# Detection of Tumor Necrosis Factor Gene by Piezoelectric Nucleic Acid Sensor

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**A piezoelectric nucleic acid sensor was constructed for detection of tumor necrosis factor gene. Two methods were employed for immobilization of nucleic acid probe on gold electrode of piezoelectric crystal. The results show that polyethyleneimine adhesion and glutaraldehyde cross-linking method has higher sensitivity, stability and selectivity than protein A method. The solid-phase nucleic acid hybridization of oligo nucleotides and tumor necrosis factor target gene sequence were monitored using this sensor. Tumor necrosis factor gene sequence (580 bp) was detected by this nucleic acid sensor for the first time.**

**Keywords** nucleic acid sensor, quartz crystal microbalance, tumor necrosis factor

## Introduction

The gene diagnosis technology, also called nucleic acid probe technology, has become an attractive method in the fields of biochemistry and clinical medicine. It is proved to be convenient and safe using non-radioactive reagents with electrochemical and optical detector. Especially, the polymerase chain reaction (PCR) technique has made gene diagnosis convenient and highly sensitive.[1,2] But some serious demerits still have existed in the gene detection technique including PCR. It is difficult to develop an automation system and the techniques are not readily available. Some information has been reported on developing of the convenient sensor type gene detection techniques based on the solid-phase hybridization with immobilized DNA probes.[3-5] These sensor-type DNA detection methods are suitable for developing an automatic instrument for quantitative analysis.

A piezoelectric quartz crystal microbalance (QCM) has been used as a mass sensor for a wide field of applications in biochemistry and environmental, food and clinical analysis.[6,7] Its resonant frequency decreases with the increase of mass on the QCM electrode in a nanogram level.[8] Recently, QCM has been employed for investigation of various DNA interactions, real-time detection of hybridization of various complementary strands,[9] real-time detection of protein-DNA binding process,[10] the intercalation of dye molecules into DNA,[11] and the enzymatic cleavage of nucleic acid.[12]

Tumor necrosis factor (TNF) was defined as an endotoxin-induced serum factor, which produced necrosis of tumors both *in vitra* and *in vivo*[13] with the advance of molecular biology. A variety of effects were discovered for TNF, such as growth promotion, growth inhibition, angiogenesis, cytotoxicity, inflammation and immunomodulation.[14] Recent studies suggest that TNF may also play a central role in obesity modulating energy expenditure fat deposition and insulin resistance.[15] Therefore, it is important to detect the TNF gene in clinical medicine. To our knowledge, there is no report on the utility of QCM for detecting TNF gene.

In this paper, we report the immobilization of nucleic acid probe on the gold electrode of QCM to construct the novel piezoelectric nucleic acid sensor. The solid-phase hybridization of nucleic acid and detection of TNF

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target sequence using this sensor are described.

## Experimental

*Materials*

The 20-mer oligonucleotides, oligo $(dA)_{20}$, oligo $(dT)_{20}$, oligo $(dG)_{20}$ and [olig $(dC)_{20}$] were purchased from Shanghai Sangon Company (Shanghai, China). Polyethyleneimine (PEI), glutaraldehyde (Glu) and phosphate buffered saline (PBS, pH = 7.2) were obtained from Sigma Chemical Company (U. S. A), and bovine serum albumin (BSA) and protein A were obtained from Shanghai Institute of Biological Products (Shanghai, China). TNF target gene (500 bp) and TNF nucleic acid probe (300 bp) were purchased from Tongji Medical Company (Wuhan, China). BSA and protein A were dissolved and diluted with PBS (0.01 mol/L, pH = 7.2) solution. Other reagents and solvents were of analytical grade. The concentration of the hybride consisted in buffer is 50% (*V*/*V*) deionized methylacetamide (5 × SSC, 1.25 × FPG, 31 mmol/L $KH_2PO_4$, 0.25% SDS 31 μg/mL). Deionized ultrapure water (DW) was used throughout.

*Equipment*

The piezoelectric crystal used in this study was gold deposited AT-cut with a basic resonance frequency of 9 MHz from Beijing Chengguang Co. (Beijing, China). The crystal consists of a 12.5 mm (diameter, thickness) quartz wafer, placed between 6 mm (diameter) gold electrodes; the oscillator circuit was constructed from a transistor-transistor logic integrated circuit (TTL-IC). The frequency was monitored with a high-resolution frequency counter (CN 3165, Sampo Co., Taiwan, China). The crystals were washed in an ultrasonic cleaner (B2000, Branson Co., U. S. A). The thermostat bath was a model CS501-3 from Chongqing Instrument Co. (Chongqing, China).

*Immobilization with* PEI-Glu

The crystals for immobilization were cleaned and activated as described elsewhere,[16] and dipped in 40 g/L PEI methanol solution for 15 s. After air drying at 35 ℃, the crystals were washed with ethanol and DW. Then the crystals were immersed in Glu aqueous solution (0.3 mol/L) at 30 ℃ for 30 min, washed with DW and dried in air. The stable frequencies ($F_1$) of the crystals were measured in the test chamber.

Oligo $(dT)_{20}$ *immobilization* A 10 μL of oligo-$(dT)_{20}$ solution was dropped on the surface of the gold electrode of the crystal at 30 ℃ for 40 min. Then the electrode and crystal were washed with PBS and DW, dried in air and immersed in a solution (0.1 g/L BSA or glycine) to block all unreacted aldehyde groups. The crystals were subsequently rinsed with PBS and DW, and then dried in air.

TNF *nucleic acid probe immobilization* The solution of TNF nucleic acid probe was denatured at 100 ℃ for 5 min, then rapidly reannealed on the ice for 10 min. A 15 μL of denaturing solution of TNF nucleic acid was dropped on the surface of the gold electrode of the crystal at 30 ℃ for 40 min. The crystals were washed with 2 × SSC and DW, dried in air, then immersed in a solution of BSA (0.1 g/L) to black all unreacted aldehyde groups. The crystals were subsequently rinsed with 2 × SSC and DW, and then dried in air.

*Immobilization of protein* A

The crystals were cleaned and activated as above, immersed in protein A solution (0.1 g/L, in 0.01 mol/L PBS, pH = 7.2) at 30 ℃ for 30 min, washed with PBS and DW, and then dried in air. The stable frequencies ($F_1$) of the crystals were measured in the test chamber. The following treatment was the same as that of PEI-Glu immobilization.

*Measurement procedures*

The stable frequencies ($F_2$) of the crystals immobilized with oligo $(dT)_{20}$ probe were measured. A 15—20 μL of PBS solution containing oligo $(dA)_{20}$, oligo $(dG)_{20}$, or oligo $(dC)_{20}$ was dropped on the surface of the gold electrode which had been immobilized with oligo $(dT)_{20}$ probe, and kept in a bath with thermostat at 50 ℃ for 30 min under moist condition. The crystals were subsequently rinsed with PBS and DW, and then dried in air. The new frequencies ($F_3$) were measured. The frequency changes ($\Delta F = F_2 - F_3$) were related to the amount of complementary oligonucleic acid target which had been hybridized with oligo $(dT)_{20}$ or TNF immobilized on the

crystals.

A purified TNF target sequence (580 bp) was denatured at 100 ℃ for 5 min, then rapidly reannealed on the ice for 5 min and diluted with hybride buffer solution. A 20 μL of denatured solution of TNF target sequences was dropped on the surface of the gold electrode, which had been immobilized with TNF nucleic acid probe, and kept in the thermostat bath at 64 ℃ for 60 min under moist condition. The crystals were subsequently rinsed with 2 × SSC and DW, and dried in air, and the new frequencies ($F_3$) were measured.

## Results and discussion

*Comparison between the immobilized* oligo(dT)$_{20}$ *procedures of* PEI-Glu *and protein* A

Two different methods for immobilized oligo(dT)$_{20}$ probe are compared in Table 1. It seems that the better results in terms of bound amounts and reproducibility were obtained with PEI-Glu method. This is because PEI-Glu member can provide more binding sites for the immobilized nucleic acid probe, whereas protein A can complex with gold to form protein A-Au complex stably, and the nucleic acid probe immobilized to protein A could wash out by buffer solution and DW easily. The PEI-Glu method was adopted for the following experiments.

**Table 1** Comparison of the two immobilization procedures[a]

| Immobilization method | $\Delta F_1$ | $\Delta F_2$ | $\Delta F_3$ |
|---|---|---|---|
| PEI-Glu | 540 ± 15 | 370 ± 8 | 295 ± 4 |
| Protein A | 115 ± 4 | 45 ± 2 | — |

[a] The frequency variation of the coating with PEI-Glu or protein A ($\Delta F_1$), the coating with oligo(dT)$_{20}$ probe ($\Delta F_2$), the hybridization target sequence oligo(dA)$_{20}$ ($\Delta F_3$) are compared. Each value ($\Delta F$) represents the average of five experiments (± standard deviation).

*Amount of nucleic acid probe immobilized on crystal*

Table 2 displays the relationship between the injected and immobilized amount of nucleic acid probe on the crystal. It is noticed that the nucleic acid probe immobilized comes to saturation when the injected amount of nucleic acid probe is up to 880 ng. The results are similar as ref. 17.

*Comparison of* BSA *blocking and glycine blocking*

The non-specific adsorption of nucleic acid increased the mass of crystal and caused the frequency variation of the crystal. It was caused by the unreacted aldehyde groups of the PEI-Glu member and should be prevented when this sensor is being used. Two reagents, BSA and glycine, were selected as blocking reagents for binding the aldehyde groups of PEI-Glu member after the immobilized nucleic acid probes. The blocking comparison of two reagents is shown in Table 3. The results show that the frequency shift caused by BSA blocking could match the Sauerbrey equation,[8] but it could not match by glycine blocking. Maybe the glycine formed stable complex and could be washed out by PBS easily. BSA was selected as the blocking reagent in the following experiments with nucleic acid probe immobilized on crystal.

*Hybridization of* oligo(dT)$_{20}$

Oligo(dA)$_{20}$, oligo(dG)$_{20}$ and oligo(dC)$_{20}$ act as target nucleic acid to hybridize with oligo(dT)$_{20}$. The specialty and reproducibility of hybridization are shown in Table 4. A larger frequency shift can be obtained with oligo(dA)$_{20}$ rather than oligo(dG)$_{20}$ or oligo(dC)$_{20}$ as target nucleic acid. The results show that the sensor has good specialty and reproducibility.

Fig. 1 shows the relationship between the frequency shifts and the amounts of target nucleic acid. The frequency shift of hybridization has a quite good linear relationship with the amount of target nucleic acid for amounts below 770 ng. However, above 770 ng, the frequency changes no longer depend on the injected amounts of target nucleic acid.

**Table 2** Relationship between the inject amount and immobilization amount of nucleic acid probe

| Injected amount (ng) | 110 | 220 | 330 | 440 | 550 | 660 | 770 | 880 | 990 | 1100 | 1210 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta F$ (Hz) | 56 | 131 | 176 | 223 | 246 | 300 | 345 | 380 | 370 | 400 | 375 |
| Immobilized amount (ng) | 86 | 201 | 271 | 343 | 379 | 462 | 513 | 585 | 570 | 616 | 578 |
| Immobilized ration | 0.78 | 0.91 | 0.82 | 0.78 | 0.69 | 0.79 | 0.69 | 0.67 | 0.57 | 0.56 | 0.47 |

**Table 3** Comparison of the two blocking procedures[a]

| Blocking procedure | $\Delta F$ |
|---|---|
| BSA | 20 ± 4 |
| Glycine | 124 ± 56 |

[a] $\Delta F$ represents the frequency changes of the immobilized oligo-$(dT)_{20}$ probe crytal after blocking with BSA or glycine. Each value ($\Delta F$) represents the average of five experiments ( ± standard deviation).

**Table 4** Specialty and reproducibility of oligo$(dT)_{20}$ hybridization[a]

| Hybridization | $\Delta F$ |
|---|---|
| Oligo$(dA)_{20}$ | 48 ± 5 |
| Oligo$(dG)_{20}$ | 2 ± 3 |
| Oligo$(dC)_{20}$ | 1 ± 3 |

[a] $\Delta F$ represents the frequency changes of the immobilized oligo-$(dT)_{20}$ probe crystal after hybrided with oligo$(dA)_{20}$, oligo$(dG)_{20}$ or oligo$(dC)_{20}$. Each value ($\Delta F$) represents the average of five experiments ( ± standard deviation).

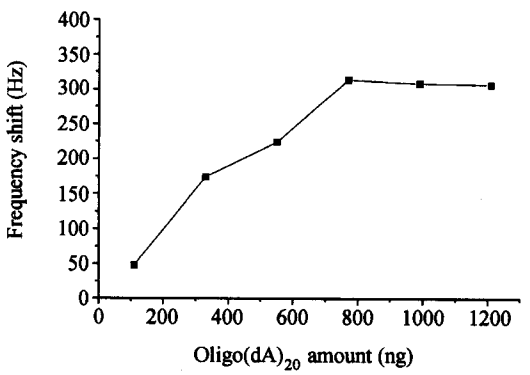


**Fig. 1** Relationship between the frequency shifts and the quantity of hybridized target nucleic acid oligo$(dA)_{20}$ with oligo$(dT)_{20}$ nucleic acid probe amount 880 ng.

*Detection of* TNF *target sequence*

After the hybridization of the TNF nucleic acid probe (300 bp) immobilized on the QCM with various amounts of TNF target gene sequence (580 bp) under optimum conditions, the frequency was measured and the frequency shifts were calculated. The correlation between the concentration of TNF target sequence in the range of 100—500 ng and the sensor response is shown in Fig. 2. This result suggests that this sensor can be used to detect TNF target sequences quantitatively.

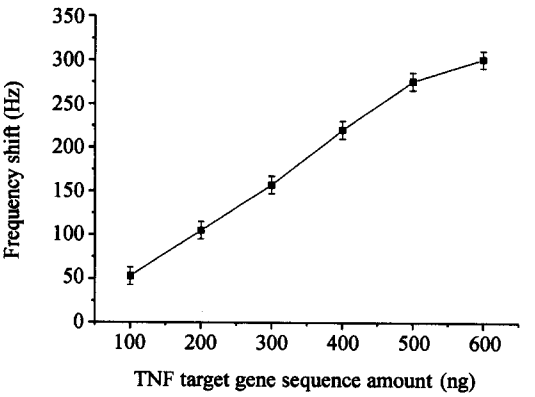


**Fig. 2** Correlation between the frequency response of sensor and the quantity of hybridized TNF target gene sequence. Each point represents the average of five assays with a 60 min reaction time at 64 ℃.

The selectivity of the sensor was evaluated by dropping the solutions of oligo$(dA)_{20}$ (0.11 μg/mL), oligo-$(dT)_{20}$ (0.55 μg/mL), oligo$(dG)_{20}$ (0.11 μg/mL), and oligo$(dC)_{20}$ (0.11 μg/mL), respectively. The measurement steps were exact as previously described for TNF gene. The frequency variations are within 20 Hz, and have little influence to the measurement of TNF gene, which suggests that these nucleic acids do not interfere with the detection significantly.

## Conclusions

QCM has been demonstrated to be a very valuable tool for monitoring of solid-phase hybridization of nucleic acid and detection of TNF target sequence. The PEI-Glu immobilization of nucleic acid and BSA for blocking of unreacted aldehyde groups are stable, reproductive and alternative. The above results promise a good future for further practical application in the detection of TNF gene.

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