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Development of QCM biosensor to detect a marine derived pathogenic bacteria *Edwardsiella tarda* using a novel immobilisation method

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ABSTRACT

QCM technology offers a real time output, simplicity of use and cost effectiveness in addition to high sensitivity. Sensitivity of QCM immunosensor can be enhanced by improving the immobilisation procedure on the quartz surface. The immobilisation strategy should be able to control both the amount and the orientation of the antibody (immunoglobulin; IgG) on the transducer for high affinity to antigens. This study introduced a new methodology recruiting oxidised IgG to expose aldehyde group in Fc region to cross-link to hydrazide conformed on self assembled monolayer (SAM) and compared with three conventional methods. Consequently, it was proved that considerable amount of antibody was immobilised and the sensitivity of new methodology was higher than other methods while ability of new methodology to immobilise IgG was lower than the conventional methods. The frequency shifts following bacterial cell injection were positively related to the frequency shifts after the injection of IgG and the amounts of bacterial cells, revealing that the frequency shifts after bacterial cell injection fully represented the weight change by specific attachments of bacterial cells to the IgG cross-linked on the gold surface. Specificity was tested on different bacteria including *E. coli*, *V. vulnificus* and *A. hydrophila* and showed no significant non-specific affinity on the tested bacteria. It was also demonstrated that the prepared sensor chip was stable enough to withstand repeated surface regeneration. Indeed, polyclonal antibody was more effective to detect antigen than monoclonal antibody which binds to only one epitope of antigen. Conclusively, the new methodology is appeared to be more sensitive than conventional methods tested and reusable for 10 times.

1. Introduction

Piezoelectric devices have been proposed as probes 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). This technology offers a real time output, simplicity of use and cost effectiveness. The first use of antigens as coating in quartz crystal microbalances (QCM) was proposed by Shons et al. (1972). Since then, many reports have been published using piezoelectric sensors for a wide range of applications in the food industry, environmental monitoring, clinical diagnostics and biotechnology. Consequently, QCM has been developed as an extremely sensitive mass sensor, capable of measuring subnanogram levels (Clark et al., 1987) by improving the detection limits using crystals of higher frequencies (>10 MHz) (Bunde et al., 1998; Lin et al., 1993) and improving the immobilisation procedure on the quartz surface (Clark et al., 1987).

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). The strategy chosen to create the sensing layer should enable to control both the amount and the orientation of the antibody on the transducer while preserving its bioactivity (Briand et al., 2007). Thus many published papers showed an immobilisation technique based on *Staphylococcus aureus* protein A coating (Babacan et al., 2000; Galli Marxer et al., 2003; Grubor et al., 2004; Kaur et al., 2004; Michalzika et al., 2005).

However, even though there is the benefit of protein A coating for a proper orientation of the antibody (Lu et al., 1996), it is not convenient to detect a particular bacteria over several samples by regeneration of a sensor chip since antibody against the bacteria should be reimmobilised to protein A for every detection. It was also known that covalent coupling of antibody increased the stability against degradation during the regeneration process (Uttenthaler et al., 1998).

Concerning the orientation of antibody for the best antigen binding capacity and efficient regeneration procedures, in this

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study, a direct immobilising technique was introduced using oxidised IgG over the gold electrode of the quartz crystal. After oxidation to expose the aldehyde residues of the carbohydrate moiety in the Fc region, the IgG can be easily immobilised by cross-linking through hydrazine cross-linked to the self assembled monolayer (SAM).

This study applied this methodology to detect a marine derived bacterium *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, 1977; Amandi et al., 1982; Kubota et al., 1982). Currently, time consuming bacterial culture followed by biochemical profiling is the most popular method for identification of an unknown bacterium including *E. tarda*. However, several researchers are seeking for quick and sensitive method to identify various pathogenic bacteria. Yu et al. (2004) reported that high-performance capillary electrophoresis (HPCE) has been applied to the identification, separation, and quantification of intact bacteria in marine environment. 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.

This study investigated a new methodology recruiting oxidised IgG to cross-link to hydrazide conformed on NHS activated SAM via exposed aldehyde group in Fc region of IgG to increase affinity and stability and for the convenient regenerations. The new methodology was compared with conventional immobilisation methodologies which directly immobilise IgG by cross-linking carboxylic group or amino group at the ends and indirectly immobilise Fc region of IgG via protein G cross-linked to SAM. This study also aims to introduce QCM biosensor system to detect a fish pathogen for the first time.

2. Materials and methods

2.1. Preparation of formalin killed cells of E. tarda and its antibody

In this study, formalin killed cells (FKC) was used to prepare antiserum and to test sensor system instead of using live bacteria for a convenience of use and safety for researchers. *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 FKC, formalin was added at a final concentration of 1% and the mixture was incubated for 24 h at 4 °C. 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 polyclonal antiserum was taken when the titer reached to 1:25,600–51,200. 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. Monoclonal antibody against *E. tarda* was purchased from AbD Serotec.

2.2. Oxidation of IgG

The purified polyclonal antibody or monoclonal antibody 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 μl 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). Oxidised IgG was stored at −70 °C.

2.3. QCM biosensor system

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. It was mounted on a well holder made with acryl and connected to a home-made oscillator module. The analogue frequency signals from the oscillator were converted to the digital ones in a frequency counter (Daga electronics, Korea). The in situ signal was stored in personal computer and performed data analysis using Microsoft excel program.

2.4. Immobilisation of the antibody

Piezoelectric quartz crystal was cleaned using piranha solution (H_2SO_4:H_2O_2 = 7:3) at 60 °C for 5 min and rinsed with absolute ethanol and distilled water. Self assembled monolayer was conformed by treating the quartz crystal with 10 mM of MPA (3-mercaptopropionic acid, Sigma) overnight and then activated with 46 mM of EDC (*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide, Sigma)/NHS (*N*-hydroxysulfosuccin-imide, Sigma) for an hour to produce carboxyl chip. After activation of SAM, following steps was proceeded for each scheme.

(1) Immobilisation of N-terminal of IgG on carboxyl chip
One hundred microliter of IgG was added at the dose of 150 μg/ml, incubated for an hour to be cross-linked to the activated gold surface via its N-terminal amino group. Uncross-linked residues were blocked by 1 M ethanolamine-HCl (Sigma) and a blocking buffer containing 1 mM EDTA, 0.25% BSA, and 0.05% Tween-20. All reaction time was 1 h and sensor chip was cleaned using distilled water between the reactions.
(2) Immobilisation of C-terminal of IgG on hydrazine chip
Hydrazine chip was prepared by converting carboxyl group on SAM to hydrazide group by treating with 5 mM carbohydrazide before IgG immobilisation. Blocking and washing steps were same as above.
(3) Immobilisation of IgG on protein G chip
Protein G chip was prepared by cross-linking protein G on the activated gold surface at the concentration of 5 mg/ml and uncross-linked residues were blocked by above methods. IgG was added to the protein G chip and incubated for an hour.
(4) Immobilisation of oxidised IgG on hydrazine chip
Oxidised IgG in buffer A was added and immobilised on the hydrazine chip prepared by above methods. 0.1 M sodium cyanoborohydride in 0.1 M sodium acetate, pH 4 was added to strengthen the interaction between oxidised IgG and hydrazide. Blocking and washing steps were same as above.

2.5. Comparison of sensitivity

To test sensitivity of four immobilisation methods, 100 μl 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 FKC (1 mg/ml) in the reaction cell. After washing out unbound substances with PBS several times, 100 μl of PBS was added into the reaction cell and the steady-state reso-

nant frequency (F2) was read again to calculate the frequency shift (frequency shifts = F1 − F2).

2.6. Dose response

Serially diluted bacterial cells were added onto the sensor chip prepared by immobilising oxidised IgG on hydrazine chip at the doses of 1000, 500, 100, 50 μg/100 μl, incubated for 20 min, and washed out unbound cells by PBS for four times. One hundred microliter of PBS was added again and the frequency was read at the steady state. The steady state was obtained within 10 min in both cases before or after bacterial cells injection. The sensitivity of polyclonal and monoclonal antibodies was compared at the doses of 500 μg/100 μl. Oxidised IgG was immobilised at the concentration of 75 μg/ml for both polyclonal and monoclonal antibodies.

2.7. Specificity of the sensor chip

Specificity of the sensor chip was studied by testing the affinity to different bacteria, *E. coli*, *V. vulnificus*, and *A. hydrophila*. These bacterial cells were prepared as FKC by the method mentioned above and applied onto the sensor chip at the doses of 5 mg/ml.

2.8. Regeneration of sensor system

The reusability of the prepared sensor chip was tested using a dissociation buffer containing 0.2 M Tris–glycine, 0.6 M NaCl and 1% DMSO, pH 2.3. One hundred microliter of PBS was added and the frequency was read at the steady state. Bacterial cells were added onto the sensor chip at the doses of 5 mg/ml, incubated for 20 min, and unbound cells were washed out by PBS for four times. One hundred microliter of PBS was added again and the frequency was read at the steady state. For regeneration, 100 μl of dissociation buffer was added, incubated for 20 min, and washed out detached bacterial cells by PBS. The same procedure was performed for 10 times on one sensor.

2.9. Statistical analysis

One-way ANOVA was followed by Duncan's multiple range test to rank the groups, if significant ($P < 0.05$) differences were found using the SPSS program. All tests were performed triplicates.

3. Results

3.1. Effects of immobilisation methods on IgG immobilisation ability and sensitivity

Frequency shifts following IgG immobilisation represented that all tested immobilisation methods were efficient enough to immobilise considerable amount of IgG (Fig. 1). Frequency shift was highest following N-terminal of IgG immobilisation while C-terminal immobilisation showed 30% less frequency shift after IgG immobilisation revealing that immobilisation of N-terminal of IgG is more effective than immobilisation of C-terminal of IgG. Frequency shifts were less than above two but similar to each other after immobilising IgG on protein G chip and oxidised IgG immobilisation on hydrazine chip.

The effect of immobilisation methods on the sensitivity of a sensor chip was studied by analysing frequency shifts following bacterial cell injection. Unlike IgG immobilisation ability, the sensitivity was highest in the sensor chip made by immobilising oxidised IgG on hydrazine chip as frequency shift was 93.5 ± 2.4 Hz (mean ± S.E., $n = 3$). Frequency shifts in other sensor chips made by immobilising IgG on protein G chip, N- or C-terminal of IgG were

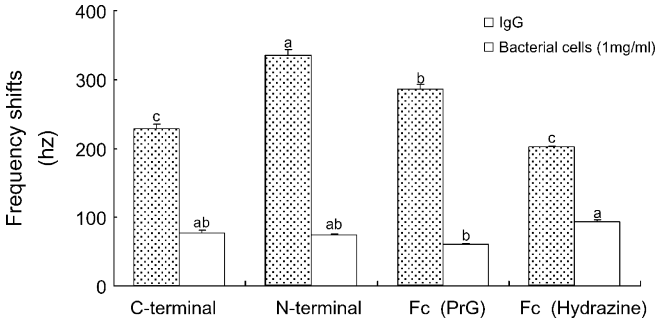

Fig. 1. Comparison of the efficiency and sensitivity of IgG immobilisation methods. The frequency change was measured after IgG immobilisation and addition of 1 mg/ml of *E. tarda* FKC. Values (mean ± S.E., $n = 3$) with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

60.6 ± 1.83, 74.5 ± 0.7, 76.33 ± 5.3 Hz (mean ± S.E., $n = 3$), respectively (Fig. 1).

3.2. Relationship between IgG immobilisation and sensitivity

Relationship between IgG immobilisation and sensitivity was examined by plotting frequency shifts following IgG immobilisation and frequency shifts following bacteria injection. The frequency shifts following the injection of IgG were highly related to the frequency shifts after bacterial cell injection at the dose of 1 mg/ml in a positive manner, $R^2 = 0.90$ (Fig. 2).

3.3. Effect of antibody property on sensitivity

In this study, most of the experiment was performed using polyclonal antibody. Monoclonal antibody was also oxidised and immobilised on hydrazine chip and the sensitivity was compared with polyclonal antibody. When the frequency shift was similar after IgG immobilisation, the frequency shift following bacteria injection was 1.7 times higher in polyclonal sensor chip (Fig. 3).

3.4. Dose responses

To test dose response of the sensor chip prepared by immobilising oxidised IgG on hydrazine chip, bacterial cells were injected at different concentrations ranged between 10 and 0.5 mg/ml.

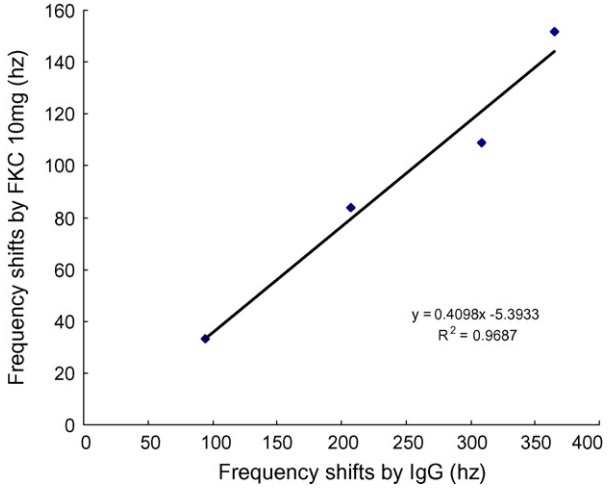

Fig. 2. Relationship between the efficiency of IgG cross-linking and the sensitivity of QCM biosensor. The frequency change was measured after IgG cross-linking and addition of 100 μg/100 μl of *E. tarda* FKC.

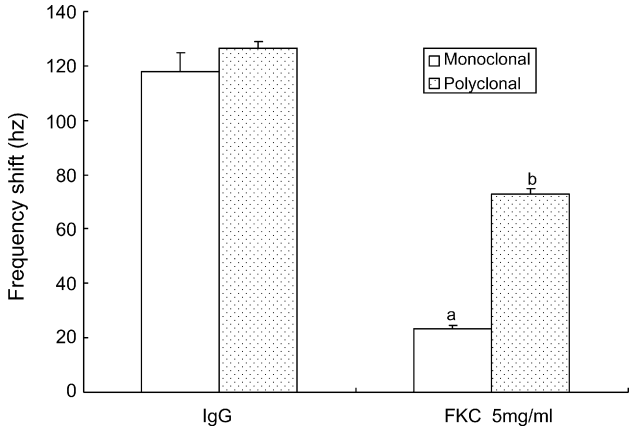

Fig. 3. Comparison of affinity of polyclonal and monoclonal antibodies. Both pf polyclonal and monoclonal antibodies were oxidised and immobilised on hydrasine chip. The frequency change was measured after IgG cross-linking and addition of 5 mg/ml of *E. tarda* FKC. Values (mean ± S.E., $n = 3$) with different letters are significantly different ($P < 0.05$) by *t*-test.

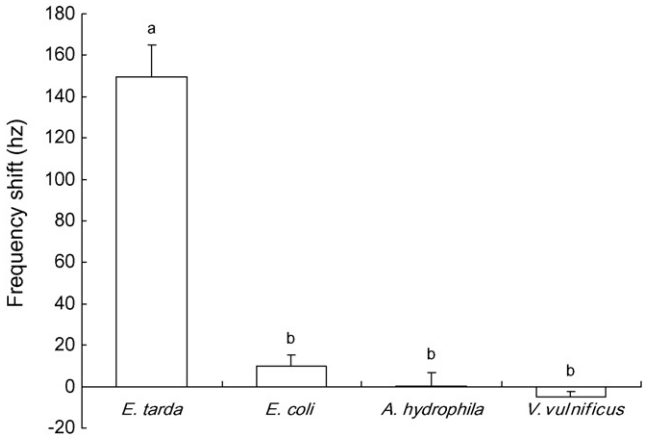

Fig. 5. Specificity test of the sensor chip. The specificity of the sensor chip was studied by testing the affinity to different bacteria i.e., *E. coli*, *V. vulnificus*, *A. hydrophila* at the doses of 5 mg/ml. Values (mean ± S.E., $n = 3$) with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Injection of bacterial cells at doses of 10, 5, 1, 0.5 mg/ml caused the frequency shift of 187 ± 7.75, 133 ± 1, 101.1 ± 1.17, 24.7 ± 7.5 Hz (mean ± S.E., $n = 3$), respectively (Fig. 4), when the injection of IgG caused the frequency shift of 288.8 ± 11.4. When the injection concentration of bacterial cells was reduced to 0.1 mg/ml, significant unstable signal was obtained. Thus the limitation of the QCM biosensor system made in this study seems to be 50 μg corresponding to 5×10^7 cells.

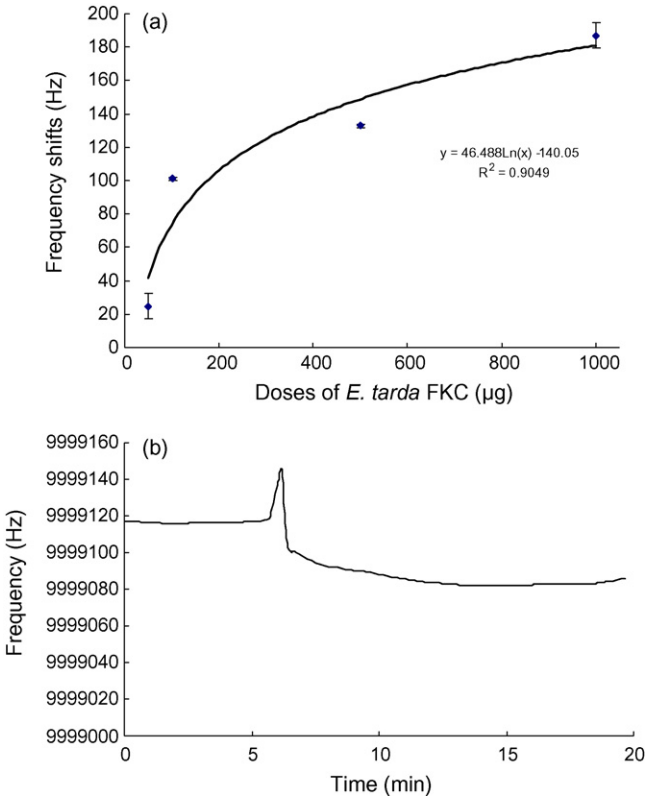

Fig. 4. (a) Dose responses of QCM biosensor for the detection of *E. tarda*. Serially diluted bacterial cells were added onto the sensor chip at the doses of 1000, 500, 100, 50 μg/100 μl. (b) A typical response curve is shown. One hundred microliter of PBS was added and read the frequency at the steady state and bacterial cells were incubated for 15 min.

3.5. Specificity of the sensor chip

Specificity of the sensor chip was studied by testing the affinity to different bacteria including an enterobacterium (*E. coli*) and other waterborne pathogenic bacteria (*V. vulnificus* and *A. hydrophila*) at the doses of 5 mg/ml. As a result, a significant affinity to those bacteria was not observed (Fig. 5).

3.6. Effect of immobilisation methods on regeneration

Regeneration was repeated for 10 times on one sensor chip. Sensor chips made by immobilising oxidised IgG on hydrazine chip appeared to be stable enough to withstand 10 times regeneration and to be more effective than sensor chips prepared by immobilising N- or C-terminal IgG since they showed higher sensitivity over 10 times regeneration (Fig. 6). Even though the signal was reduced after one use, the signal was sustained at the level able to detect the presence of *E. tarda* in a sample over 10 times reuses. It was also shown that regeneration efficiency was reduced by removing salt in the dissociation buffer (Fig. 6).

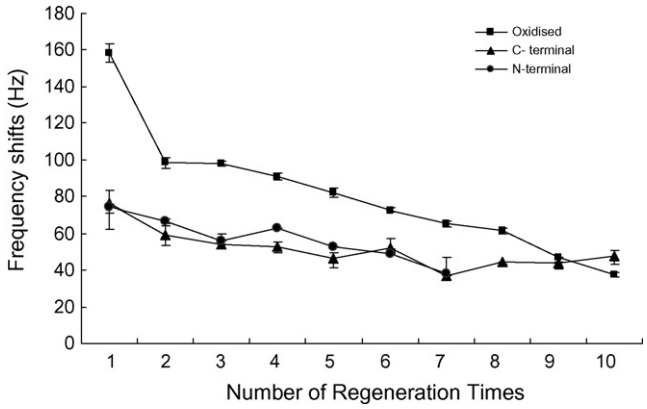

Fig. 6. Regeneration efficiency test of the sensor chip. The reusability of the prepared sensor chip was test using a dissociation buffer containing 0.2 M Tris–glycine, 1% DMSO and 0.6 M NaCl, pH 2.3. The regeneration procedure was performed for 10 times on one sensor chip using 5 mg/ml of bacterial cells. Frequency shifts were observed at each generation in oxidised IgG (square), C-terminal (triangle) and N-terminal (circle) immobilisation methods.

4. Discussion

4.1. Effects of immobilisation methods on IgG immobilisation ability and sensitivity

Many published papers showed that immobilisation techniques based on direct adsorption or on protein A coating, resulted in appropriate sensor signals, but only cross-linker 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 (Uttenthaler et al., 1998).

This study introduced a direct immobilising methodology accompanying right direction of IgG without using protein A for a convenient regeneration of sensor chip by recruiting oxidised IgG which expose aldehyde group in carbohydrate moiety in Fc region located at opposite side of antigen binding site. Aldehyde residues in the Fc region of oxidised IgG can be cross-linked to hydrazide conformed on NHS activated SAM. This immobilisation method was compared to other methods which immobilise IgG by directly cross-linking its N- or C-terminal or by binding its Fc region on protein G cross-linked to sensor chip. All tested immobilisation strategies were found to be efficient enough to immobilise considerable amount of antibody.

However, it was suggested that the more IgG on the sensor chip did not bring the higher sensitivity since the sensitivity was higher in the sensor chip prepared by immobilising oxidised IgG which showed lower IgG immobilisation. This result represents that the orientation of IgG may affect the sensitivity of sensor chip since oxidised IgG can expose the antigen binding site while IgG cross-linked to carboxyl chip hides the antigen binding side, causing a difference in sensitivity.

Nevertheless, in this study, the sensor chip prepared by immobilising IgG on protein G chip showed lowest sensitivity even lower than two other conventional methods, even though the orientation of IgG should be right direction since protein G specifically binds to Fc region of IgG. This could be due to the reason that protein G can bind IgG via constant region of Fab as well as Fc region in IgG with a 10 fold less affinity than determined for the Fc region (Sjobring et al., 1991). Since constant region of Fab is close to antigen binding site which exists in variant region, in the case of immobilisation of IgG on protein G via constant region of Fab, antigen binding site can be hided, causing less sensitivity. It could be also suggested that IgG immobilised on protein G chip does not stand up like the IgG directly immobilised on sensor chip thus all of them are not exposing their antigen biding site in upside.

4.2. Relationship between IgG immobilisation and sensitivity, and effect of antibody property on sensitivity

It was demonstrated in this study that the frequency shifts following the injection of IgG are positively related to the frequency shifts after bacterial cell injection, 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 immobilisation efficiency of IgG is an important factor affecting the sensitivity of sensor chips. It was also revealed that polyclonal antibody was more effective to detect antigen than monoclonal antibodies which bind to only one epitope of antigen. Assumingly, using a couple of monoclonal antibodies to various epitopes may help to increase the affinity of sensor chips, causing higher sensitivity.

4.3. Dose responses

In this study, frequency shift was changed by various doses of bacterial cells in dose dependant manner at the range of 10–0.5 mg/ml. Since decrease of bacterial cell dose down to 0.1 mg/ml caused significantly unstable signal (data not shown), the limitation of the QCM biosensor system made in this study seems to be 50 μg. This amount is corresponding to 5×10^7 cells. This sensitivity is lower than the other report by Yu et al. (2004). Yu et al. demonstrated that *E. tarda* was identified within 10 min after direct injection into fish fluid by CE blue light-emitting diode (LED)-induced fluorescence using SYTO 13 (488/509 nm), a cell-permeable green nucleic acid stain, to stain the cells and the limit of detection was found to be 4.2×10^4 cells/ml. However, HPCE method does not provide advantages of simple use and low price like QCM sensor.

Moreover, our result is in agreement with other reports which have illustrated the theoretical basis of piezoelectricity and the practical application of PZ sensors for the determination of various kinds of microorganisms (Ivnitski et al., 1998). According to the previous studies dealing with QCM immunosensors detecting microorganisms, the detection ranges are varied in 10^6–10^9 cells/ml for various microorganisms i.e., *C. albicans* (Muramatsu et al., 1986), *S. epidermidis* (Bao et al., 1996), *S. typhimurium* (Prusak-Sochaczewski et al., 1990; Ye et al., 1997), *C. trachomatis*, *E. coli*, *Y. pestis*, *S. dysenteriae* (Koenig and Gratzel, 1993a,b; Plomer et al., 1992). Unfortunately, there has been no report about PZ sensor detecting marine pathogenic organisms until present.

4.4. Specificity of the sensor chip

Specificity of the sensor chip is a crucial factor in developing a microorganism detection tools. Hence, it was studied by testing the affinity to different bacteria including an enterobacterium (*E. coli*) and other fish pathogenic bacteria (*V. vulnificus* and *A. hydrophila*) at the doses of 5 mg/ml. As a result, a significantly high affinity to those bacteria was not observed. Relatively higher affinity to *E. coli* than other two bacteria might be due to two reasons: the phylogenetic relevance between *E. tarda* and *E. coli* caused low level of antigenic similarity as both of them belong to the same genus 'enterobacteria' and the dose (5 mg/ml) of tested bacterial cells was too high compared to natural amount in fish organ samples except intestines. Affinity to *E. coli* can be significantly reduced or may not be detected, if the dose of tested bacterial cells was reduced to less than 5 mg/ml.

4.5. Effect of immobilisation methods on regeneration

In this study, the regeneration procedure was performed for 10 times on one sensor chip and found reduction of signals. Even though the signal was reduced during regeneration, it was sustained at the level able to detect the presence of *E. tarda* in a sample over 10 times reuses. Reduction of signal during regeneration might be resulted in the harsh condition of the dissociation buffer containing 0.2 M Tris–glycine and 1% DMSO, pH 2.3 since low pH of dissociation buffer can change the property of amino acid in the antigen binding site thus reducing the antigen binding ability of IgG on the sensor chip.

The regeneration efficiency of oxidised IgG sensor chip was compared with unoxidised IgG through N- or C-terminal and a higher signal reduction in oxidised IgG sensor chip was observed after the first regeneration. Such a reduction of signal was not observed in two other sensor chips. It can be suggested that the reduced sensitivity after the first regeneration in the sensor chip prepared

by oxidised IgG can be due to the affinity reduction by oxidation damage of IgG. According to Uesugi et al. (2000), oxidation of the affinity-purified rabbit Ab by exposing to hypochlorous acid (HOCl) or peroxynitrile (ONOO) resulted in a concentration-dependent loss of binding capacity. In spite of the reduced signal after the first regeneration, the sensitivity of oxidised IgG sensor chip was sustained at higher or similar level to unoxidised IgG sensor chips over 10 times reuses.

5. Conclusions

In conclusion, it was demonstrated that the prepared sensor chip was able to detect *E. tarda* cells in dose dependent manner at detection limit of less than 50 μg and the new methodology was appeared to be more sensitive than other tested methods. The prepared sensor chip was reusable over 10 times but sensitivity was reduced after the first use even though still higher sensitivity than other chips was sustained. Some experiments can be carried out to produce more stable sensor chips or to improve the regeneration performance in the future.

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