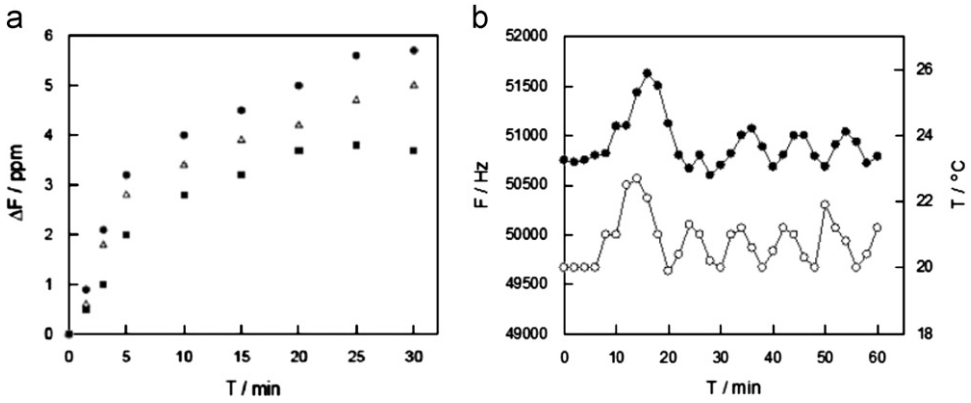

Fig. 2. (a) Stability evaluation of ceramic resonators at different resonance frequencies within 30 min at 25 °C (● for 70 MHz resonator; ▲ for 50 MHz resonator and ■ for 40 MHz resonator): percentage of frequency shift; (b) Frequency shift (-●-) of a 40 MHz ceramic resonator with environmental temperature fluctuation between 20 °C and 22 °C (-○-).

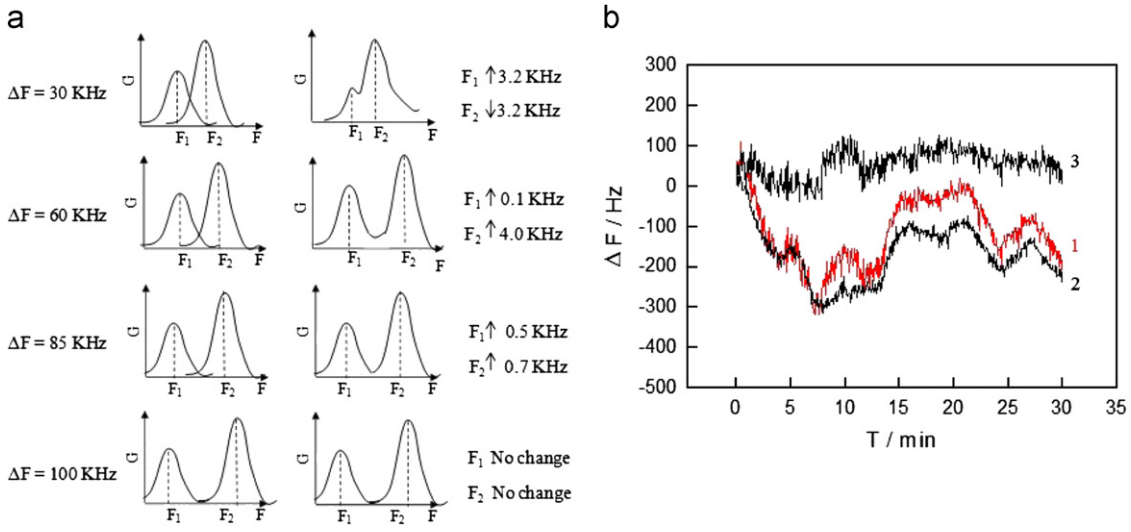

Fig. 3. (a) Frequency shift before and after parallel connection of two ceramic resonators with different resonance frequency differences; (b) Frequency shift of two parallel connected 40 MHz resonators (line 1 and line 2) and frequency shift difference between these two resonators (line 3) within 30 min when the environmental temperature shifts about 1°. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

resonators at the same frequency level (e.g. 40 MHz piezoelectric ceramic resonators used in this study) may have cross-talks between each other when they are oscillating at the same time. As shown in Fig. 3a, when the resonance frequencies of the two ceramic resonators were approximately the same, large interference was found that would affect the frequency readout. By increasing the difference between the two resonance frequencies, the interference was reduced or even eliminated. Therefore, different resonance frequency between the ceramic resonator pairs was tested to find the threshold. As in Fig. 3a, when the resonance frequency difference increased to 100 KHz, the interference could be eliminated. Thus this parameter was used in the design and development of the ceramic biosensor.

The feasibility of using the parallelly connected resonators to compensate the large frequency change induced by environmental influences was investigated. Two resonators were connected in parallel and their frequency readings were recorded for 30 min. The environmental temperature shifted about 1° during this period. As shown in Fig. 3b, the frequencies of both individual resonators were changed and the shifts were similar, i.e. about 450 Hz and 400 Hz for line 1 and line 2, respectively. However, by subtracting one frequency shift from another, their frequency shift difference was only about 130 Hz (Fig. 3b, line 3). These results indicated that parallel connection of the resonators could reduce the frequency shift at ambient conditions. The same experiment was repeated several times on different pairs of parallelly connected resonators and the difference of the frequency shifts were all below 150 Hz. Therefore, this parallel connection design of ceramic resonators was suitable for use in biosensor development and the baseline noise level was about 150 Hz for sensing application.

3.4. Biosensor development based on dual resonator chip

After fabrication of the dual piezoelectric ceramic resonator chip, coating of a linker group (chemical or protein) on the gold surface was carried out to immobilize antibodies for antigen capturing (e.g. cancer marker). The gold plated ceramic resonator was tested further for its ability to resonate after attaching linker groups such as protein A and nafion for antibody immobilization and antigen capturing.

3.4.1. Immobilization of antibody through protein A

Protein A was chosen as the bridging linker because it can form a strong complex (k_a=108 L/mol) with electrode gold surface by physical adsorption and has high affinity towards Fc region of antibodies. Using Protein A to immobilize antibody will orient the functional Fab of the antibody to capture its antigens in the sample solution (Deshpande, 1996).

In this study, the development of the ceramic resonator-based biosensor was demonstrated by using prostate cancer marker, prostate specific antigen (PSA) (Brawer, 2000a, 2000b). Existing methods for PSA detection often require a radioisotope, enzyme, fluorescence or colloidal gold-labeled antibody or antigen, which may suffer from drawbacks of requiring skilled personnel, time-consuming procedures and expensive chemicals (Malan, 2001). For the dual resonators, after Protein A incubation, one resonator was immobilized with PSA antibody as described in the Method part, while the other resonator as control. PSA antigen at different concentrations (0.025, 0.25, 2.5, 25, 2.5×10^2, 2.5×10^3 ng/ml) was added onto both resonators in serial and incubated for 1 h. The frequency change was measured by the impedance analyzer and plotted against the log concentration of the antigen (Fig. 4). The result showed that PBS did not lead to obvious frequency shift, while specific antibody–antigen interaction resulted in

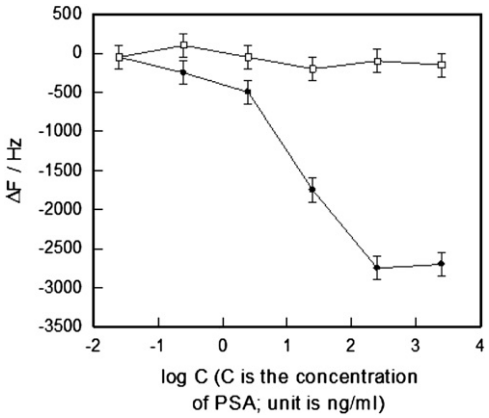

Fig. 4. Frequency changes against the log concentration of the PSA. PSA detection plot (-●-) vs. PBS control plot (-○-). The concentrations of PSA solutions are 0.025, 0.25, 2.5, 25, 2.5×10^2, 2.5×10^3 ng/ml.

Table 2
Evaluation of the recovery of prostate specific antigen (PSA) from normal human serum. Data were presented as mean ± S.D. from three independent tests.

Amount of PSA added to the serum (ng/ml)	2.5	10	50
Amount of PSA detected by ceramic sensor (ng/ml)	2.38 ± 0.28	9.75 ± 0.78	54.36 ± 0.35
Recovery rate (%)	95.2%	97.5%	108.7%

significant frequency changes, with only small amount of samples required (1 μl). The biosensor has a detection range from 0.025 to 2.5×10^2 ng/ml for PSA. The sensitivity is compatible to that required by clinical measurement as PSA has a normal level below 4 ng/ml and the threshold level is 10 ng/ml (Ilic et al., 2011). The calculated detection limit is 295 pg/ml, which is in the same order as that of reported ELISA method (193.5 pg/ml) (Cheow et al., 2010).

Biosensors have several potential advantages over other methods of cancer analysis, especially increased assay speed and flexibility (Rasooly and Jacobson, 2006). In this study, in order to investigate the utility for real sample analysis, the frequency change of normal serum spiked with additional PSA, (2.5, 10 and 50 ng/ml) were evaluated and the recovery rate was calculated. As shown in Table 2, the recovery rate of PSA from human serum samples ranged from 95.2 to 108.7%, which demonstrated the flesibility of this ceramic biosensor for clinical measurement.

3.4.2. Immobilization of antibody through nafion

Another linker group for antibody immobilization, e.g. nafion membrane, was utilized in our study. Nafion is a polyanionic perfluorosulfonated ionomer that is chemically inert, nonelectroactive and hydrophilic, with suitable properties for preparation of modified electrodes (Deng et al., 2005). The thickness of nafion film is an important element that influences the performance of the immunoassay. We noted that 1% nafion coating solution was optimum for preparation of nafion-modified gold electrode surface on ceramic resonators based on reported protocols (Chetcuti et al., 1999; Deng et al., 2005; Zhou et al., 2003).

After the formation of nafion membrane on the ceramic resonators, one resonator was immobilized with PSA antibody, while the other as control to evaluate non-specific binding. As shown in Fig. 5a, after 1 h incubation with 1 μl of 2 ng/ml PSA antigen, the antigen binding to the antibody-immobilized

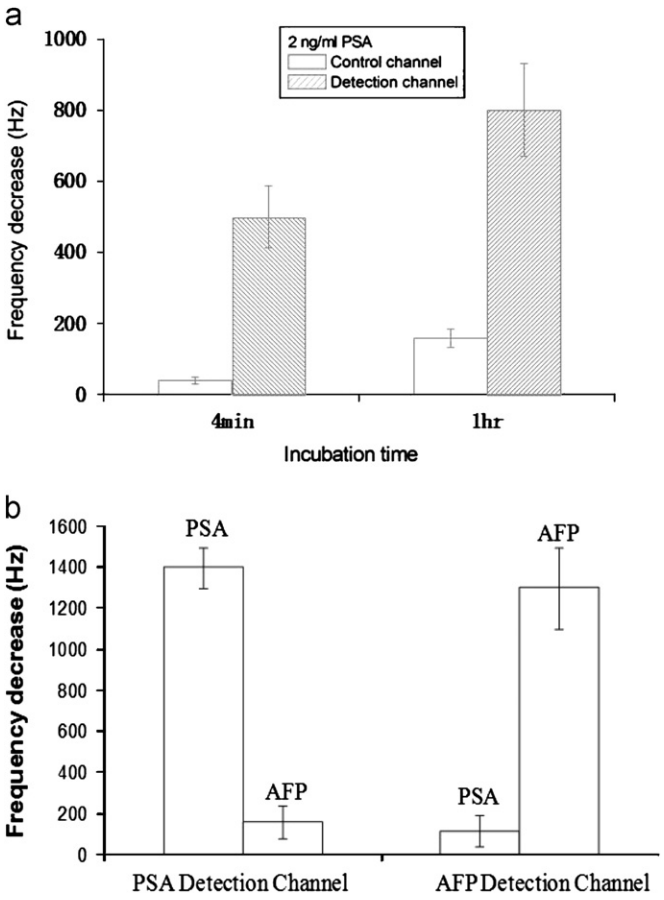

Fig. 5. (a) The frequency changes of the binding of PSA (2 ng/ml) to PSA antibody-immobilized resonator (detection channel) or control channel (without PSA antibody) at 4 min and 1 hr were compared. (b) Specific antigen binding evaluation on PSA and AFP detection channels, where the PSA and AFP solutions are 10 ng/ml and 1 μg/ml respectively.

resonator (the detection channel) caused a frequency drop of −800 Hz, which was greater than that of the control channel with a frequency drop of −160 Hz. It should be noted that at the first 4 min of incubation, there was already binding signal on the detection channel ($\Delta F = -500$ Hz), while the control channel only gave 40 Hz frequency change which was within the noise level (Fig. 5a). Therefore, the detection time could be shortened based on the kinetics binding.

The specificity of the dual resonators was tested by immobilizing two different antibodies on the two channels respectively. For this purpose, one resonator was immobilized with PSA antibody and the other with AFP antibody. As shown in Fig. 5b, specific binding signal was observed with a frequency drop of −1400 Hz on the PSA antibody immobilized channel when applying 1 μl of 10 ng/ml PSA solution to both channels, while the AFP immobilized channel only decreased by around 120 Hz. Similarly, when 1 μl of 1 μg/ml AFP solution was added to both channels, the AFP channel recorded a −1270 Hz frequency shift while the PSA channel recorded a −94 Hz shift, indicating the specificity of the biosensor.

4. Conclusion

A novel biosensor using PZT ceramic resonators as transducer for label-free, low cost, and direct detection of cancer biomarkers was developed. We have selected suitable ceramic resonators with high resonance frequency (40 MHz) and designed a parallelly connected dual ceramic resonators scheme, in which one resonator was used as the sensing unit and the other as the control unit, in order to eliminate environment influences including temperature fluctuation and to achieve the required frequency stability for biosensing applications. The detection of selected cancer biomarkers, such as prostate specific antigen (PSA) and α-fetoprotein (AFP) was carried out to evaluate the performance of the developed biosensor. The dual biosensor device showed high sensitivity (LOD 0.25 ng/ml) and fast detection time (within 30 min) for PSA with small amount of sample (1 μl), which is compatible to that required by clinical measurements. The results also showed that the ceramic resonator-based piezoelectric biosensor could be combined with different chemical interfaces, and the miniaturized size of the ceramic resonators makes it suitable for fabricating sensor arrays for multiplex detection.

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