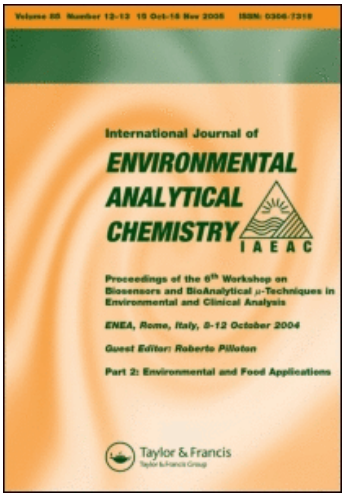

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STUDIES ON BIOTIN–AVIDIN INDIRECT CONJUGATED TECHNOLOGY FOR A PIEZOELECTRIC DNA SENSOR

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Because of their high sensitivity, piezoelectric sensor techniques are extremely useful for environmental or clinical analysis. We developed a piezoelectric crystal DNA biosensor for the detection of the hybridization reaction based on the self-assembled monolayer technology and biotin–avidin system. 3,3′-Dithiopropionic acid was applied to form a self-assembled monolayer (SAM) on the gold surface of the quartz crystal. Avidin was coated on the gold electrode conjugated with 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), and then biotinylated nucleotide acids were immobilized on the gold electrode surface through the specific interaction of biotin and avidin. Our results indicated that, using this immobilization method, the piezoelectric DNA sensor shows a higher sensitivity and specificity in detecting the hybridization reaction. The sensor can be used repeatedly by electrode regeneration.

Keywords: Piezoelectric DNA sensor; Biotin–avidin system; Self-assembled monolayer

INTRODUCTION

Sequence-specific hybridization between nucleic acids, either in solution or immobilized on a fixed support, is widely used for the detection and analysis of genetic material in diverse applications such as the identification of genetic diseases and disorders, and the detection and characterization of viruses, bacteria and parasites. Because of their piezoelectric properties, quartz crystals are used as microbalances (QCM). They can function as an extremely sensitive mass sensor capable of measuring subnanogram levels of mass changes. In addition, they are inexpensive, chemically, physically and mechanically durable systems, and as no label is required, interest in the use of piezoelectric devices for hybridization analysis has increased markedly in recent years [1,2].

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The principal limitation of the QCM is nonspecific adsorption of molecules present in real matrices; QCM is a mass sensor, and any molecule binding to the surface is a potential interferent. To effectively minimize the nonspecific binding, experiments are focused on immobilization methods to improve the selectivity and sensitivity of the piezoelectric biosensor.

Biotin can be easily activated, and can bind rapidly and specifically with avidin. In addition, the biotin–avidin complex is stable, and the biotin–avidin system (BAS) [3] can attach to nucleic acids with high conjugation rates without interfering with the activities of nucleic acids. Hence, the BAS has been widely used in studies of nucleic acids. In this study, we used BAS to improve the performance of piezoelectric DNA biosensors for hybridization to minimize nonspecific adsorption on the gold surface and to orientate the immobilized probe. The gold electrode was treated with 3,3′-dithiopropionic to form a self-assembled monolayer (SAM). 1-Ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were added to form SAM functional groups, and then avidin was stabilized on the gold electrode by the functional groups of SAM. Ethanolamine was used to block out any sites not attached to avidin. Finally, 169-bp biotinylated nucleotide acids were immobilized on the gold electrode surface by the specific interaction of biotin and avidin (see Fig. 1).

Staphylococcal enterotoxins (SEB) are a population of seven major serological types of heat stable emetic enterotoxins, produced by *Staphylococcus aureus*, one of the major causes of bacterial food poisoning. Because of its remarkable toxicity and stability, SEB is considered a prime threat, i.e. a biological weapon of mass destruction [4]. Thus, it is very important to determine SEB in food hygiene and clinical analysis. So far, several methods have been established to determine SEB, such as immunoassays, hybridization techniques and polymerize chain reactions (PCR) [5,6]. However, these methods have several disadvantages, such as nonspecific reactions and the fact that they are qualitative, not quantitative, analyses.

In this work, we describe a piezoelectric DNA sensor for detecting the SEB gene. Biotinylated nucleic acid probe (169-bp DNA fragment through PCR) was immobilized on the avidin-coated gold electrode. The immobilized probe was then reacted in solution with the target SEB gene with complementary sequences. Hybridization could then be detected through the quartz crystal frequency shift according to Sauerbrey's equation [7].

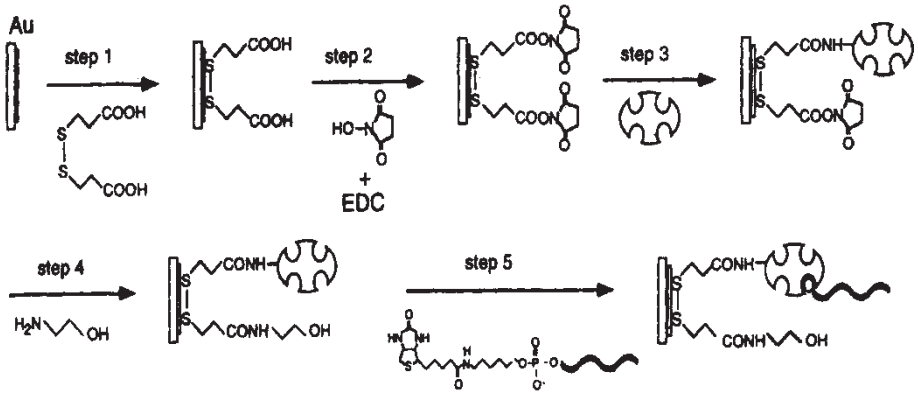

FIGURE 1 SEB DNA probe immobilized on the Au electrode by using an avidin–biotin interaction.

EXPERIMENTAL

Apparatus

The following materials were used: AT-cut quartz piezoelectric crystals (10 MHz, or 3.58 MHz, diameter 8.5 mm or 5.5 mm), gold electrodes (obtained from the Institute of Piezoelectric Crystal, P.R. China); PCR DNA thermal cycler (PE-2400, USA), the network analysis, cymometer (model 8610B, P.R. China); and oscillator generator (self-designed). The quartz crystal used in this study was gold-deposited AT-cut quartz with a 10-MHz resonance frequency (from the Institute of Piezoelectric Crystal, Beijing, China). The crystal consisted of an 8.5-mm diameter, 0.2-mm thick quartz wafer, placed between two 5.5-mm gold electrodes. The oscillator circuit was home-made. The frequency was monitored with a high-resolution frequency counter.

A staphylococcal enterotoxin B gene was amplified by the PCR from a standard strain of *S. aureus*. The quartz crystal was housed inside a methacrylate cell such that only one side of the crystal was in contact with the solution in the cell well. The frequency variations were continuously recorded using a quartz crystal analyzer. The frequency shifts reported in the article are the differences between two stable frequency values (±1 Hz). Temperature was controlled (±1°C) by an air thermostat, and fluctuations gave negligible frequency variations. The electrode surface was cleaned with a boiling solution consisting of H_2O_2 (33%), NH_3 (33%) and deionized ultrapure water in a 1 : 1 : 5 ratio. The crystals were then immersed in the solution for 10 min, thoroughly washed with distilled water and used immediately afterwards.

Reagents

3.3′-Dithiopropionic acid, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and the avidin were purchased from the Fluka Co. (USA). Ethanolamine was obtained from Yili Chemical Reagent company (Beijing, China). Standard strains 1000A (staphylococcal entertoxin A, SEA), 1093C (SEC), 494D (SED) and 216B (SEB) were bought from Wisconsin University (USA). Biotin-labeled dUTP and Taq DNA polymerase were purchased from Sigma Co. (USA). Biotin-5′-TGATATTAATTCGCATGAAACTGAC-3′ was purified by HPLC (obtained from Sigma). The composition of the buffers used for the experiments was as follows:

- PCR buffer: TE buffer, 10 mM Tris–HCl, pH 8.0, 1 mM NaAc, 1 mM EDTA, pH 8.0
- Immobilization buffer: 300 mM NaCl, 20 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4.
- Hybridization buffer: 150 mM NaCl, 20 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4.

Each 50-μL PCR reaction was a mixture of 4 μL of 0.05 μmol/L mixed primers, 2 μL of 0.025 μmol/L 4dNTP mix, 5 μL of 10× PCR reaction buffer; 1 μL of 5 μg/L template, 4 U of Taq DNA polymerase and 38 μL of sterile deionized water. The electrode surface was cleaned with a boiling solution consisting of H_2O_2 (33%), NH_3 (33%) and deionized ultrapure water in a 1 : 1 : 5 ratio. The crystals were immersed in the solution for 10 min, thoroughly washed with distilled water and used immediately afterwards. All other reagents were purchased from Sigma (Beijing). Deionized ultrapure water was used in all of the experiments.

Procedures

Preparation of the Biotinylated SEB Gene Segment (169bp) by PCR

The bacterial DNA was extracted from different *S. aureus* strains and amplified by PCR. The synthetic oligonucleotide primers used were SEB1 and SEB2 (5′-TAC-GTAGATGTGTTTGGA-3′ and 5′-AGGGATCCTCACTTTTTCTTTGTCG-3′) to amplify a 169-bp fragment of the *seb* gene of *S. aureus* [6]. PCR products were examined by gel electrophoresis. To investigate the hybridization reaction with the real samples, the optimized procedure was as follows: 20 μL of the solution of the DNA fragments obtained from the amplification by PCR was diluted with 80 μL of hybridization buffer (final total volume, 100 μL); the sample was then denatured by heating at 95°C for 3 min and then freezing the sample in ice for 30 s. The 100-μL sample was then added to the cell well.

The hybridization reaction was allowed to proceed for 20 min, and the crystal was then washed with the hybridization buffer. The frequency value was recorded, and the difference between this value and that displayed before the hybridization was evaluated. After the hybridization reaction, the probe could be regenerated by treatment for 1 min with 1 mM HCl, as for the reaction with the standard solution of the oligonucleotides, and another cycle of hybridization–regeneration could be performed.

Immobilization of the Nucleotide Acid Probe

The gold-plated quartz crystal was cleaned with a solution of 25% H_2O_2/75% H_2SO_4 (piranha solution), to eliminate any impurities such as organic compounds from the gold surface, and rinsed with deionized ultrapure water several times. The immobilization procedure was adopted from Storri *et al.* [8,9]. The freshly cleaned quartz crystal was dipped in 3,3′-dithiopropionic acid for 20 min at room temperature, followed by the addition of NHS then EDC. After 30 min, carboxylated quartz crystal was dipped into 3 mL of buffer (pH 7.9, 10 μg of avidin, 10 mmol of Tris–HCl, 0.2 mol/L NaCl) for 1 h, washed with the same buffer and then dipped in 1 mol/L ethanolamine solution for 30 min. The avidin was then immobilized on the surface of the crystal. Next, the avidin crystal was immersed in biotinylated nucleotide acid solution for immobilization.

Hybridization of Biotinylated DNA Probe on the Surface of the Quartz Electrode

This work used a quartz harmonic oscillator (10 MHz) as a sensitive unit and 169-bp biotinylated probe, which was amplified by PCR as a molecular identification unit. The probe was then immobilized on the surface of the gold electrode by the specific interaction between biotin and avidin. Figure 2 shows the frequency changes in hybridization, indicating that the adsorbate impurity on the surface of the electrode was eliminated after being treated with piranha solution. Because of the decreasing mass on the surface, the frequency of the QCM was increased. In contrast, after avidin settled on the electrode, the mass on the surface increased, thus leading to a decrease in frequency according to the Sauerbrey equation [7]. The same phenomenon occurred after avidin conjugation with biotin on the DNA probe and after hybridization between the DNA probe and the single-strand DNA (ssDNA). This suggested, therefore, that the DNA probe had been immobilized on the electrode and that the effect of hybridization is distinct.

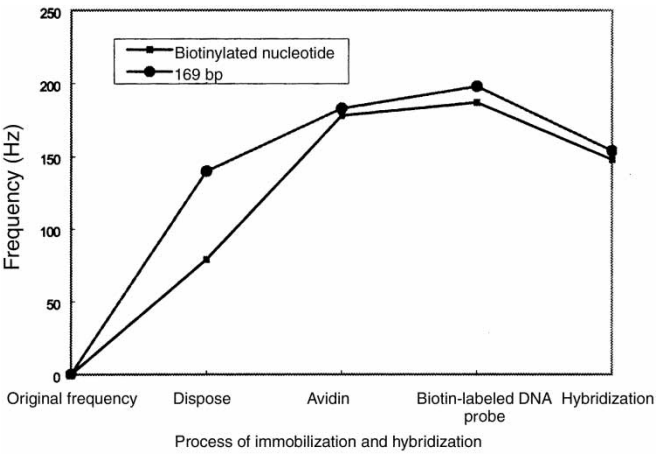

FIGURE 2 Process of immobilization and hybridization.

Measurement Method

The probe was immobilized by adding 80 μL of the 169-bp DNA fragment in the immobilization buffer to the cell well. The reaction was monitored for 20 min, and then the crystal was washed with the immobilization buffer to remove the unbound DNA. The frequency was recorded until it reached a stable value ($F1$). Then, the immobilized probe sensor was placed in hybridization buffer containing ssDNA. After hybridization for 2 h, the crystal was taken out and rinsed with the same buffer. The crystal was rinsed with deionized water to remove any effects resulting from nonspecific adsorption. Finally, the crystal was placed into a desiccator, allowed to dry completely, and its resonant frequency ($F2$) measured. The frequency change was calculated from the equation $\Delta F = F1 - F2$, and all the experiments were performed at room temperature (~25°C).

RESULTS AND DISCUSSION

Static Measurement

Biotinylated SEB DNA probe, amplified by PCR, was immobilized on the quartz harmonic oscillator (10 MHz) and hybridized with ssDNA. The impedance shift measured before and after immobilization by network analysis was found to occur not only during immobilization but also during hybridization (Table I). This indicated that the biotinylated DNA probe was immobilized on the quartz harmonic oscillator and that hybridization took place on the electrode. The results of hybridization with the complementary sequence of target gene are also illustrated in Fig. 3 ($y = 32.6x - 34.3$, $r^2 = 0.990$).

Consistency Test of Immobilization

To determine which immobilization method is better, we immobilized the biotinylated DNA probe on the surface of the electrode of the quartz harmonic oscillator (10 MHz), then hybridized this with complementary ssDNA in the group and inter-groups

TABLE I Impedance shift in immobilization and hybridization of SEB DNA probe by PCR

Impedance shift[a]	*Golden electrode of crystal quartz*					
	1	*2*	*3*	*4*	*5*	*6*
ΔQ1 (Ω)	9.187	10.653	10.106	9.233	11.024	9.241
ΔQ2 (Ω)	9.276	10.989	10.436	9.624	11.243	9.684

[a]ΔQ1: Impedance shift due to immobilization by biotin-labeled DNA probe; ΔQ2: impedance shift due to DNA hybridization.

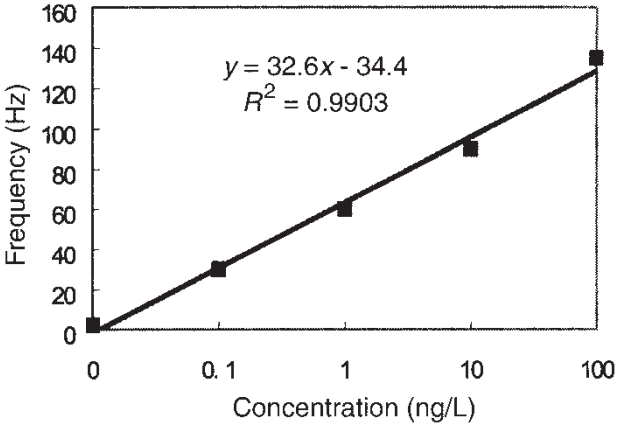

FIGURE 3 Linear relationship between hybridization concentration and frequency shift.

TABLE II Consistency of the immobilization process

Immobilization substance	ΔF ($n=6$) (*inter-group*)	*S* (*inter-group*)	ΔF ($n=6$) (*in group*)	*S* (*in group*)
169-bp ssDNA	128 Hz	4.21	121 Hz	4.56
Biotinylated nucleotide probe	123 Hz	6.21	119 Hz	8.45

(see Table II). Table II shows that the inter-group consistency is better than that in group in the probe-immobilization process, which might be caused by experimental error in the course of electrode regeneration. Also, the immobilization effect of the 169-bp DNA probe was better than that of the synthesized biotinylated nucleotide probe.

Specificity Test of the PZ DNA Sensor

Two different DNA probes were immobilized (one, a 169-bp DNA fragment from PCR from the *seb* gene of *S. aureus*, the other a synthesized 23mer oligonucleotide according to a 169-bp DNA fragment) on the gold electrode of the quartz crystal (3.58 MHz), and ssDNA of 1000A, 1093C and 494D, respectively, were added to the hybridization solution and hybridized (Table III). The differences in original frequency and immobilized frequency for the two group data were analyzed statistically and were found to be distinctly differentiated, with biotinylated DNA probe having been immobilized on the electrode. The two data groups of immobilized frequency and hybridization

TABLE III Specificity of the piezoelectric DNA sensor

Frequency (Hz)	*Gold electrode of crystal quartz*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Original frequency	3 582 442	3 582 231	3 583 968	3 581 622	3 581 172	3 580 541	3 582 654	3 580 273
Immobilized frequency	3 582 291	3 582 079	3 583 806	3 581 469	3 581 271	3 580 381	3 582 100	3 580 110
Hybridization frequency	3 582 289	3 582 081	3 583 809	3 581 471	3 581 268	3 580 379	3 582 102	3 580 111

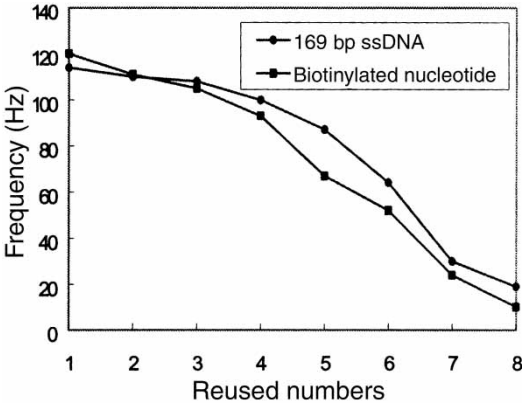

FIGURE 4 Reused numbers of the piezoelectric DNA sensor.

frequency were analyzed using the SAS software. A P value of 0.6622 (i.e. >0.05) shows that there are no differences between them, i.e. there is no specific hybridization on the gold electrode between the two probes. However, when ssDNA was added to standard strain 216B, which could produce SEB, the two data groups showed significant differences (Table III), thus indicating that the two different DNA probes have a specific reaction on the surface of gold electrode. Therefore, this test shows that this PZ sensor was highly specific.

The Reuse Number of the PZ Sensor

The quartz crystal was soaked in 0.25 mol/L hydrochloric acid and then immersed in 0.5 mol/L sodium hydroxide to remove any extraneous substances from the crystal surface. The quartz crystal could be used repeatedly without any resultant damage by ensuring that the correct cleaning procedure was followed (Fig. 4). This showed that the biotinylated probe produced by PCR amplification could be used several times, and the consistency was good from the first time to the fourth. However, after eight times, the activity of biotin would have been lost completely. The PZ sensor produced from the synthesized biotinylated oligonucleotide could be used three times. After five times, the frequency shift decreased markedly. Figure 4 also shows that the PZ sensor in both probes performed well, but considering that the probe produced by PCR amplification was abundant, cheap and easy to make, we carried out further studies on the biotinylated 169-bp DNA probe.

Comparison with Other Methods

We detected 10 clinical samples with PCR, blotting hybridization and PZ sensor produced using biotinylated 169-bp DNA probe. Eight out of 10 were positive; the others were negative, indicating that two strains did not produce SEB. The piezoelectric sensor, which used biotin–avidin indirect conjugation technology, seems to be suited for gene detection. Compared with the PCR method [7], which is well known for its time-saving and sensitivity features, could detect 2 h later, and the response curve tended to reach saturation 7 h later. In addition, the piezoelectric sensor is less sensitive than that of PCR. However, compared with blotting hybridization [10], because there is no pre-hybridization, the piezoelectric sensor has its advantages such as its simplicity and short amount of time required. Also, its sensitivity is equivalent to that of blotting hybridization. Hence, the piezoelectric DNA biosensor is an alternative to current gene diagnostics and separation methods.

CONCLUSION

In this study, we have described a piezoelectric biosensor for hybridization detection and used the system to analyze SEB samples amplified by PCR. The method of coupling the biotin–avidin indirect conjugation on the gold surface of the crystal may provide reproducible results and minimal nonspecific adsorption effects from non-complementary strands.

Acknowledgments

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References

[1] F. Caruso, E. Rodda, D.F. Furlong and K. Niikura, *Anal. Chem*, **69**, 2043–2049 (1997).
[2] X.C. Zhou, L.Q. Huang and S.F. Li, *Biosens. Bioelectron.*, **16**, 85–95 (2001).
[3] N. Wrobel, W. Deining, P. Hegemann and V.M. Mirsky, *Colloids and Surfaces B: Biointerfaces*, **32**, 157–162 (2003).
[4] Z.R. Lei, *Bacteriotoxin Molecular Biology*, pp. 88–94. Science and Technology Press, Beijing (1993).
[5] F.J. Schmiz, M. Steiert, B. Hofmann and H.P. Heinz, *J. Med. Microbiol.*, **47**, 335–340 (1998).
[6] O.D. Brakstad, K. Aasbakk and J.A. Maeland, *J. Clin. Microbiol.*, **30**, 1654–1660 (1992).
[7] G. Sauerbrey, *Z. Phys.*, **155**, 206–209 (1959).
[8] S. Storri, T. Santoni and M. Mascini, *Anal. Lett.*, **31**, 1795–1799 (1998a).
[9] S. Storri, T. Santoni and M. Mascini, *Biosens. Bioelectron.*, **13**, 347–351, (1998b).
[10] Z.X. Gao and F.H. Chao, *Chin. J. Med. Lab. Sci.*, **22**, 281–283 (1999).