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QCM DNA biosensor for the diagnosis of a fish pathogenic virus VHSV

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ABSTRACT

Viral haemorrhagic septicaemia (VHS) is one of the most serious viral diseases damaging both fresh and marine fish species. VHS caused by VHSV and diagnosis of VHSV has been dependent on the conventional methods, such as cell culture and RT-PCR, which takes a few days or several hours. This study demonstrates a rapid and sensitive QCM biosensor for diagnosis of VHSV infection in fish. The QCM biosensor was developed to detect a main viral RNA encoding G protein in VHSV using the specific DNA probe. To maximize the sensitivity of the biosensor, we prepared three different DNA probes which modified 3′ end of DNA by thiol, amine, or biotin and compared three different immobilisation methods on quartz surface coated with gold: immobilisation of thiol labelled probe DNA on naked gold surface, immobilisation of amino labelled probe DNA on gold surface prepared as carboxyl chip using MPA followed by EDC/NHS activation, and immobilisation of biotin labelled probe DNA on gold surface after immobilising avidin on carboxyl chip prior to biotin. As a result, immobilisation method using avidin–biotin interaction was most efficient to immobilise probe DNA and to detect target DNA. The QCM biosensor system using biotinylated probe DNA was stable enough to withstand 32 times of repeated regenerations and the detection limit was 0.0016 μM. Diagnosis using the QCM biosensor system was more sensitive and much faster than a conventional RT-PCR analysis in detecting the viral RNA.

1. Introduction

Many researchers have developed QCM biosensor as an extremely sensitive mass sensor capable of measuring sub-nanogram levels and applied it on the extensively wide ranges, for example, in the food industry, environmental monitoring, clinical diagnostics and biotechnology. Among them, a clinical diagnosis for infectious diseases using QCM sensor has several great advantages as a label free detection system including a rapid detection, easy manipulation and low cost by minimising reagents and efforts. Biological pathogens can be detected by attachment to the receptors immobilised on the surface of the biosensor chip. The receptors to detect pathogens can be any substances which specifically bind to ligands of pathogens.

Antibodies are the most popular receptors to detect pathogens for biosensor systems. However, proteins like antibodies have limitations in storage and repeating regeneration process since they are unstable and easily degraded at room temperature or under harsh regeneration conditions using very low or high pH solution. In our previous study, a significant decrease of frequency was found during regeneration process, indicating a damage of antigen binding site in antibody [1]. Thus, researchers are recently seeking more stable receptors for example peptides, DNA, PNA, and aptamers [2,3].

DNA has useful features to be used in biosensor system due to its stability and specificity. DNA can be produced in short time and easily manipulated to increase affinity and specificity by changing the sequences. Indeed, DNA can be easily immobilised on sensor chip by modifying end of DNA with reactive residues, for example, thiol which crosslinks to Au directly or biotin. The detection of specific DNA sequences has been applied to many areas including clinical, environmental and food microbiology diagnosis and plays fundamental role in rapid detection of genetic mutation, indicating that it is possible to make a reliable diagnosis of diseases. QCM DNA biosensors have already been developed by several researchers on the purpose of genetic diagnosis [4], detection of genetically modified organisms [2], and bacteria or virus detection [5,6].

QCM DNA sensors can be used to detect target DNA or RNA by triggering a direct signal caused by hybridisation of target DNA to DNA probe immobilised on the surface of sensor chip. Immobilisation of probe DNA on sensor chip surface is the first step and very important in developing a DNA sensor since it could determine the sensitivity of the sensor. DNA must be attached to the solid support, retaining native conformation, and binding activity. According to Tombelli et al. [5], the detection limits and the analytical performances of the biosensor can be improved by optimising

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the immobilisation of the receptor on the transducer surface. DNA probe is single stranded and immobilised by covalent attachment, utilization of self-assembled monolayer or electro polymerisation that provide chemically stable recognition layers [7]. Tombeli et al. [5] also previously reported that DNA immobilisation strategies on gold surface of quartz using biotinylated probe and thiolated probe were successfully applied to develop genetically modified organism (GMO) detection QCM sensor. They demonstrated that developed DNA sensor was able to detect the target DNA which has sequences within a promoter widely used in GMO.

Biotinylated probe or thiolated probes can be purchased from companies producing oligonucleotides. Another probe which can be purchased from those companies and can be applied for DNA immobilisation on gold surface is amine labelled probe DNA. Amine labelled DNA can be immobilised on carboxylic self-assembled monolayer conformed on gold surface in cheaper price than thiol or biotin labelled probe DNA. In this study we tried three different immobilisation strategies using biotinylated, thiolated, and amine labelled probe DNA and compared their efficiency of probe DNA immobilisation as well as the sensitivity of the prepared sensor chips in detecting same amount of target DNA.

Viral haemorrhagic septicaemia (VHS) is one of fish viral diseases causing substantial economic losses for public and commercial aquaculture ventures each year. It is caused by a single strand RNA virus, VHSV [8] which belongs to the genus Novirhabdovirus of the family Rhabdoviridae [9]. VHS is the economically most important disease in farmed salmonids worldwide and also in marine fish species including olive flounder [8,10,11]. This disease is one of the fish diseases notifiable to OIE (The World Organisation for Animal Health, http://www.oie.int/eng/maladies/en_classification 2009.htm). It is an emerging need to develop a fast, reliable, and cost-effective method for VHSV detection to reduce economic risk in aquaculture and fish trading.

Like other viruses, VHSV has been detected by traditional cell culture detection method or relatively newly developed RT-PCR or Q-PCR analysis method. Traditional cell culture detection method takes several days, and RT-PCR and Q-PCR analysis themselves take only a couple of hours [12]. However, in the case of RNA virus like VHSV, RT-PCR and Q-PCR analysis need a reverse transcription step of purified RNA before PCR analysis. RNA purification and reverse transcription steps take additional a few hours and need highly skilled techniques for each step. However, QCM DNA sensor can give us a promise to detect VHSV easily and in shorter time since the purified RNA sample can be directly detected on the QCM DNA sensor without reverse transcription and amplification steps.

This study aimed to develop a QCM DNA sensor to detect VHSV using oligo DNA probe specific to a main gene, i.e. G protein, in VHSV. We first investigated three different methods to immobilise probe DNA on quartz surface coated with gold: immobilisation of thiol labelled probe DNA on naked gold surface [5,3], immobilisation of amine labelled probe DNA on gold surface prepared as carboxyl chip using MPA followed by EDC/NHS activation, and immobilisation of biotin labelled probe DNA on gold surface after immobilising avidin on carboxyl chip prior to biotin [5,13]. We also investigated the sensitivity, specificity, and reusability of the prepared DNA sensor system in addition to application to detect the viral RNA in RNA sample purified from fish tissues.

2. Materials and methods

2.1. QCM biosensor system

A 10 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 used as the transducer of the QCM biosensor system. It was mounted on a well holder made with acryl and connected to a homemade oscillator module. The well holders were made in two types i.e., batch and flow type. Processes until probe DNA immobilisation were performed in batch type well while later steps were done in flow type well. While flow type well was used, PBS (phosphate buffered saline, pH 7.4) was continuously flowed by a syringe pump (NE-1600, New era pump system) at the flow rate of 1.5 ml/h. After obtaining a stable signal, 100 μl of targets (DNA or RNA) was injected into flow type well through injection valve (V450, Upchurch). 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.

2.2. Preparation of probe DNA and target DNA

Three different probe DNAs were designed from the sequence of VHSV G protein [8] and prepared to have biotin (P1), thiol or amine residues at the 5′ end. The immobilisation efficiency and sensitivity of different immobilisation schemes were tested. Sequence of probe DNA was 5′-X-CTGGTGACTGATAGCGGGT-3′ (X: thiol, amine, biotin). Target DNA (T1) was designed as complementary to probe DNA and another target DNA (T(F)1) was designed to have same DNA sequences with T1 but labelled with FAM (5-carboxy fluorescein) at the 5′ end to make easy recognition of target DNA on the sensor chip. Sequences of probes and target DNA (T1) corresponded to positions 260–277 of VHSV G gene (T1: 5′-ACCCGCTATCAGTCACCAG-3′).

2.3. Immobilisation of probe DNA

Piezoelectric quartz crystal was cleaned using piranha solution (H_2SO_4:H_2O_2 = 7:3) at 60 °C for 1 min and rinsed with absolute ethanol and distilled water. Three different methods were attempted to immobilise probe DNA on gold surface of the quartz:

(1) Direct immobilisation of thiol labelled probe DNA on naked chip
5 μM of thiol labelled probe DNA was directly crosslinked on the cleaned gold surface by interaction between thiol and Au. Uncrosslinked Au residues were then blocked by incubation with 10 mM of MPA (3-mercaptopropionic acid, Sigma).
(2) Immobilisation of amine labelled probe DNA on carboxyl chip
First, carboxyl chip was prepared by conforming self-assembled monolayer (SAM) by treating the quartz crystal with 10 mM of MPA overnight and then activated with 0.4 M of EDC (*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide, Sigma)/0.1 M NHS (*N*-hydroxysulfosuccin-imide, Sigma) for an hour. After activation of SAM, 5 μM of amine labelled probe DNA was crosslinked and uncrosslinked residues were blocked by 1 M ethanolamine–HCl (Sigma).
(3) Immobilisation of biotin labelled probe DNA on avidin chip
Avidin chip was prepared by crosslinking 5 μM of avidin (Sigma) on the activated carboxyl chip prepared by above method and uncrosslinked residues were blocked by 1 M ethanolamine–HCl. 5 μM of biotinylated probe DNA was added and incubated for an hour.

2.4. Comparison of sensitivity

To test the sensitivity of three sensor chips, the frequency shifts (mean ± S.D., n = 3) were recorded after adding 1 μM of target DNA (T1) on each sensor chip. PBS was flowed continuously from syringe pump connected to a cell which contains the prepared sensor chips and measured the resonant frequency until a steady-state baseline

was obtained (F1). One hundred μl of target DNA (T1) was added through injection valve onto the sensor chip. PBS was flowed again and the steady-state resonant frequency (F2) was read again to calculate the frequency shift (frequency shifts = F1 − F2). The steady state was obtained within 5 min at the both cases before or after target DNA injection.

2.5. Dose responses of the sensor chip immobilised with biotinylated DNA

To test the dose responses of sensor chip prepared by immobilising biotinylated DNA probe, T1 was serially diluted in PBS and added at the concentrations of 1, 0.2, 0.04, 0.008, 0.0016 μM. Unbound T1 was washed out by PBS and the frequency was read at the steady state. To measure the amount of target DNA bound on the sensor chip precisely, serially diluted T(F)1 was added at the same concentrations, dissociated from the sensor chip using 0.1 M glycine–HCl, collected in 96-well plate (PS black, Porvair), and measured fluorescent intensity (excitation 355, emission 535) using a fluorometer (Mithras LB 940, Berthold). The attached T(F)1 on the sensor chip was visualised by capturing the image using a fluorescence microscope (BX51, Olympus).

2.6. Specificity test of the sensor chip immobilised with biotin labelled DNA

The specificity of the prepared sensor chip was tested using a mismatched DNA. The mismatched DNA (MT1) was designed as a part of scinderin gene, which is constitutively expressed and abundant in most cells, with a similar size to target DNA (MT1: 5′-GAGCCTCCCCACCTGATGA-3′). One μM of complementary DNA and mismatched DNA were applied onto the sensor chip.

2.7. Regeneration test of the sensor chip immobilised with biotin labelled DNA

The reusability of the prepared sensor chip was tested using a dissociation buffer containing 0.8 M Tris–glycine, pH 2.3. PBS was flowed continuously from syringe pump connected to a cell which contains the sensor chip and the frequency was read at the steady state. One hundred μl of target DNA (T1) was added through injection valve onto the sensor chip at the concentration of 1 μM. PBS was flowed again and the frequency was read at the steady state. For regeneration, 100 μl of dissociation buffer was added, and washed out DNA by PBS. The same procedure was performed for 32 times on one sensor.

2.8. RNA extraction

RNA samples were prepared from three fish infected by VHSV and subjected to the sensor system. Since head kidney is the major site of VHSV infection in fish, RNA was extracted from aseptically collected head kidney using TRIzol (Invitrogen) and dissolved in DEPC treated water. RNA concentration was determined by optical density reading at 260 nm using NanoDrop (Thermo, USA), and integrity was verified by ethidium bromide staining of 28 S and 18 S ribosomal bands on a 1% agarose gel. RNA was stored at −80 °C until used.

2.9. cDNA synthesis, PCR, and Q-PCR

cDNA was synthesised for PCR and Q-PCR. RNA was reverse-transcribed into cDNA using (Promega) and MMLV reverse transcriptase (Promega). Three μg of total RNA in 13 μl DEPC-water was incubated with 1 μl of a random primer (Promega) for 10 min at 70 °C. Then, 1 μl of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega), 4 μl 5× first strand buffer (Promega), 1 μl of 10 mM dinucleoside triphosphate (dNTP) mix (Promega) were added and the mixture incubated at 42 °C for 2 h. The reaction was terminated by heating to 94 °C for 15 min and cooled down at 4 °C. Total volume of cDNA was 20 μl and stored at −20 °C until used.

Routine PCR (reverse transcription-polymerase chain reaction) was carried out to decide if the fish infected by VHSV or not and to compare the sensitivity with the QCM biosensor. PCR was performed using 0.5 μl of cDNA (corresponding to 0.1 μg of RNA) as a template for one and half hours: 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, at 56 °C for 30 s and at 72 °C for 30 s, and 1 cycle at 72 °C for 10 min. Electrophoresis of the PCR products was followed on a 1% agarose gel containing ethidium bromide for 30 min and the gel image was captured under UV light. The intensity of bands on the gel indicates the severity of infection.

2.10. Detection of viral RNA using QCM DNA sensor

To test whether the prepared sensor system is efficient enough to diagnose VHSV infection in fish or not, RNA was subjected to the sensor system. The concentration of RNA was 28 μg/ml and 100 μl was injected. RNA samples were heated at 65 °C to remove secondary structure and cool down on ice to prevent hindrance effect from the secondary structure of RNA. Cooled RNA sample was immediately injected to the sensor chip. Copy number of viral RNA in the RNA samples was determined by Q-PCR.

3. Results and discussion

3.1. Comparison of three probe DNA immobilisation strategies

The present study examined three different DNA immobilising methodologies using thiol, amine and biotin labelled probe DNAs. Many published papers showed immobilisation techniques based on crosslinking procedures using thiols or the interaction between avidin and biotinylated molecules [5,3,13]. In this study we additionally tried to immobilise amine labelled DNA probe on a carboxyl chip since it is much cheaper to produce than biotin and thiol labelled DNA probe. The price of amine labelled oligonucleotide is half of biotin labelled oligonucleotide and one sixth of thiol labelled oligonucleotide in Korea. Moreover, biotin labelled DNA probe can only bind to avidin or streptavidin immobilised on sensor chip prior to biotin labelled DNA probe immobilisation, which are very expensive proteins.

In this study, all tested immobilisation methods were efficient enough to immobilise considerable amounts of probe DNA (Fig. 1). However, while frequency shifts were similarly high following biotin or thiol labelled probe DNA immobilisation, amine labelled probe DNA immobilisation showed 30% less frequency shifts, revealing that amine labelled probe DNA is less effectively immobilised than other two probes. Less immobilisation of amine labelled probe DNA was thought to be due to the low reactivity of amine interacting with carboxyl group on gold surface under the experimental condition in this study. Further study is needed to optimise the immobilisation of amine labelled DNA probe by changing the immobilisation conditions including reaction time and pH.

The sensitivity of three immobilisation methods was varied. The sensitivity of amine labelled DNA probe immobilised sensor chip was the lowest. Even though similar amount of DNA probe was immobilised, the sensitivity of biotinylated DNA probe immobilised sensor chip was higher than thiolated DNA probe immobilised sensor chip since the frequency shift in biotinylated DNA probe sensor chip was bigger (Fig. 1). This is an agreement with a pre-

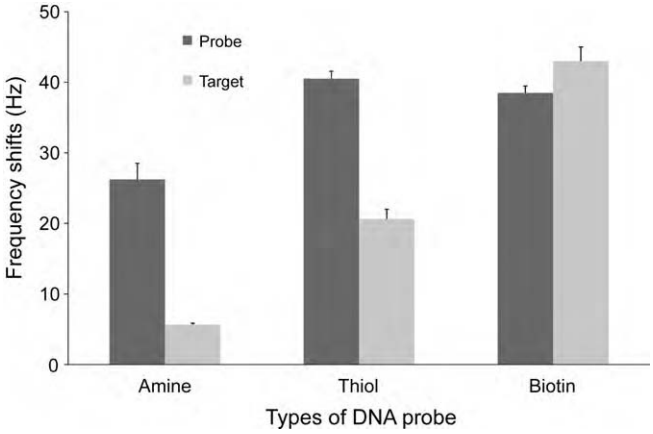

Fig. 1. Effects of probe DNA types on the efficiency of probe immobilisation and the sensitivity of sensor chips. 5 μM of amine labelled, thiolated or biotinylated probe DNA was immobilised on carboxyl, naked, or avidin chip, respectively, and the frequency shifts (mean ± S.D., $n = 3$) were recorded to compare the immobilisation efficiency of three methods.

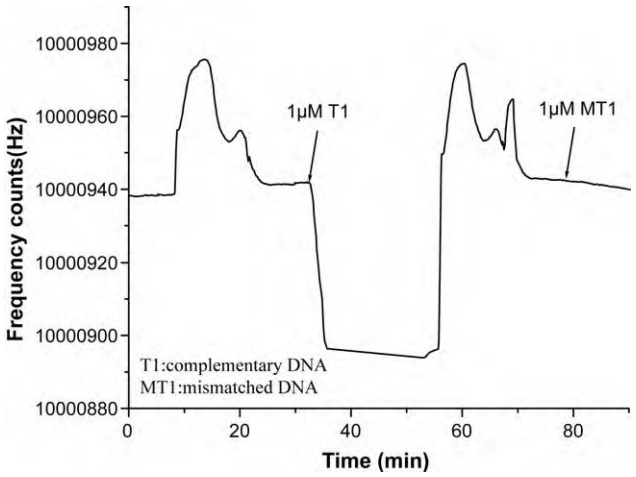

Fig. 3. Specificity test. Complementary DNA (T1) and mismatched DNA (MT1) were applied on to the sensor chip at the same concentration, 1 μM. T1 was incubated first and MT1 was incubated after regenerating the sensor chip by 0.1 M glycine–HCl.

vious study where reported that immobilisation method using streptavidin–biotin interaction was better in terms of sensitivity and reproducibility than direct immobilisation method using thiolated probes [3].

Difference in the sensitivity between biotinylated probe and thiolated DNA probe immobilised sensor chips might be due to the conformational difference between immobilised DNAs. It is known that avidin is a tetrameric glycoprotein which can bind up to four molecules of biotin simultaneously, thus four biotin labelled probes can be immobilised on one avidin molecule [14].

3.2. Dose responses

Dose responses of the sensor chip prepared by immobilising biotinylated probe DNA was tested in this study. Injection of target DNA (T1) at the doses of 1, 0.2, 0.04, 0.008, and 0.0016 μM caused the dose dependent increase of frequency shift from 40.6 ± 2.08 to 1.33 ± 0.58 Hz (mean ± S.E., $n = 3$) (Fig. 2). Attachment of target DNA to the probe DNA immobilised on the sensor chip was confirmed by

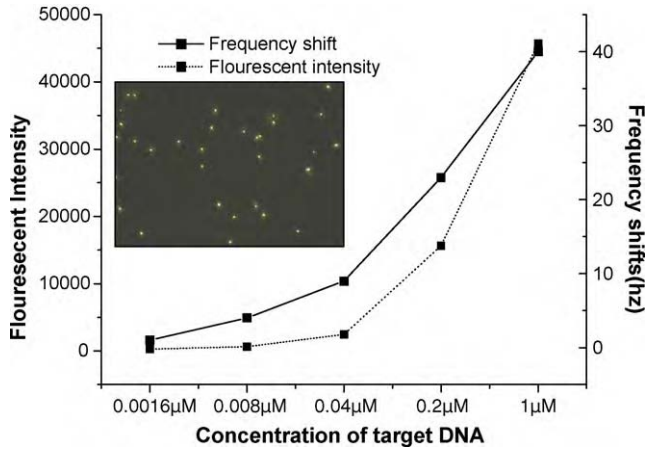

Fig. 2. Dose responses of biotin labelled DNA probe immobilised sensor system. To test the dose responses of sensitivity of sensor chip prepared by immobilising biotin labelled DNA probe, the frequency shifts (mean ± S.D., $n = 3$) were recorded after adding serially diluted target DNA (T1) at the concentrations of 1, 0.2, 0.04, 0.008, 0.0016 μM. Serially diluted T(F)1 was also incubated and measured the fluorescent intensity (ex. 355 nm, em. 535 nm) after dissociating from the sensor chip using 0.8 M Tris–glycine, pH 2.3. The box in the graph shows the image of T(F)1 on the sensor chip captured by fluorescent microscope and shown as green bright spots.

observing the presence of fluorescent target DNA under fluorescent microscope (Fig. 2). The amount of attached target DNA was verified by measuring the fluorescent intensity after dissociating from the sensor chip. Consequently, the changes of fluorescent intensity were dependent to injected amounts of target DNA, revealing that the amounts of attached target DNA were correspondent to the amount of injected target DNA (Fig. 2). Moreover, the frequency shifts following the injection of target DNA injection are positively related to the changes of fluorescent intensity. This reveals that the frequency shifts after target DNA injection fully represent the weight change by attachment of target DNA to the probe DNA crosslinked on the gold surface.

Even though Cosnier and Mailley [7] suggested that the desirable detection limit of DNA sensors is as low as 1 ato M for medical diagnostic application, it is technically very difficult to satisfy their suggestion at the moment. The detection limit of our QCM system was supposed to be about 8 nM. Our result is similar to the report from Pang et al. [15] but lower than other studies where reported detection limits from 0.46 nM [4] to 8.6 pg/l [6]. However, the detection limit of our system can be lower than 8 nM when detecting viral RNA from infected fish. Since QCM sensors measure the weight change on the sensor surface target DNA is much shorter and 80 times lighter than the viral RNA encoding G protein, the predicted detection limit of our system is to be about 0.1 nM for the detection of real target viral RNA

3.3. Specificity test of the sensor chip

Specificity of the sensor chip is a crucial factor in developing a biosensor for the detection of microorganisms or their genomes. In this study the non-specific affinity of the sensor chip to the mismatched DNA was tested using the partial sequences of a constitutively expressed, abundant and ubiquitous gene (scinderin like gene). As a result, a significant affinity to the mismatched DNA was not observed (Fig. 3).

Even though it was known that avidin sometimes causes background problems in some histochemical applications and flow cytometry caused by a non-specific binding of DNA or RNA on avidin on the sensor chip since avidin is a highly cationic glycoprotein and its oligosaccharide component (heterogeneous structures composed largely of mannose and *N*-acetylglucosamine) can interact non-specifically with negatively charged cell surfaces and nucleic acids [14], non-specific binding was not found in this study.

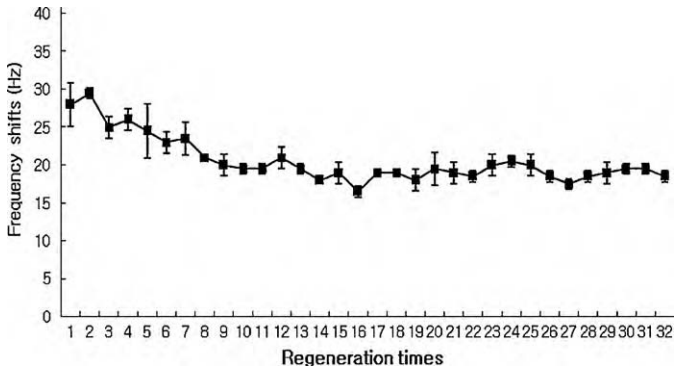

Fig. 4. Regeneration test. One μM of target DNA (T1) was added onto a sensor chip and the sensor chip was regenerated using a dissociation buffer containing 0.8 M Tris–glycine, pH 2.3. Regeneration was performed for 32 times in triplicates.

3.4. Regeneration test

The regeneration procedure was performed for 32 times on one sensor chip. Sensor chips made by immobilising biotinylated probe DNA appeared to be stable enough to withstand more than 30 times regeneration since the signal was not significantly reduced over 30 times regeneration (Fig. 4). This is in agreement with a previous study by Tombelli et al. [3] which demonstrated that a sensor chip immobilised with a biotinylated probe DNA was able to be used for 25 cycles when regenerated by 1 mM of HCl. These results implicate that DNA sensor is more stable than antibody sensors during regeneration.

3.5. Detection of RNA

The developed DNA sensor system was applied to detect viral RNA isolated from VHSV infected fish without any additional steps for reverse transcription and amplification. Three fish was confirmed to be infected by VHSV using RT-PCR and infection degrees were varied at high, middle, and low level. When the viral gene amplified by RT-PCR was visualised on agarose gel under UV lamp after stained using ethidium bromide, the bands were very faint or almost undetectable in the sample infected at middle level or at low level, respectively, while the band was very thick and dark in the sample infected at high level (Fig. 5). The frequency shifts following the injection of RNA samples were proportional to the degree of infection and clearly indicate the viral infection in the fish sample (Fig. 5). Comparing with RT-PCR, diagnosis of VHSV using QCM DNA sensor was more sensitive and much faster. We could monitor the binding of viral RNA to the probe DNA after RNA sample injection in real time and the reaction was finished within 10 min, while RT-PCR took more than 3 h for reverse transcription, PCR and electrophoresis for PCR.

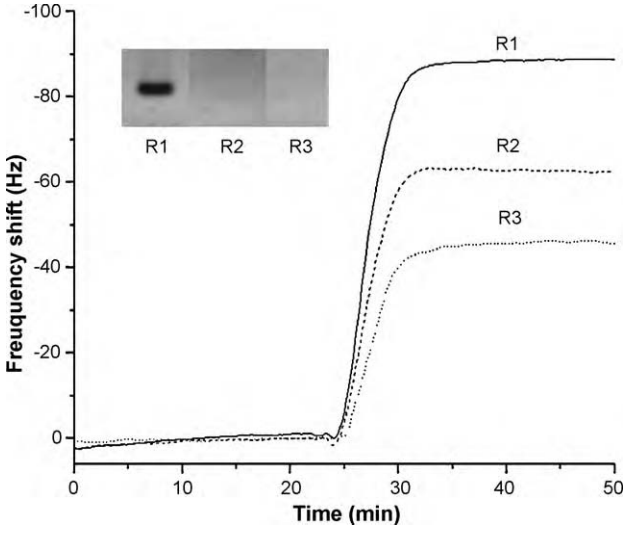

Fig. 5. Detection of viral RNA. RNA from three fish infected by VHSV was subjected to the sensor system and measured frequency shifts. RNA was extracted from head kidney and 2.8 μg RNA in 0.1 ml of PBS was applied onto the sensor chip. The gel image of RT-PCR products is shown and implicates that the severity of infection is highest in R1 and lowest in R3.

4. Conclusions

Among three immobilisation schemes using thiol, amine, or biotin labelled probe DNA, the immobilisation method using biotin labelled probe DNA was the most efficient to detect target DNA and the attachment of target DNA labelled with FAM was visualised under fluorescent microscope. The prepared sensor system was able to detect target DNA as well as RNA samples from VHSV infected fish in dose dependent manner. The dose limit of the sensor system to detect T1 target DNA was less than 0.0016 μM. This QCM system showed specificity to complementary target DNA but not to mismatched DNA. Also this QCM system was able to withstand regeneration procedure until more than 32 times without any significant reductions of signal. Furthermore, this QCM system was efficient enough to detect to detect viral RNA with high sensitivity and was an easier and faster method than conventional RT-PCR.

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