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Application of a flow type quartz crystal microbalance immunosensor for real time determination of cattle bovine ephemeral fever virus in liquid

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Abstract

Bovine ephemeral fever (BEF) is a viral disease of cattle. A flow type quartz crystal microbalance (QCM) immunosensor was developed for the real time determination BEF virus (BEFV) that is suitable for clinical point-case diagnosis. Self-assembled monolayer (SAM) of thiols and sulphides by the cystamine–glutaraldehyde method was used for the immobilization of BEFV monoclonal antibody on the gold surface of a quartz crystal microbalance (QCM). A positive correlation was found between the virus concentration and frequency changes ($R^2 = 0.9962$) on this QCM system. The reproducible rates for the 50 and 10 μg/mL samples were 4 and 13.9%, respectively. There was no interference from non-specifically adsorbed phage. Using this flow type QCM immunosensor, BEFV could specifically be detected with sensitivity comparable to a conventional enzyme-linked immunosorbent assay (ELISA). The measurement could be obtained directly, within several minutes, rather than hours as required visualizing the results of ELISA. In addition, the observation of reproducible and constant changes after successive additions of BEFV suggests that a QCM immunosensor in a flow cell could be developed for automated or continuous real time operation.

Keywords: Immunosensors; Bovine ephemeral fever; Quartz crystal microbalance; Biosensor; Virus detection

1. Introduction

Bovine ephemeral fever is a viral disease of cattle and water buffaloes which also has sub clinical involvement in a variety of ruminant species. The disease has major economic significance as it causes great economic losses due to decreased production in dairy herds, reduction in the condition of prime animals, disruption of stock movement, and disruption of markets [1]. Ephemeral fever caused by BEF virus, a single stranded negative sense RNA virus that is a member of the Rhabdovirus family [2]. Ephemeral fever is usually diagnosed based on history and clinical signs. Diagnosis can be made based on the sudden onset of febrile reactions lasting for 2–5 days with spontaneous recovery. Seasonal occurrence and symptoms of oropharyngeal secretions, joint pains and stiffness are characteristic manifestations of the disease. However, a confirmatory diagnosis can be obtained by isolation of BEFV from blood collected in heparin or EDTA anticoagulant during fever or by demonstration of a rising titer of neutralizing and complement fixing antibodies in paired sera collected during illness and 2 or 3 weeks later [3,4]. This study used a biochemical method based on enzyme-linked immunosorbent assay (ELISA) for BEFV antibodies detection [5]. Generally, two blood samples were tested for each object one collected during the initial stage of the illness, and the other collected 3 weeks later. If BEFV is responsible for

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the illness, BEFV antibody levels will be much higher in the second test than in the first. This method has several advantages, including high selectivity and lower detection limit, but the procedure is somewhat complicated and requires several hours for a single measurement. Besides, this method cannot distinguish between naturally inspected titre and vaccination titre.

The BEFV is present in nasal discharge and/or respiratory secretions in infected cattle. Possibly, a diagnosis of BEF can be made by detecting BEFV in cattle sera or saliva. However, to the knowledge of the authors, no previous studies have reported the threshold of virus concentration required to cause clinical symptoms. To avoid the spread of BEFV infection, a rapid and real time screening method was required for detecting BEFV in cattle. A method using a biosensor is a promising strategy for dealing with problems such as those associated with conventional ELISA methods. An assay system which uses biosensor can achieve a selective and sensitive detection because the bio-molecules used in the biosensor have a specific and strong affinity to the target molecule [6–10]. A quartz crystal microbalance (QCM) system [10,11] has been shown to be an effective transducer to construct an immunobiosensor, and is highly sensitive and suitable use in real time and automated monitoring [12–15]. Such a piezoelectric device has the advantages of very small size, minimal electrical and electronic circuitry requirements, adaptability to micro-fluidic techniques, inexpensive fabrication, utility in a flow cell, and sequential refinement of positive signals [12,13]. The sensing part of the QCM consists of a thin quartz disk with electrodes plated on it [11]. The application of an external electrical potential to a piezoelectric material produces internal mechanical stress. A resonant oscillation is achieved by including the crystal in an oscillation circuit which has electric and the mechanical oscillations near to the fundamental frequency of the crystal. The fundamental frequency depends on the thickness of the wafer, its chemical structure, shape and mass [16]. Several factors can influence the oscillation frequency, including the physical properties of the adjacent media [11] (density or viscosity of air or liquid). As shown by Sauerbrey (1959), changes in the resonant frequency are simply related to the mass accumulated on the crystal [17]. Thus for detection in air the frequency change is simply related to the change in mass:

$$\Delta F = -2.3 \times 10^6 F_0^2 \frac{\Delta M}{A}$$

where ΔF is the measured frequency shift (Hz), F_0 the resonance frequency of the crystal, ΔM the change of the mass deposited and A the area coated.

As the analyte binds to the coated surface, there is an increase in mass at the interface, which will decrease the oscillation frequency. The observed frequency change is directly proportional to the increase in mass and can, therefore, be correlated with the amount of analyte binding.

The first QCM immunosensor was developed by Shons et al. for the detection of BSA antibodies [18]. The crystal was precoated with nyebar C and BSA using the 'dip-and dry' method. Although the adsorption of antibodies is a widely used method for the detection of various analytes [19–21], including HIV antibodies [15,22], it is not particularly reliable due to the complexity of sample composition, handling, and lack of automated, continuous operation.

On the other hand, a system using a QCM immunosensor in a flow cell might be developed for automated or continuous operation. The relationship between the oscillation frequency change of a quartz resonator in contact with liquid and accumulated mass had first realized by Kanazawa and Gordon (1985) who derived the following equation [23]:

$$\Delta F = -F_0^{3/2} \left(\frac{\rho_1 \eta_1}{\pi \rho_q \mu_q} \right)^{1/2}$$

where F_0 is the resonance frequency of the crystal, ρ_1 and η_1 the absolute viscosity and density of the solution, ρ_q and μ_q the density and shear modulus of the quartz crystal, respectively. The details of the characteristics of the quartz sensor in the liquid phase were reviewed by Thompson et al. [24]. The resonance frequency is influenced by viscosity, density, conductivity, specific gravity, surface roughness, surface stress, etc. [25–30]. This observation opened up possibilities for applying the QCM immunosensor for developing a new type of signal for biosensor design [24].

In this study, a flow type quartz crystal microbalance immunosensor for real time determination of BEFV was developed. Self-assembled monolayer (SAM) of cystamine and glutaraldehyde were used for the immobilization of antibodies to the gold surface of quartz crystals [16,31–35]. The thiol–gold interaction has a higher binding energy than an electrostatic interaction [36–38] as measured by X-ray photoelectron spectroscopy (XPS). It is, therefore, a reasonably stable surface treatment. SAMs of antibodies have been achieved using antibody–thiol complexes [39]. A frequency decrease was detected as the immuno-reaction between the BEFV and its antibodies. In this paper, we show that the QCM measurement is useful for the determination of the BEFV.

2. Experimental

2.1. Reagents and materials

Bovine serum albumin (BSA), glutaraldehyde and glycine were purchased from Sigma Chemical Co. (St. Louis, USA). The anti-BEFV monoclonal antibodies (IgG1, K), BEFV and rabies virus were provided by Mr. S.S. Liu of the Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan. The BEFV was isolated from the blood of a cow suffering ephemeral fever. The virus has 7 passages in the brain of suckling mice, 34 passages in baby hamster kidney-21 (BHK21) cell and the plaque was purified in Vero cells to produce a high titre seed stock. The seed stock of the virus was stored in liquid nitro-

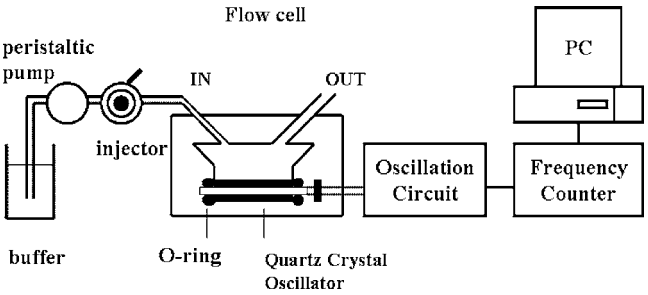

Fig. 1. Schematic illustration of the QCM setup. Films are prepared on the gold electrode (4 mm in diameter).

gen vapour. The virus suspension was purified by a number of centrifugation steps where the virus was clarified, pelleted and equilibrated on a 15–60% (w/w) sucrose gradient. The procedure of BEFV monoclonal antibody preparation and characterization was by the method described by Cybinski et al. [40]. The bovine sera from 10 cattle were used to evaluate the QCM immunosensor. Those cattle were from herds in Pingtung, Taiwan, an area known to be free of BEF. The running buffer for assay is 100 mM phosphate buffer saline (PBS). 100 mM phosphate buffer were prepared by mixing stock standard solution of K_2HPO_4 and KH_2PO_4 and adjust the pH with NaOH. The common chemicals used for preparation of buffers, etc., were of analytical reagent grade. All the solutions were prepared with deionized distilled (DD) water.

2.2. Apparatus

A schematic illustration of the QCM setup is depicted in Fig. 1. The QCM measurement system (ANT P2000, Taipei, Taiwan) is constructed by attaching the flow cell in which temperature is controlled by circulating water. The quartz crystal oscillator employed in this study was 9 MHz AT-cut quartz, with Au electrodes deposited on both sides (4 mm in diameter). The FIA system consists of a fluid circuit with a peristaltic pump, an injection valve and a plug-in mount for the sensor chips (i.e. QCM mounted on the Teflon carrier). The electronic components comprise an oscillator circuit integrated in the lid of the plug-in mount, a frequency counter, an A/D converter, a personal computer and electronic controls for the pump and the injection valve. The output frequency was continually monitored by a HP 53181A universal counter (Boise, ID, USA) and finally transferred to a computer using a GPIB interface. Frequency output was programmed using the LabVIEW 6.1 software package (National Instruments, Austin, TX, USA). Before measurement, the crystals were cleaned by immersion in 1.2 M NaOH for 10 min, and 1.2 M HCl for 5 min. After each step, the crystal was thoroughly washed with distilled water. The freshly cleaned QCM crystals were soaked overnight in 25 mM cystamine. The derivative QCM was washed with distilled water to remove soluble unreacted reagents.

2.3. ELISA

The purified anti-BEFV monoclonal antibody was diluted to 200 μg/mL in 100 mM sodium carbonate buffer pH 9.6 and then 100 μL solution were added to each well of a polystyrene microtitration plate overnight at 4 °C [5]. After decanting antibody solution, unbound sites were blocking with 1% gelatin in PBS (pH 7.0) containing 0.05% Tween 20 at room temperature for 2 h. The wells were then incubated with various concentrations of horseradish peroxidase label goat anti-mouse IgG antibodies. All incubation steps were done at 37 °C for 1 h and the microtitration plates were washed with PBS containing 0.05% Tween 20 three times when proceeded from one incubation steps to another. The specific binding was evaluated by reacting with chromogenic peroxides substrate (Cappel ICN Pharmaceuticals, Inc., Ohio, USA) and the absorption was measured by ELISA reader (Dynateck, MR 5000) at 492 nm.

2.4. Immobilization of antibodies on the gold electrode

A peristaltic pump combined with a pulse-dampening module developed specifically for this system guaranteed a continuous flow of buffer liquid with small fluctuations and a flow rate of 70 μL/min through the flow system. Unless otherwise stated, the cystamine modified-coated gold surface was upside and in contact with the flowing solution. The regent and sample were injected into the flow system. An injection valve with a 100 μL sample loop was employed for injection of the samples. All experiments were carried out at 35 °C. Vacuum degassed PBS (pH 7.0) was pumped over the crystal surface until baseline with noise below 0.2 Hz was achieved.

For each experiment a single injection of 20 mM glutaraldehyde was introduced into the flow stream and allowed to interact with the cystamine modified-coated gold surface. Then the sensor chip was incubated with anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine. Unless otherwise state, 200 μg/mL anti-BEFV antibodies were used.

2.5. BEFV measurements in test samples

After the immobilization of antibodies and blocking, various concentrations of BEFV solution were injected into the test flow stream using a syringe when a steady-state baseline frequency of the testing system had been obtained. The response frequency was displayed and simultaneously logged by the computer until a steady-state was achieved. The difference between the baseline and the steady-state frequency was used to calculate the concentration of BEFV. Calibration curves to determine sensor sensitivity were obtained by measuring the BEFV concentration from 1 to 300 μg/mL. All the solutions used in the BEFV measurements were prepared with 100 mM sodium phosphate buffer (pH 7.0) solution.

3. Results and discussion

3.1. Typical binding curve registered with the flow type QCM immumosensor

Self-assembled monolayer on gold surfaces have become an increasingly important method for molecular immobilization [36,37] The S–Au association is quite stable, and can be used as a first layer in the development of a self-assembled tether for derivatization of the QCM surface. We chose to pursue an SAM having a thiol-terminated linker which attaches it to the gold surface and a derivatizable terminus for the attachment of antibody. The cystamine–glutaraldehyde method was used in the following experiment (Fig. 2). The reaction of glutaraldehyde with a primary amino group to form a Schiff base bond has been investigated and applied to covalently binding compounds and antibody-containing amino groups. As reported elsewhere, the gold electrode surface was functionalized with primary amino groups by chemisorptions of cystamine [41]. The amino tails of SAMs and the anti-BEFV antibody reacted with the aldehyde groups of bifunctional reagent glutaraldehyde. The electrode modification process is illustrated in Fig. 2.

After installation in the FIA system, the cystamine coated sensor surface was then concurrently reacted with 20 mM glutaraldehyde, 200 μg/mL anti-BEFV antibodies, 100 mM glycine and various concentrations of BEF virus in order. Fig. 3 shows a typical binding curve registered with the

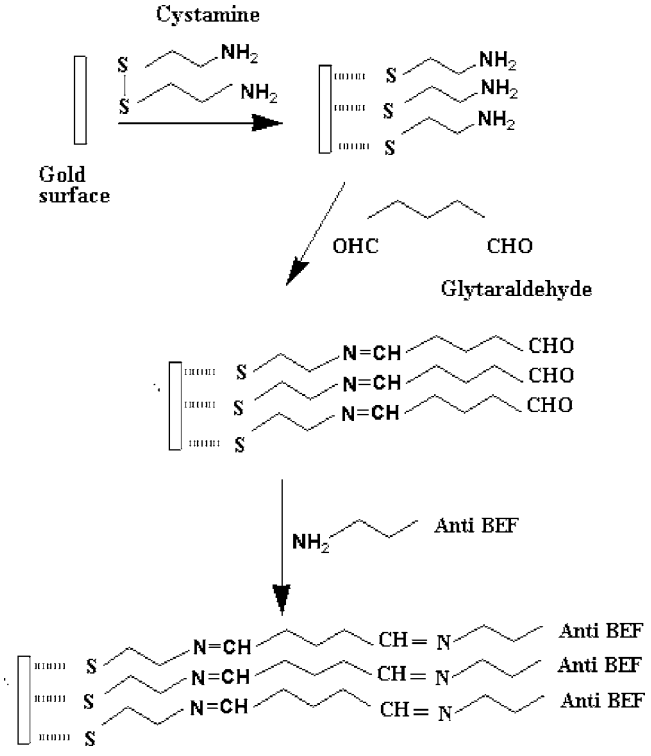

Fig. 2. The chemical steps of BEFV antibody immobilization by the cystamine–glutaraldehyde method.

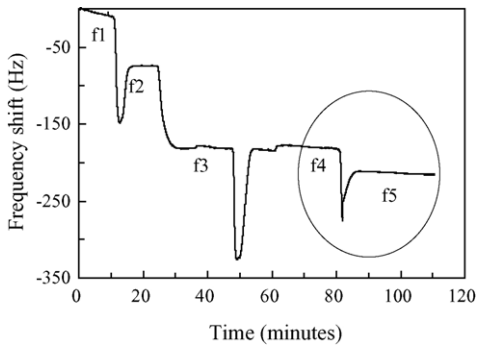

Fig. 3. Example of a binding curve: (1) coated surface in equilibrium with phosphate buffer; (2) the frequency decrease indicates a Schiff base binding between the glutaraldehyde and cystamine; (3) the frequency decrease indicates the binding of glutaraldehyde and the antibody; (4) no frequency shift after the blocking process; (5) the frequency decrease indicates binding between the BEFV and anti-BEFV immobilized on the electrode surface. At equilibrium, the frequency reaches a stable value.

QCM. The frequency shifts were calculated as the difference between two stable frequency values, that is to say $f_2 - f_1$, $f_3 - f_2$, $f_4 - f_3$ and $f_5 - f_2$. The first decrease in frequency ($f_2 - f_1$) at 10 min is due to the S–Au association of cystamine and glutaraldehyde. The first large drop in frequency, at 25 min ($f_3 - f_2$), is due to the binding of the glutaraldehyde and anti-BEFV antibody. The un-reactive area of the glutaraldehyde was blocked by the addition of glycine followed by the addition of anti-BEFV antibody, resulting in no significant change in frequency at 45 min ($f_4 - f_3$). The frequency decrease at 80 min ($f_5 - f_4$) was due to the binding between the BEFV and anti-BEFV antibody immobilized on the electrode surface. At equilibrium the frequency reaches a stable value. Two commonly used methods exist for generating surface presenting ligands for use in immunosensor studies [42]: (1) commercially available carboxymethyl dextran-coated substrates (CM5 chips) with protein, and (2) SAM techniques used on gold surfaces. The dextran-coated substrates are convenient to use because a variety of chemical methods for immobilizing proteins to dextran have been developed [33]. Whereas, the dextran-coated substrates are disadvantages, such as those associated with non-specific binding, the exclusion of large proteins from the interior of the gel and high cost. SAMs made from thiols presenting ligands are useful for immunosensor studies. SAMs are structurally well characterized on a molecular scale, and can be tailored to be highly resistant to non-specific adsorption. Furthermore, they lack the ambiguities associated with the interfacial partitioning of large proteins because the binding occurs at the surface of the SAMs.

3.2. Determination of BEFV concentration and the regeneration of the coated crystal

Optimal conditions for antibody immobilization were investigated after varying the antibody concentration. A QCM coated with cystamine and glutaraldehyde was tested with

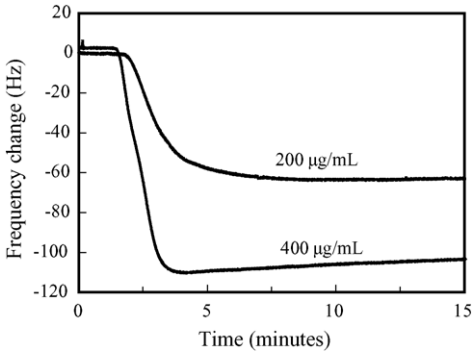

Fig. 4. Typical time course of frequency change of QCM in contact with various concentrations of anti-BEFV antibody.

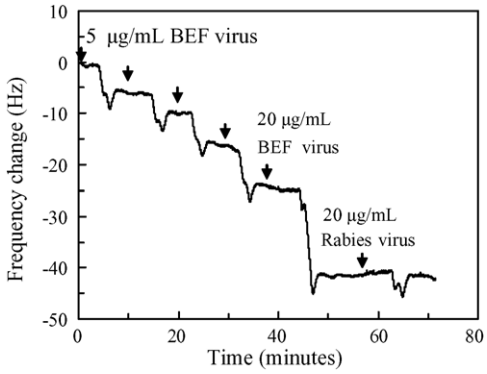

Fig. 5. Measurement curve for the QCM immunosensor. BEFV in a concentration of 5 and 20 μg/mL was injected four times and one time, respectively, on the quartz crystal microbalance. The sensor chip was incubated with 200 μg/mL anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine.

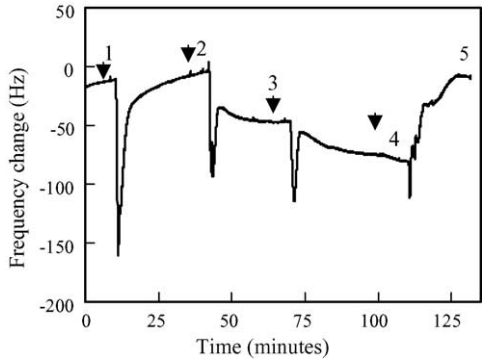

Fig. 6. Binding and regeneration curve of the BEFV immunosensor: (1) the frequency decreased but rapidly returned to the initial baseline when 60 μg/mL BSA was injected; (2) 41 Hz residual frequency change observed after the first injection of the 60 μg/mL BEFV; (3) only 25 Hz of frequency change occurred after the second injection of the same concentration of BEF virus. The less residual frequency changes indicated that the amount of BEFV exceeds the linear detection range of the sensor for two consecutive injections of 60 μg/mL BEFV sample; (4) the surface was treated with a solution of 80 mM glycine HCl to break the antibody–antigen bond and regenerate the surface in order to perform a new experiment; (5) successful regeneration by glycine HCl brings the frequency back to its original frequency. The sensor chip was incubated with 200 μg/mL anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine.

200, 400 and 500 μg/mL anti-BEFV antibody resulting in frequency differences of 60, 108 and 111 Hz, respectively (Fig. 4). After the adding of 200 μg/mL anti-BEFV antibody, the frequency decreases with time due to the Schiff base binding of glutaraldehyde and anti-BEFV antibody. A stable value was reach at the seventh minute. However, when 400 μg/mL of anti-BEFV antibody was added, the frequency decrease is seen to be more rapid and trends to saturate quickly, indicating most antibody molecules have bound to the glutaraldehyde. The frequency increased slowly 4 min after the injection of anti-BEFV antibody and could not reach a stable frequency. It was possible that some non-binding antibody mass also contributes to some frequency shift in the solution, but is removed during the rinse. The slow frequency increase was due to the dissociation of some non-binding anti-BEF antibody. When the electrodes were treated with 400 μg/mL anti-BEFV antibody, the electrode can bind with anti-BEFV antibody to 97% of complete binding. The frequency differences were 106, 107, 108 and 110 Hz for four injections of 400 μg/mL anti-BEFV antibody to the cystamine and glutaraldehyde modified electrode. Good reproducibility (CV = 1.6%) was achieved. Although the electrode could couple more antibodies when 400 μg/mL anti-BEFV antibodies were used, such antibodies have disadvantage associated with overloading the crystal which decrease the detection range of the sensor (data not shown). Therefore, crystals coated with 200 μg/mL of antibody solution were used for subsequent studies.

After blocking, different dilutions of the analyte were injected onto the sensors. Fig. 5 shows the measurement signal for four consecutive injections of BEF virus 5 μg/mL and one injection of BEF virus 20 μg/mL onto the sensor. In this case the mass deposition effect was slightly over-lapped by the viscosity effect of the sample. Therefore, the frequency change is determined after the temporary viscosity peak and, because of this, the frequency change only corresponded to the permanent frequency change due to analyte adsorption. To confirm that the interaction between the virus and the surface was specific, a control experiment has performed by injecting rabies virus on the sensor. No significant response was observed (Fig. 5) after injection of 20 μg/mL rabies virus, confirming that virus binding was mediated by interactions between the anti-BEFV antibody and the BEFV. Fig. 6 shows the measurement signal of the immunosensor after blocking with glycine. The sensor surface was then concurrently reacted with 60 μg/mL BSA, two consecutive injections of BEF virus 60 μg/mL, and 80 mM glycine/HCl (pH 2.8) in order. With BSA reaction, the frequency slowly returned to the initial baseline value compared to the frequency change observed in the first injection of 60 μg/mL BEFV leading to the 41 Hz residual frequency changes described. The lack of response to BSA shows that the responses observed were not due to non-specific binding. Only a 25 Hz frequency change was found after the second injection of the same concentra-

tion of BEFV. The less residual frequency changes indicated that the amount of BEFV exceeds the linear detection range of the sensor for two consecutive injections of 60 μg/mL BEFV sample. A further study on the linear detection range of the sensor is presented below. For repeat use of the sensor, the bound analyte should be dissociated from the antibody-coated sensor. We tested 8 M urea, 80, 100, 500 and 1000 mM of glycine/HCl as dissociating agents. The concentration of 80 mM glycine/HCl (pH 2.8) was found to be the best for use as a dissociating agent (data not shown), as it restored the frequency to 80% of its original value. In literature, glycine/HCl was demonstrated to be an effective dissociation agent for anti-BSA and human TNF-α monoclonal antibody [33,43], but fails in human IgG antibody [33]. In the present experiments only 20% of the original response could be restored following three assays. These results indicate the infeasibility of the system used in these experiments because of the denaturing of BEFV antibody [44]. Consequently, the detection was started with the cystamine modified electrode rather than with the regenerated sensing phase in the following experiments. Further work is required to create a system which can remove previously bound antibodies for continuous monitoring. A system using capture agent [45] or stabilizers [44] to immobilize antibodies on QCM immunosensors would be useful for reducing the BEFV antibody denaturing.

3.3. Calibration curve and the reproducibility of the BEF immunosensor

If the measurement was performed immediately following the blocking process, a frequency change of 7 Hz was observed for 5 μg/mL of BEFV (Fig. 5). However, the frequency change reduced to 3 Hz when the measurement was performed at 1 week after the blocking process. It indicated that the electrodes presented here demonstrate a degradation of reactivity within the first week. This performance agreed with the findings of Ben-Dov et al. and Park et al. [33,41,42,44,46] in similar experiments. The explanation is as follows: the stability of cystamine/glutaraldehyde/anti-BEFV antibody is controlled not only by the stability of the Schiff base bond (–C=N–), but also by the stability of the SAM itself. The roughness of the electrodes [47] and the purity of glutaraldehyde [48] may influence the SAM stability. Although the electrodes discussed here exhibit a rapid degradation during the first week, they reveal slowly reducing activity in next 4 weeks. This result agreed with the findings of Park et al. in similar experiments [44]. To improve the regression and reproducibility results, in the following experiments, BEFV detection was performed 1 week later following the blocking process.

Response curves obtained by exposing the sensor to buffer solutions containing different concentrations of BEF virus were characterized by fast reaction and very low non-specific binding after exposure to rabies virus. In Fig. 7A, line 1, the frequency changes are plotted as a function of BEF virus concentration from 1 to 300 μg/mL. The interaction of the sensor with BEF virus is specific because the sensor does not respond to the rabies virus (Fig. 7A, line 2). The calibration curves for the BEF sensor are depicted in Fig. 7B. The dose response is linear over BEF virus concentrations ranging from 1 to 100 μg/mL ($R^2 = 0.9962$), but begins to plateau at concentration greater than 100 μg/mL with the increase in concentration (Fig. 7A, line 1). We obtained a detection limit of 5 μg/mL (frequency change = 3 Hz; S/N > 3) for the determination of BEFV.

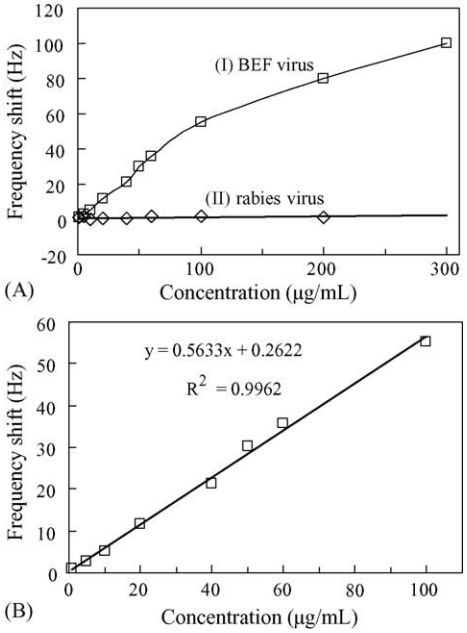

Fig. 7. (A) Specificity of the BEFV sensor. Line 1 represents the frequency change as a function of BEFV concentrations from 1 to 300 μg/mL (□). Line II shows the dose responses of the sensor exposed to rabies virus. (B) Calibration graph of the BEFV sensor. The sensor chip was incubated with 200 μg/mL anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine.

Table 1 shows the steady-state frequency shifts measured after repeated affinity adsorption of BEFV on to the crystal. The coefficient of variability (CV%) values of inter-assay with 10, 20 and 50 μg/mL virus concentration ($n = 5$) were 13.9, 8.3 and 4.0%, respectively, whereas those of 20 and 50 μg/mL were low enough to show good reproducibility. These results indicate that the proposed BEFV immunosensor could provide reproducible determination of BEFV.

Table 1
Frequency shift and reproducibility of the BEF virus immunosensor

Concentration (μg/mL)	Frequency shift (Hz) (mean[a] ± S.D.)	CV[b] (%)
10	4.72 ± 0.66	13.9
20	9.34 ± 0.78	8.3
50	31.2 ± 1.25	4.0

The data represent the average valves of five measurements.
[a] Average valves of five measurements.
[b] Coefficient of variability values.

3.4. Comparison of conventional immunoassay (ELISA) with QCM immunosensor for BEFV determination

Comparison of the result obtained from conventional ELISA with that of QCM immunosensor for BEFV determination revealed that the former method obtained a lower detection limit for BEFV (Fig. 8). This result demonstrates that the QCM method was less sensitive than the ELISA, which is consistent with the findings of Hengerer et al. [49] in a similar experiment. Since the flow rate in this experiment was 70 μL/min, with 100 μL of sample injected, there was insufficient time to complete the immunological reaction. The sensitivity of the immunosensor can be improved by either reducing the flow rate, using a more specific buffer solution, using a chemically milled technique to establish a thin quartz membrane [12], employing electrochemical pretreatment or plasma modification of the gold electrode surface to increase their surface functionalities and smoothness or remove surface contaminant to immobilize large amounts of antibodies [50,51], or coupling glycosylated monoclonal antibody with oxidized sugar to enhance the sensor sensitivity [45].

Although the QCM immunosensor was less sensitive than the conventional ELISA method, the QCM biosensor possesses several advantages over conventional ELISA: (1) QCM immunosensor requires only a small quantity of reagents and does not require any special preparation. (2) Antibody labeling or a second reagent for immunocomplex detection is not required for QCM immunosensor. (3) A real time and a complete automation procedure are possible in combination with a sample collector. (4) The measurement cycle of the QCM immunosensor can be completed within 10 min, compared to 4 h for the ELISA. (5) The QCM immunosensor enables users to verify the results posteriorly by injecting a positive control following each measurement cycle.

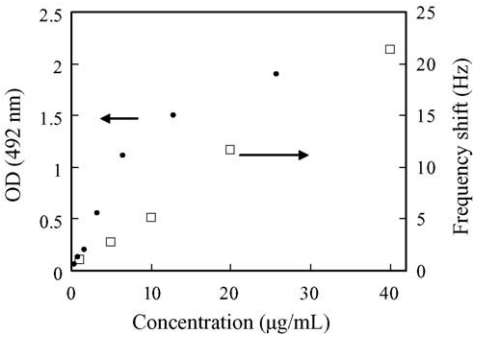

Fig. 8. Comparison of absorbance values measured in the conventional ELISA (●) and frequency shift obtained by the QCM immunosensor (□). The sensor chip was incubated with 200 μg/mL anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine.

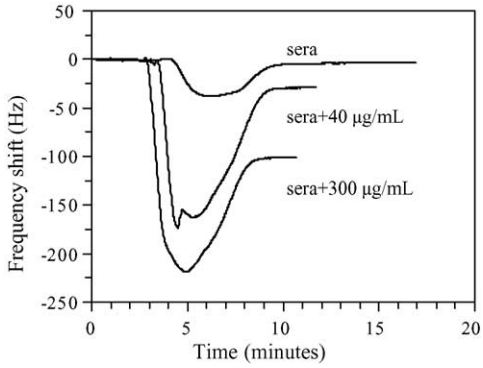

Fig. 9. Measurement curve for the QCM immunosensor. BEFV in various concentrations was injected on the quartz crystal microbalance. The sensor chip was incubated with 200 μg/mL anti-BEFV antibodies at 35 °C and allowed to interact with the glutaraldehyde followed by blocking the crystal surface with 100 mM glycine. BEFV levels were conducted by spiking various levels of BEFV into bovine sera with a dilution faction of 50.

3.5. Determination of the BEFV in the sera sample

The feasibility of applying the proposed immunosensor to measure BEFV levels in a complex matrix was studied. This experiment was performed by spiking various levels of BEFV into bovine sera with a dilution faction of 50 [4]. Unlike BSA and rabies virus, bovine sera induced a 5 Hz background residual frequency change (Fig. 9), which was owing to the non-specific adsorption of protein in serum. The difference between the background and steady-state frequencies following the addition of serum was used for calculating the response. The results show that the response was proportional to the quantity of BEFV in the sera samples. A 113 (24.3 Hz) and 96% (96.3 Hz) recovery was achieved for the 40 and 300 μg/mL samples, respectively. A more effective immobilization method (such as mixed SAMs [42] or the monolayer were further modified a sub-layers [41] to resistant to non-specific adsorption) is required in future studies.

4. Conclusions

The results showed that this flow type QCM immunosensor can operate in liquids and shows a constant frequency decrease corresponding to the increase in BEFV concentration. No significant response was observed after 20 μg/mL rabies virus or 60 μg/mL BSA was injected, confirming that virus binding was mediated by interactions between anti-BEFV antibody and BEFV. A detection limit of 5 μg/mL was obtained. The sensitivity of the proposed QCM immunosensor is inferior to the conventional ELISA. While these preliminary results using the QCM immunosensor were obtained in less than 10 min, compared to 4 h for the standard ELISA. The reproducible and constant changes for successive additions of BEFV found in this investigation make it feasible to develop an automated or continuous real time operation biosensor. The lower sensitivity of the proposed sensor may reflect the

lack of optimization of the synthetic system. The sensitivity of the QCM biosensor can be expected to be improved by optimizing the buffer compositions, pH, flow rate and by using a more effective immobilization method. The optimized QCM immunosensor can be further developed for the application of clinical diagnosis. The BEFV can be found in nasal discharge and/or respiratory secretions of infected cattle. Possibly, BEF can be diagnosed based on real time detection of BEFV in cattle saliva using the QCM immunosensor. Further work is in progress in our laboratory to exploit these possibilities.

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References

[1] S. Nandi, B.S. Negi, Immunol. Microbiol. Infect. Dis. 22 (1999) 241.
[2] A. Delte-Porta, F. Brown, J. Gen. Virol. 44 (1979) 99.
[3] S. Tzipori, J. Brit. Vet. 131 (1975) 720.
[4] D.H. Cybinski, Vet. Microbiol. 13 (1987) 1.
[5] H. Zakrzewski, D.H. Cybinski, P.J. Walker, J. Immunol. Meth. 151 (1992) 289.
[6] F. Scheller, F. Schubert, D. Pfeiffer, R. Hintsche, I. Dransfeld, R. Renneberg, U. Wollenberger, K. Riedel, M. Pavlova, M. Kuhn, H.G. Muller, P. Tan, W. Hoffmann, W. Movitz, Analyst 114 (1989) 653.
[7] C. Wrotnowski, Genet. Eng. News 2 (1998) 38.
[8] K.U. Chang, W.L. Hsu, H.Y. Chen, C.K. Chang, C.Y. Chen, Anal. Chim. Acta 481 (2003) 199.
[9] X. Cui, F. Yang, Y. Sha, X. Yang, Talanta 60 (2003) 53.
[10] G.G. Guilbault, J. Jordan, CRC Rev. 19 (1988) 1.
[11] C.K. O'Sullivan, G.G. Guilbault, Biosens. Bioelectron. 14 (1999) 663.
[12] E. Uttenthaler, M. Schraml, J. Mandel, S. Drost, Biosens. Bioelectron. 16 (2001) 735.
[13] B.D. Spangler, E.A. Wilkinson, J.T. Murphy, B.J. Tyler, Anal. Chim. Acta 444 (2001) 149.
[14] H.C. Lin, W.C. Tsai, Biosens. Bioelectron. 18 (2003) 1479.
[15] C. Kőβlinger, S. Drost, F. Aberl, H. Wolf, P. Woias, S. Koch, Sens. Actuators B 18–19 (1994) 271.
[16] J. Rickert, A. Brecht, W. Gopel, Biosens. Bioelectron. 12 (1997) 567.
[17] G.Z. Sauerbrey, J. Physik 155 (1959) 206.
[18] A. Shons, F. Dorman, J. Najarian, J. Biomed. Mater. Res. 6 (1972) 565.
[19] G. Sakai, T. Sakai, T. Uda, N. Miura, N. Yamazoe, Sens. Actuators B 42 (1995) 89.
[20] K. Yun, E. Kobatake, T. Haruyuma, M. Laukkaned, K. Keinanen, M. Aizawa, Anal. Chem. 70 (1998) 260.
[21] J.L.N. Harteveld, M.S. Nieuwenhuizen, E.R.J. Wils, Biosens. Bioelectron. 12 (1997) 661.
[22] C. Kőβlinger, F. Drost Aberl, H. Wolf, S. Koch, P. Woias, Biosens. Bioelectron. 7 (1992) 397.
[23] K.K. Kanazawa, J.G. Gordon, Anal. Chem. 57 (1985) 1771.
[24] M. Thompson, A.L. Kipling, W.C. Duncan-Hewitt, L.V. Rajakovic, B.A. Cavic-Vlasak, Analyst 116 (1991) 881.
[25] T. Nomura, M. Maruyam, Anal. Chim. Acta 147 (1983) 365.
[26] T. Nomura, T. Nagamuns, Anal. Chim. Acta 155 (1983) 231.
[27] J.E. Rodederer, G.J. Bastians, Anal. Chem. 55 (1983) 2333.
[28] T. Nomura, T. Mimatsu, Anal. Chim. Acta 143 (1982) 237.
[29] T. Nomura, K. Tsuge, Anal. Chim. Acta 169 (1985) 257.
[30] S. Bruckenstein, M. Shay, Electrochim. Acta 30 (1985) 1295.
[31] Y. Cohen, S. Levi, S. Rubin, I.J. Willner, Electroanal. Chem. 417 (1996) 65.
[32] J. Horacek, P. Skladal, Anal. Chim. Acta 347 (1997) 43.
[33] S. Storri, T. Santorini, M. Minunni, M. Mascini, Biosens. Bioelectron. 13 (1998) 347.
[34] S. Storri, T. Santoni, M. Mascini, Anal. Lett. 31 (1998) 1795.
[35] R. Vaughan, C.K. O'Sullivan, G.G. Guilbault, Anal. Chem. 364 (1999) 54.
[36] P. Fenter, A. Eberhardt, P. Eisenberger, Science 266 (1994) 1216.
[37] K.L. Prime, G.M. Whitesides, Science 252 (1991) 1164.
[38] J.M. Tour, L. Jones II, D.L. Pearson, J.J.S. Lamba, T.P. Burgin, G.M. Whitesides, D.L. Allara, A.N. Paarikh, S.V. Atre, J. Am. Chem. Soc. 117 (1995) 9529.
[39] I.S. Park, N. Kim, Biosens. Bioelectron. 13 (1998) 1091.
[40] D.H. Cybinski, P.J. Walker, K.A. Byrne, H. Zakrzewski, J. Gen. Virol. 71 (1990) 2065.
[41] I. Ben-Dov, I. Willner, E. Zisman, Anal. Chem. 69 (1997) 3506.
[42] J. Lahiri, L. Isaacs, J. Tien, G.M. Whitesides, Anal. Chem. 71 (1999) 777.
[43] Y. Liu, W. Zhang, X. Yu, H. Zhang, R. Zhao, D. Shangguan, Y. Li, B. Shen, G. Liu, Sens. Actuators B 99 (2004) 416.
[44] J.W. Park, S. Kurosawa, H. Aizawa, S.I. Wakida, S. Yamada, K. Ishihara, Sens. Actuators B 91 (2003) 158.
[45] B.D. Spangler, B.J. Tyler, Anal. Chim. Acta 399 (1999) 51.
[46] C. Ruan, F. Yuan, C. Lei, J. Deng, Anal. Chem. 70 (1998) 1721.
[47] M. Steizle, G. Weismuller, E. Sackmann, J. Phys. Chem. 97 (1993) 2974.
[48] S. Kirkeby, P. Jakobsen, D. Moe, Anal. Lett. 20 (1987) 303.
[49] A. Hengerer, J. Decker, E. Prohaska, S. Hauck, C. Kőβlinger, H. Wolfs, Biosens. Bioelectron. 14 (1999) 139.
[50] G. Cui, J.H. Yoo, J.S. Lee, J. Yoo, J.H. Uhm, G.S. Cha, H. Nam, Analyst 126 (2001) 1399.
[51] H. Hiratsaka, K.I. Kojima, H. Suzuki, H. Muguruma, K. Ikebukuro, I. Karabe, Analyst 126 (2001) 658.