RESEARCH COMMUNICATION

A Novel and Cost-Effective Method for Early Lung Cancer Detection in Immunized Serum

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Abstract

Background: The aim of this study was to detect early lung cancer rapidly with a novel and cost-effective quartz crystal microbalance (QCM) immunosensor. Materials and Methods: Murine Lewis lung carcinoma LL/ 2 cells were first cultured onto the surface of 10MHz 3rd overtone AT-cut quartz crystals in Dulbecco's modified Eagle medium, and then the serum sample of LL/2 cell immunized rabbits was also dripped onto the quartz crystal surface center by micro-injector. In addition, non-immune rabbit serum was used as a negative control. The additional mass of the crystal which caused by specifically adsorbing antibody results in a change in resonant frequency. A frequency counter was employed to monitor the frequency variation. Then the antibody content of the LL/2 cell can be detected rapidly through changed frequency. Results: The antibody contents of the LL/2 adsorbed on the surface of six quartz crystals were 155ng, 55ng, 55ng, 32ng, 32ng, 0ng, respectively. The results showed that the LL/2 antibodies could be detected if they exist in serum at nanogram level with a high detection precision and a positive detection rate of above 80%. Conclusions: Our test results reveal that the proposed method has potential application in detection of early lung cancer.This novel detection method might be particularly suited for health screening of the general population.

Keywords: Quartz crystal microbalance - immunosensor - early lung cancer - detection

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Introduction

Lung cancer is one of the most common cancers in the world. The incidence rate is the highest among the various malignant tumors and it is the leading cause of cancer death, meanwhile, the mortality from lung cancer is increasing substantially in recent years. The majority of patients have already belonged to a later period while diagnosing because of insidious onset, low content and low concentration of cells in early lung cancer, and making prognosis poor. Five-year relative survival rate is less than 15%(Jemal et al., 2004) and patients with symptoms are even lower. However, the prognosis of surgical treatment among the lung cancer found on patient in early period is obviously improved compared with middle or late period, and the survival rate can approach 80% (Klose et al., 1995). Therefore, the detection of early lung cancer plays a vital role in prognosis.

Immune assay is a common method for early lung cancer detection in clinical laboratory. Now there exist several immunoassay methods, such as radioimmunoassay (RIA), chemiluminescence immunoassay (CLIA), and enzyme-linked immunosorbent assay (ELIA). Although they are comparatively sensitive and precise, the instruments and materials involved are expensive. Furthermore, the detection procedure requires more time and complex operations with labeling steps, especially can not determine diverse lung cancer-associated antibodies or antigens in serum at the same time (Zhang et al., 2007). Although a number of other ways for early lung cancer detection in clinical diagnosis, there is still no satisfactory method in terms of precision, safety, specificity, detection cost, detection speed and stability. These disadvantages can be addressed by QCM immunosensor.

QCM immunosensor is an ultra-sensitive mass biosensor that combines the high sensitive piezoelectric effect of AT-cut quartz crystal and high specific recognition of immune reaction. The adsorption of materials, such as mass, on the surface of the crystal changes its frequency. The relationship between the change in mass and the corresponding change in the oscillation frequency has been shown by the well-known Sauerbrey equation as follows (Sauerbrey, 1959; Hlavay et al., 1977):

$$\triangle F = -2.3 \times 10^{-6}\, n\, F_0^2\, \triangle m/A \quad (1)$$

where $\triangle m$ is the changed mass on the crystal surface, in g, $\triangle F$ is the change in resonance frequency of the coated crystal, in Hz, F_0 is the fundamental resonance frequency of the crystal, in Hz, n is the overtone number; A is the area of electrode surface, in cm^2.

It is the most biosensor type used in biomedical

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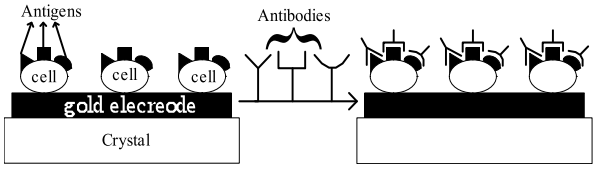

Figure 1. Schematic of Illustration of Adsorption

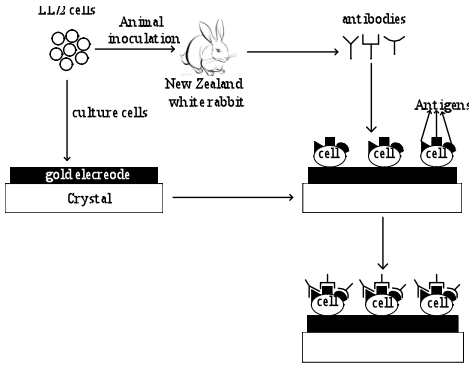

Figure 2. Schematic Protocol of the Experiment

application due to its advantages in specificity, swiftness, low cost, portability, and highly mass sensitivity at the picogram level. Shons et al. was the first to construct a QCM biosensor in 1972 to determine bovine serum albumin antibody activity (Shones et al., 1972). Since then, hepatitis viruses (Konig et al., 1995), atrazine (Steegborn et al., 1997), SARS (Zuo et al., 2004) and carcinoembryonic antigen (Shen et al., 2005) etc. were successfully detected by the QCM immunosensor. However, the QCM immunosensor has not, to the best of our knowledge, been applied to detect lung cancer.

In this paper, murine Lewis lung carcinoma LL/2 cells were first cultured onto the surface of the QCM, then we employed polyclonal antibodies recognize antigens (Mu et al., 2011)in order to increase the sensitivity and specificity of lung cancer to detect serum samples of immunized rabbit. When antigens on the crystal surface specifically adsorbed antibodies in the serum, the additional mass on crystal surface is linearly related to the resulting frequency shift. The adsorption schematic is shown in Figure 1.The experiment schematic is shown in Figure 2. Using this method, we succeeded in measuring the antibody content of the LL/2 cell in rabbit serum, and thus achieve the early detection of lung cancer. In addition, we employed the negative control to verify that the research has significance for early lung cancer detection.

Materials and Methods

Animals and Cell line

New Zealand white rabbit were purchased from the West China Experimental Animal Center (Chengdu, China). Animal protocols for these experiments were approved by the West China Hospital Cancer Center's Animal Care Committee. Murine Lewis lung carcinoma LL/2 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and was cultured onto the surface of crystal using Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, N.Y.) containing 10% fetal calf serum (Gibco BRL, Grand

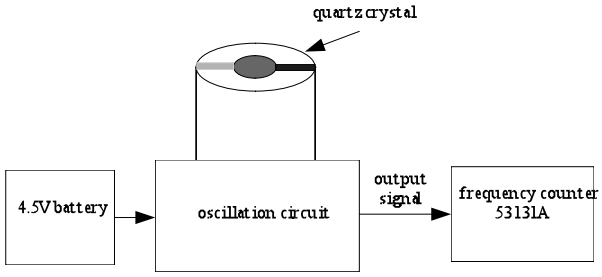

Figure 3. Test Diagram

Island, N.Y.) with 100 units/ml Amikacin, in a humidified 5% CO_2 incubator at 37°C.

Prepare of antibody of LL/2 cells

The antibody was generated by immunizing New Zealand white rabbits with LL/2 cell using 107 per injection. The primary inoculation was with Freund's complete adjuvant (Sigma) at day 0, followed by two boosts with Freund's incomplete adjuvant (Sigma) at day 14 and 28. The serum was pooled a week after the last injection. Blood was allowed to clot overnight at 4°C and serum was removed from the top of the mixture after centrifugation at 12000g. Immunoglobulin (IgG) was isolated using an affinity chromatography system (AKTA explore, GE, USA) and freeze dried (Rlphr 1–4 LSC freeze drier, Christ, German). Antibodies were kept frozen at -80°C until used.

Equipment and apparatus

All QCM crystals used were 3rd overtone AT-cut, 5mm gold electrode with a resonance frequency of 10 MHz from Tangshan JingYuan YuFeng Electronics co., Ltd (Hebei, China). Subtle distinction exists in resonance frequency among the crystals since the crystals are not calibrated. A high resolution frequency counter (Agilent 53131A) from Agilent Technologies (US) was employed to display frequency shift. A self-made gate crystal oscillator with 74HC04 chip and 4.5v dc power supply was used. The test diagram is shown in Figure 3. Micro-injector (range from 0.2ul to 10ul) was obtained from KeXiao co., Ltd (Hang Zhou, China).

Measurements

The resonance frequency F1 of all crystals were measured by frequency counter at 25°C.After culturing LL/2 cells on the crystal surface, the crystal was dipped in 2% paraformaldehydy-phosphate buffered solution for 10min, then washed with phosphate buffer solution (PBS, PH 7.4) and deionized water (DW) to wash away culture medium and other interfering substances and dried in air at 25°C. These steps were repeated twice, and then the resonance frequency F2 of crystals with coated cells was subsequently counted. The gold electrode surfaces of crystals were covered with 0.6ul LL/2–associated serum by micro-injector and kept at 25°C for 2 min,the washed with PBS and DW and air-dried. Subsequently, the resonance frequency F3 of crystals at the moment was counted. The test methods and test conditions of negative control were exactly the same as mentioned above.

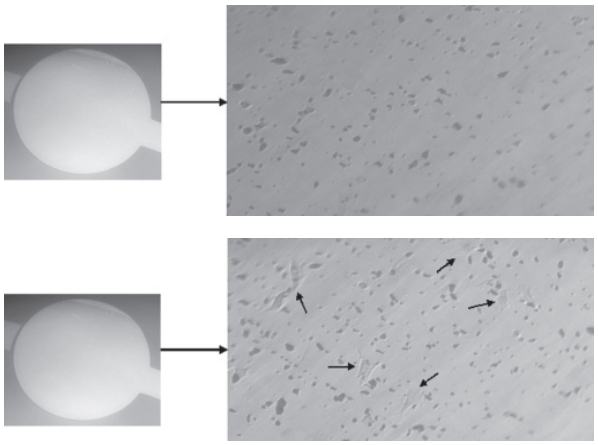

Figure 4. Comparison of Pictures of Crystals Before and After Culturing with LL/2 Cells

Table 1. Data for $\triangle F$ Values

Crystal number	F0 (Hz)	$\Delta F1$ (Hz)	$\Delta F2$ (Hz)
Positive			
1	10006805	251	154
2	10000885	446	63
3	10009180	70	98
4	10007912	50	36
5	10009640	54	37
6	10000885	121	0
Negative			
7	10007463	832	0
8	10009719	219	358
9	10000767	336	139
10	10000898	50	1
11	10008054	164	22
12	10008047	73	0

Results

Stability of test system

The stability of quartz crystal resonator is the major factor to influence the stability of test system and is the key factor to determine whether the QCM can be applied in clinical examination. 10MHz 3rd overtone AT-cut crystal employed in this study is of high quality factor and small temperature coefficient of resonant frequency at room temperature, ensuring good stability of whole test system. In addition, in order to achieve optimum frequency-temperature stability of the QCM, the measurement process is carrier out at constant temperature (25°C). As a result, the 3rd overtone AT-cut model of QCM immunosensor used in this study is of excellent accuracy with ±1Hz at 25°C in the process of measurement.

The form of antigen

The immobilization layers of crystal surface is particularly important in QCM immunosensor, since a high sensitivity and a short reaction time can only be achieved by thin hydrophobic substances carrier membrane In this study, the cells were cultured on the crystal so as to make LL/2 antigens firmly immobilized on the crystal surface. The comparison of pictures of crystal before and after culturing with LL/2 cells are shown in Figure 4. It is clear that the LL/2 cells were grown tightly on the crystal surface 24 hours later. This novel antigen immobilization method avoids the phenomenon of the waveguide, thus sharply increasing the sensitivity and shortening the whole detection time.

Table 3. Data for $\triangle m$ Values

Crystal number	Δm (ng)
1	133
2	55
3	55
4	32
5	32
6	0

Detection of antibody of the LL/2 in serum

The crude serum collected from the immunized rabbits was tested by the QCM immunosensor to detect the antibody content of the LL/2 in serum and the non-immune rabbit serum was used as a negative control. The crystal surface was covered with only 0.6ul serum at a time so as to avoid exceeding the load limit of the crystal. The detection results and negative control results were shown in Table 1 and Table 2, respectively. The frequency shift of crystal resonator caused by adsorption of cells was calculated as follows:

$$\Delta F2 = F1 - F2 \qquad (2)$$

The frequency shift of crystal resonator due to the binding of antibody of the LL/2 in serum to its homologous antigen on cells was calculated as follows:

$$\Delta F3 = F2 - F3 \qquad (3)$$

It can be easily seen from Table1 that the resonance frequency of five crystals changed significantly among six crystals after covered with 0.6ul LL/2-associated antibody serum. The high true positive rate mainly attributes to novel antigen immobilization that LL/2 cells were cultured on crystal surface. Since a variety of lung cancer-associated antigens have been identified in LL/2 cells, the applied method that polyclonal antibodies recognize antigens increased the sensitivity for tumor, and avoided the shortcoming of monoclonal antibody to identify its homologous antigen with low positive rate. In addition, the selectivity and accuracy were sharply improved.

Using equation (1) and (3), the content of antibody of the LL/2 in serum of immunized rabbit can be calculated and the results were shown in Table 2. The table clearly shows that this detection system is able to detect antibodies of the LL/2 cell at the level of nanogram. The above results show that we could make judgment of lung cancer from frequency shift if lung cancer-associated antibodies were contained in serum. Furthermore, this test method provides high accuracy with more than 80%.

Discussion

We employed a cost-effective quartz crystal microbalance immunosensor to detect early lung cancer in immunized serum. In the process of experiment, there was no change in resonant frequency of the sixth crystal which was used to test immunized serum. This can be clearly seen from Table 3. The reason for this phenomenon was the drip operation. As we all know that the sensitivity curve of crystal is Gaussian curve, the highest sensitivity is

at the centre of electrode, thus the sensitivity will sharply decreased when dripping mass loading off centre. As a result, the variation of frequency is not obvious. So when immunized serum was dripped onto the quartz crystal surface, the immunized serum was not dripped in the centre of electrode, making the variation of frequency is nearly zero. The 3rd overtone AT-cut model of QCM immunosensor increased oscillation stability, ensuring the satiability of whole test system. The use of novel antigen immobilization method not only eliminated non-specific interference but also avoided the phenomenon of waveguide. The result of the experiment shows the accuracy and possibility of the QCM immunosensor in detecting lung cancer in immunized serum in the gas phase. The measurement process proves the swiftness and simplicity in detection. In addition, the proposed method provides potential advantages, including low cost (the whole detection system costs only 1 dollar) and security (will not cause radiation damage for patients). This novel detection method is particularly suited for health screening for the general population.

Our preliminary replication experiment provides further evidence for its potential applicability in clinical practice; however, this method has the certain problem and the flaws including false-positive diagnosis and can not identify the specific lung cancer-associated antibodies in serum samples. Therefore, the further investigation will focus on increasing the accuracy, identifying the specific antibodies, and providing some experimental basis and theoretical basis for lung cancer diagnosis, treatment and prognosis.

Acknowledgements

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