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Piezoelectric immunosensor for the detection of *Edwardsiellosis*

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In this study, a QCM biosensor was made to detect *Edwardsiella tarda (E. tarda)* using a specific antibody. A 9 MHz AT-cut piezoelectric wafer layered with two gold electrodes of 5 mm diameter had a reproducibility of ±0.1 Hz in frequency response and was used as the transducer of the QCM biosensor. Self assembled monolayer (SAM) was conformed on a quartz crystal by treating with 3-mer-captopropionic acid (MPA) and activated with N-ethyl-N'-(3-dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The resulting NHS group was further converted to hydrazide by the reaction with hydrazine. Aldehyde group was introduced into the carbohydrate moiety of anti-*E. tarda* antibody by the reaction with periodic acid and was used to immobilise the antibody through the reaction with hydrazide group on the electrode surface. A baseline was established in the presence of phosphate-buffered saline (PBS) and a resonant frequency (F1) was measured. Sample was added to the sensor surface and second resonant frequency (F2) was measured after unbound substances were washed out with PBS several times. Finally, the frequency shift (ΔF) representing the mass change was calculated by subtracting F2 from F1. After adding the oxidized anti-*E. tarda* antibody to the electrode surface containing hydrazide group, frequency shift of 288.8±11.4 Hz (mean ± S.E) was observed, thus proving that considerable amount of antibody was immobilized. In the immunoassay test, the frequency shift of 187±7.75 Hz, 58±0.67 Hz, 22±1.39 Hz, 7.67±1.83 Hz (mean ± S.E) were observed at doses of 1000, 500, 100, 50 ㎍ of bacterial cells, respectively. It was also demonstrated that the prepared sensor chip was stable enough to withstand repeated surface regeneration with 0.2 M Tris-glycine and 1% DMSO, pH 2.3 more than ten times.

Key words: *Edwardsiella tarda,* QCM biosensor, Antibody

Introduction

Biosensors consist of biochemical recognition agents, like enzymes, antibodies, nucleic acids or chemoreceptors, immobilized on the surfaces of transducers that change the recognition event into a measurable electronic signal. Piezoelctric devices have been proposed as probes that are able to continuously monitor affinity reactions (antigen-antibody, DNA hybridisation reaction, etc.), without the use of any label (Minunni et al., 1995; Guilbault et al., 1992; Skladal et al., 1994). The first use of antigens as coating in quartz crystal microbalances (QCM) was proposed by Shons et al. (1972). Since then, QCM has been developed as an extremely sensitive mass sensor, capable of measuring subnanogram levels (Clark et al., 1987). The detection limits can be improved by using crystals of higher frequencies (>10MHz) (Bunde et al., 1998; Lin et al., 1993) and improving the immobilization proce-

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dure on the quartz surface (Clark et al., 1987).

Edwardsiella tarda (E. tarda), which is a member of the bacterial family Enterobacteriaceae, is an economically important pathogen and is frequently found in organically polluted water. The natural reservoir of this bacterium appears to be the intestine of animals including fishes. It has been isolated from a variety of cultured fishes, including eel *(Anguilla japonica)*, channel catfish *(Ictalurus punctatus)*, mullet *(Mugil cephalus)*, seabream *(Evynnis japonicus)*, chinook salmon *(Oncorhynchus tsawytscha)*, tilapia *(Tilapia nilotica)*, and others (Wakabayashi and Egusa 1973, Meyer and Bullock 1973, Kusuda et al. 1976, Kusuda et al. 1977, Amandi et al. 1982, Kubota et al. 1982). Currently, bacterial culture followed by biochemical profiling is the only method of *E. tarda* identification. This study demonstrates a quick and sensitive QCM biosensor system to detect *E. tarda* and its application.

Materials and methods

Preparation of formalin killed cells of *Edwardsiella tarda* and its antibody

Edwardsiella tarda (E. tarda) was isolated from diseased fish and cultured on tryptic soy agar (TSA) plate. A single colony was inoculated into 400 ml of TSB medium and cultured overnight. To prepare formalin killed cells (FKC), formalin was added at a final concentration of 1% and the mixture was incubated for 24 hours at 4℃. FKC was washed with phosphate buffered saline (PBS) several times and was used as an antigen to prepare rabbit antiserum against *E. tarda*. A rabbit was immunized with the prepared FKC and the antiserum was taken when the titer reached to 1:25600-51200. Rabbit IgG was purified using Protein G-agarose fast flow column (Sigma). Purity and protein concentration of the IgG fraction were estimated on a SDS-PAGE gel stained with Coomassie brilliant blue R250 and with BCA protein assay kit (Novagen), respectively.

Oxidation of IgG

The purified IgG was oxidised with periodic acid (Sigma) to generate aldehyde group in the carbohydrate moiety located within Fc region. Briefly, 1 ml of 0.5 mg/ml IgG purified from rabbit anti-*E. tarda* antiserum in 50 mM phosphate buffer was mixed with 20 ㎕ of 50 mM sodium m-periodate (Sigma). The mixture was incubated in the dark for 30 min. Unreacted sodium m-periodate was removed by dialysis against buffer A (100 mM sodium acetate buffer, pH 5.5). Oxidized IgG was stored in a freezer.

QCM biosensor system

A 9MHz AT-cut piezoelectric wafer layered with two gold electrodes of 5mm diameter had a reproducibility of ±0.1 Hz in frequency response and was used as the transducer of the QCM biosensor. It was mounted on a well holder made with acryl and connected to a home made oscillator module (Daga electronics, Korea). The analogue frequency signals from the oscillator were converted to the digital ones in a frequency counter. The in situ signal was stored in personal computer and performed data analysis using Microsoft excel program.

To test adsorption level of each substance, 100 ㎕ of PBS was added into the reaction cell of the well holder and measured the resonant frequency until a steady-state baseline was obtained (F1) before incubating a sample in the reaction cell. After washing out unbound substances with PBS several times, 100 ㎕ of PBS was added into the reaction cell and the steady-state resonant frequency (F2) was read again to calculate the frequency shift (ΔF = F1-F2).

Immobilization of the antibody through amino residue coupled to EDC/NHS activated SAM layer

Piezoelectric quartz crystal was cleaned using piranha solution (H_2SO_4: H_2O_2 = 7:3) at 60℃ for 5 min and rinsed with absolute ethanol and distilled water. The quartz crystal was treated with 10 mM of MPA (3-mercaptopropionic acid, Sigma) and then activated with 46 mM of EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide, Sigma)/NHS (N-hydroxysulfosuccinimide, Sigma). 5 mM Carbohydrazide (Sigma) was cross-linked on the activated gold surface and uncross-linked residues were blocked by 1 M ethanolamine-HCl (Sigma). The Fc region of periodic acid treated antibody was added and immobilised on the gold surface through amino residues of carbohydrazide with right direction. 0.1 M sodium cyanoborohydride in 0.1 M sodium acetate, pH 4 was added to strengthen the interaction between periodic acid treated antibody and carbohydrazide. Uncross-linked residues were blocked by a blocking buffer containing 1mM EDTA, 0.25 % BSA, and 0.05% Tween-20. All reaction time was 1 hour and sensor chip was cleaned using distilled water between the reactions.

Sensitivity of sensor system

One hundred ㎕ of PBS was added and read the frequency at the steady state. Serially diluted bacterial cells were added onto the sensor chip prepared by above method at the doses of 1000, 500, 100, 50 ㎍/100 ㎕, incubated for 20 min, and washed out unbound cells by PBS for 4 times. One hundred ㎕ of PBS was added again and read the frequency at the steady state. The steady state was obtained within 5 min at the both cases before or after bacterial cells injection.

Regeneration of sensor system

The reusability of the prepared sensor chip was test using a dissociation buffer containing 0.2 M Tris-glycine and 1% DMSO, pH 2.3. One hundred ㎕ of PBS was added and read the frequency at the steady state. Bacterial cells were added onto the sensor chip at the doses of 500 ㎍/100 ㎕, incubated for 20 min, and washed out unbound cells by PBS for 4 times. One hundred ㎕ of PBS was added again and read the frequency at the steady state. One hundred ㎕ of dissociation buffer was added and incubated for 20 min, and washed out by PBS to removed detached bacterial cells. The same procedure was performed for 10 times on one sensor chip and observed the point showing a significantly decreased signal.

Results and Discussion

Anti-*E. tarda* antibody immobilization by direct cross-linking

Traditional methods for antibody immobilization on the gold surface of a piezoelectric quartz crystal include coating with protein A (Minunni et al., 1995; Guilbault et al., 1992; Boveniser et al., 1998; Attili and Suleiman, 1995) or silanisation, usually with aminopropyltriethoxysilane and activation with glutaraldehyde (Muramatsu et al., 1987; Steegborn and Skladal, 1997). Different polymers have also been used including polyethyleneimine (Konig and Gratzel, 1993; Prusak-Sochaczewski et al., 1990). Generally, conventional immobilisation methods based on adsorption or covalent binding via the amino group of the protein are not site-directed and produce a random orientation of the antibody, which results in a very low binding efficiency (Vikholm et al., 1999). In this study, a direct immobilizing technique was introduced using oxidized anti-*E. tarda* antibody over the gold electrode of the quartz crystal. Concerning the orientation of anti-

body for the best antigen binding capacity, the antibody was immobilized by cross-linking through hydrazine cross-linked via EDC/NHS activation of the sulfide monolayer (MPA).

In this study, it was also demonstrated that the frequency shifts following the injection of IgG are positively related to the frequency shifts after bacterial cell injection at the dose of 1 mg (Fig.1), revealing that the frequency shifts after bacterial cell injection fully represent the weight change by specific affinity of bacterial cells to the IgG cross-linked on the gold surface, but not by non-specific affinity to the gold surface. It is also indicated that the immobilization efficiency of IgG is an important factor affecting the sensitivity of sensor chips.

Detection of *E. tarda* cells

Currently, bacterial culture followed by biochemical profiling is the only method of bacteria identification including *E. tarda*. However, several researchers are seeking for quick and sensitive method to identify fish pathogens. Yu et al. (2004) reported that high-performance capillary electrophoresis (HPCE) has been applied to the identification, separation, and quantification of intact bacteria. It was demonstrated that *E. tarda* was identified and determined (<10 min) after direct injection into fish fluid by CE blue light-emitting diode (LED)-induced fluorescence using SYTO 13 (488 nm/ 509 nm), a cell-permeable green nucleic acid stain, to stain the cells. The limit of detection and recovery were found to be 4.2×10^4 cells/ml and 70%, respectively. Byers et al., (2002) also reported that *Aeromonas salmonicida*-specific polymerase chain reaction (PCR) test have a detection limit of approximately 4×10^5 CFU/g sample.

In this study, injection of bacterial cells at doses of 1000, 500, 100, 50 μg caused the frequency shift of 187±7.75 Hz, 58±0.67 Hz, 22±1.39 Hz, 7.67 ±1.83 Hz (mean±S.E), respectively (Fig. 2), when the injection of IgG (27 μg) caused the frequency shift of 288.8±11.4 Hz. (mean±S.E, data not shown). Thus the limitation of the QCM biosensor system made in this study is 50 μg corresponding to 5×10^7 CFU. This sensitivity is less than the above reports by Yu et al. (2004) or Byers et al., (2002) and can be improved by decreasing the in-

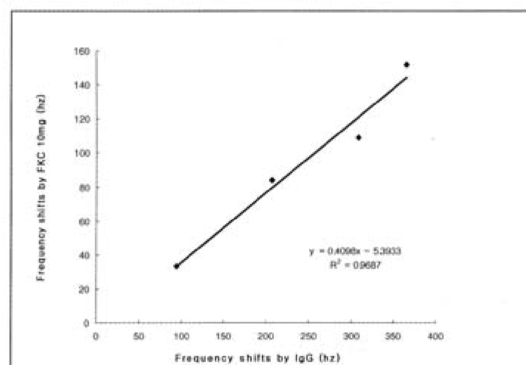

Fig.1. Relationship between the efficiency of IgG cross-linking and sensitivity of QCM biosensor. The frequency change was measured after IgG cross-linking and addition of 100 μg/100 μl of *E. tarda* FKC.

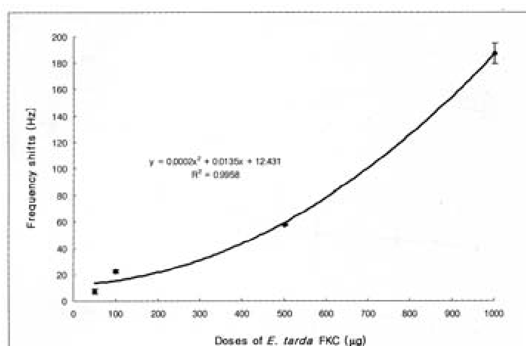

Fig.2. Dose response of QCM biosensor for the detection of *E. tarda*. One hundred μl of PBS was added and read the frequency at the steady state. Serially diluted bacterial cells were added onto the sensor chip prepared by above method at the doses of 1000, 500, 100, 50 μg/100 μl, incubated for 20min, and washed out unbound cells by PBS for 4 times. One hundred μl of PBS was added again and read the frequency at the steady state.

jection volume of sample to 20 $\mu\ell$.

Specificity of the sensor chip was studied by testing the affinity to different type of *E. tarda* strain, i.e. weak and strong pathogenic strain, 219 and KFE, or different fish bacteria, *Vibrio vulnificus*. As a result, the sensor chip showed the same affinity to *E. tarda* 219 and KFE, while the affinity to *V. vulnificus* was not observed (Data not shown). Specificity of the sensor chip against to the different serotypes of *E. tarda* should be further studied.

Regeneration of sensor chips

Many published papers showed that immobilisation technique based on direct adsorption or on protein A coating, resulted in appropriate sensor signals, but only crosslinker procedures using thiols or the interaction between avidin and biotinylated molecules, provided a long sensor lifetime. Moreover, covalent coupling increased the stability against degradation during the regeneration process (Uttethaler et al., 1998).

In this study, a direct immobilizing technique was introduced using oxidized anti-*E. tarda* antibody over the gold electrode of the quartz crystal. Concerning the orientation of antibody for the best antigen binding capacity, the antibody was immobilized by cross-linking through hydrazine cross-linked via EDC/NHS activation of the sulfide monolayer (MPA). This is important in reusability since directly crosslinked antibodies are not detached by dissociation buffer thus it is not needed to add antibodies whenever test a new sample.

In this study, the regeneration procedure was performed for 10 times on one sensor chip. Even though the signal was significantly reduced after one use, the signal was sustained at the level able to detect the presence of *E. tarda* in a sample over ten times reuses (Fig. 3). This means more than ten

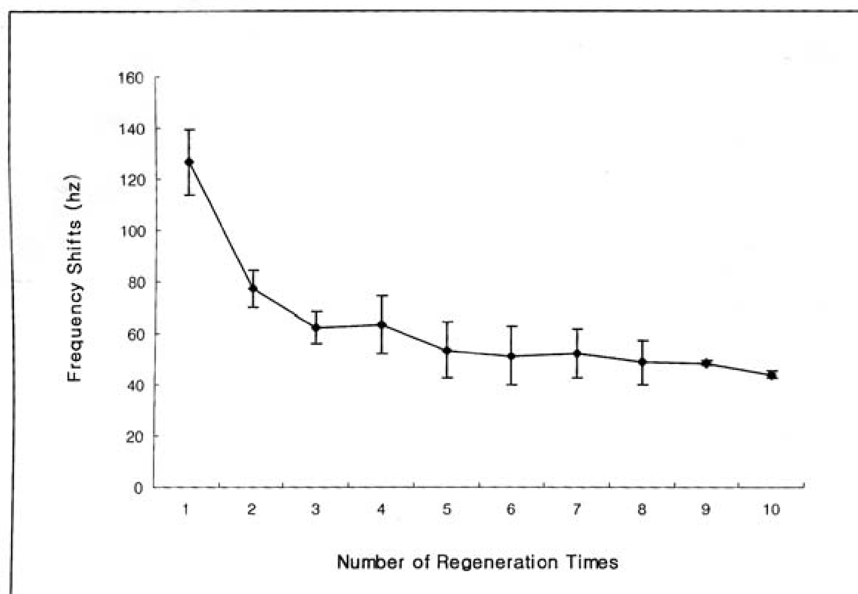

Fig.3. Regeneration experiment to test the reusability of the QCM biosensor. The reusability of the prepared sensor chip was test using a dissociation buffer containing 0.2M Tris-glycine and 1% DMSO, pH 2.3. One hundred $\mu\ell$ of PBS was added and read the frequency at the steady state. Bacterial cells were added onto the sensor chip at the doses of 500 μg/100 $\mu\ell$, incubated for 20 min, and washed out unbound cells by PBS for 4 times. One hundred $\mu\ell$ of PBS was added again and read the frequency at the steady state. One hundred $\mu\ell$ of dissociation buffer was added and incubated for 20 min, and washed out by PBS to removed detached bacterial cells. The same procedure was performed for 10 times on one sensor chip.

samples can be tested using only one chip.

In conclusion, it was demonstrated in this study that the prepared sensor chip was able to detect *E. tarda* cells in the dose dependent manner at detection limit of less than 50 ㎍ and reusable over ten times.

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