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Non-labeled, Real-time Detection of H1N1 DNA Hybridization Using the QCM-D System

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Abstract– Detection of H1N1 or other seasonal and highly contagious viruses are of vital importance, since early diagnosis from a patient’s fluid, such as saliva, would accelerate treatment and containment of the flu. Current lab tests often require cumbersome labeling steps and several hours to detect the presence of viral DNAs. The QCM-D (quartz crystal microbalance with dissipation) technology was used to detect presence as well as distinguish H1N1 and H1N5 DNAs in real-time (a few minutes) with the use of relatively small sample volume (~200-500 ul). Single stranded complementary H1N1 DNA was immobilized via Neutravidin-biotin linkage on a gold substrate where biotin was anchored through self assembled monolayers (SAM). Samples containing ss-H1N1 or H5N1 DNA were introduced to the sensor chamber at various sensing conditions. Effect of temperature, ionic strength in buffer, and flow rate were studied and discussed in this paper. The setup allowed recognition and monitoring of kinetics of up to 100 nano-molar (nM) concentrations H1N1/H5N1 DNA in real-time without cumbersome labeling steps. This result demonstrates possibility of real-time diagnosis of H1N1 or other contagious viruses in a doctor’s office.

I. INTRODUCTION

Novel H1N1 flu, popularly known as swine flu, is a respiratory infection caused by an influenza virus first recognized in recent years. In June 2009, when the infection’s spread had been verified worldwide, the World Health Organization (WHO) declared H1N1 flu a global pandemic. Avian flu, H5N1 flu, is caused by avian influenza, a virus that rarely infects humans. When bird flu does infect humans, it is often fatal.[1] The flu virus can be detected by the specific sequence of the flu’s DNA matching the presence of flu DNA in a patient’s blood or saliva sample. Many current diagnostic tools that test DNA sequence matching require 30-60 minutes of hybridization time.

The goal was to see real-time DNA hybridization for a faster detection time, using Q-Sense technology to

determine the best circumstances. In this project, the H1N1 and H5N1 specific DNA were used to monitor its concentrations without labeling and in real time. If H1N1 binding can be tracked in real-time it would mean that hospitals could use amplified saliva samples to check for the virus on-site, and with minimal cost or wait. We used the Q-Sense E1 system, sold by the Q-Sense company headquartered in Sweden. Their technology uses an oscillating/vibrating crystal coated with electrodes to measure frequency and dissipation changes as molecular bonding or disassociation occurs on the gold surface.

II. SETUP AND MATERIALS

QCM-D technology: A sensing unit of Quartz crystal microbalance with dissipation (QCM-D) sensor is a thin quartz crystal (diameter 2.5 cm, thickness 1mm) with thin electrodes coated on both sides; this experiment required gold electrodes as they make a stable surface for biological reactions. (Figure 1)

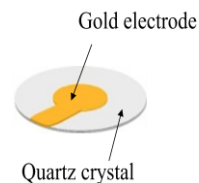


Figure 1. Model of quartz-crystal sensor with gold electrodes

The crystal is a piezoelectric resonator, meaning that it changes shape with voltage or in this case a voltage difference causes oscillation. The resonant frequency of the sensor’s oscillation varies linearly with the mass of absorbed layers on the surface. Binding to the surface results in an increase in mass which causes the crystal to oscillate slower, with the change in frequency describing the presence of binding, many times also describing the amount of DNA strands that bind to the surface. By measuring the resonance at several frequencies in conjunction with the dissipation, it becomes possible to determine whether the absorbed film on the surface is rigid or water-rich.

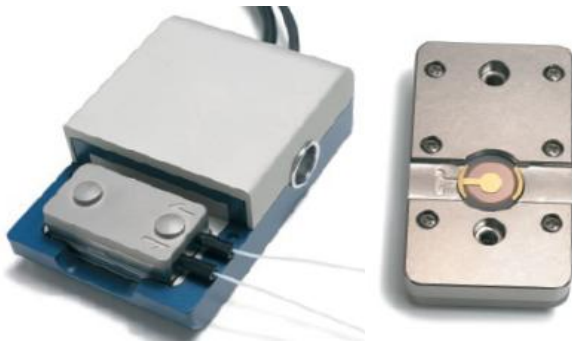


Figure 2. Product images of QCM-D sensing chamber and crystal

Dissipation, the D in QCM-D, is what sets apart this specific technology and system from other quartz crystal microbalance sensing systems. Dissipation means how long it takes for the oscillating crystal to slow down/stop moving once the power source that causes the crystal's oscillation is switched off. The dissipation gives us insight to the thickness of the binding above the surface, since surface consistencies will affect the speed at which the crystal slows. A fast dissipation means that the surface is compact and rigid, while a slow dissipation suggests that the surface binding is water-rich and has a greater volume above the sensing surface.

QCM-D frequency and dissipation readings happen incredibly fast – the system collects up to 200 data points per second. Since after amplification, saliva has very small DNA concentrations (40nM), and our experiment was to test the feasibility of using this system to work in tandem with a doctor's office where the sample provided would be amplified DNA – not an unlimited amount, it was crucial to obtain good results using low volumes and low concentrations. The speed of the system allows for measurements to be accurately compared against each other in similar trials since the low volume and low concentration samples required for these trials result in very small changes in frequency and dissipation. A couple hundred data points per second allows for an accurate average of measurements (even if the data points are not precise) and determining with confidence if the changes plotted are indeed structural changes due to binding and hybridization or just noise (a result of a mistaken reading by the sensor due to a small change in temperature, accidental moving of the equipment that could influence the reading, etc). Additionally, if the frequency changes drastically but the dissipation does reflect these same changes then it is a good indication that no binding actually occurred and it was simply an inaccurate reading.

Setup: The measurements were made using the QCM-D sensor (Q-sense AB, Göteborg, Sweden) device which has a mass sensitivity factor of 1 Hz=17.7 ng/cm², valid for thin, rigid film. (Figure 2). The quartz crystal (chromium used to bind sputtered gold to surface) was

Name	DNA sequences (5' → 3')	Comments
H1N1 probe	Biotin-TCACTGCAAACATCATGG	Immobilized on sensor surface
H1N1	CCATGAGTTTGCAGTGA	Target H1N1 DNA
H1N1-nonmatching	AACGTCTGGCTACTCTC	For control experiment
H1N1-SMM	CCATGAGTTTG <u>A</u> AGTGA	Single C-G mismatch
H5N1 probe	Biotin-AGAAGGCCAATCCAGTC	Immobilized on sensor surface
H5N1	GACTGGATTGGCCTTCT	Target H5N1 DNA
H5N1-SMM	GACTGGATTG <u>A</u> CCTTCT	Single C-G mismatch

Table 1. DNA sequences used over the course of the research

purchased from Q-sense AB. The QCM-D setup allows for subsequent measurements of up to four harmonics (15, 25, and 35 Hz corresponding to the overtones n=3, 5, and 7, respectively) of the 5 MHz crystal. The frequency and dissipation shift shown here is n=5 overtone unless otherwise stated. During the measurement, the temperature was controlled at 25 degrees Celsius unless otherwise stated and the short term noise level in f and D with liquid load was 0.3 Hz and 0.2x10⁻⁶ respectively. The flow rate was set as 75 µ/min.

Materials: The quartz crystal can be cleaned following a standard cleaning steps [2] and reused about 5 times before the sputtered gold starts to show wear. SAM chemicals were purchased from Asemblon, Inc. Single stranded DNA (ss-DNA) were custom synthesized by Biosys, Inc. The sequences are summarized in Table 1.

The H1N1 and H5N1 probes are biotin linked half-strands of H1N1/H5N1 DNA used to prepare the sensor surface. The actual target H1N1 and H5N1 were half-strands, non-labeled, that match the probes (opposite bases). To ensure that the readings were measuring hybridization and not just indiscriminate binding of the DNA to the surface, a completely non-matching strand of DNA was used. Additionally, strands of H1N1 and H5N1 with a single mismatch (SMM) were used to test the sensitivity of the QCM-D system to a single base being different from the target DNA (completely matching strands). Other chemicals were purchased from Sigma Aldrich, Inc.

III. METHODS

QCM cleaning and SAM surface preparation: To clean the QCM-D sensor, the gold surface is exposed to 10 minutes of ultra violet ozone (UVO) cleaning, 5 minutes of immersion in 5:1:1 (miliQ water: 16% ammonium hydroxide: 30% hydrogen peroxide) APM solution at

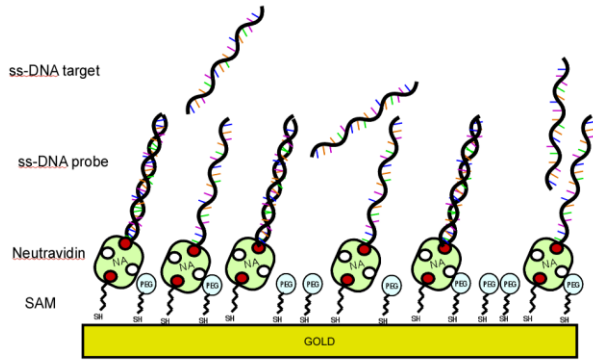


Figure 3. Representation of sensor surface (not to scale)

75°C followed by rinsing in miliQ water and dried in a stream of nitrogen gas. Previously, four methods of biomolecular immobilization were tested on the gold surface and the thiol functionalized self assembled monolayers (SAM), or biotin-neutravidin capture on the biotin-functionalized thiols, provided the most stable baseline (no changes in frequency even over long periods of time) and minimized non-specific binding (proteins will stick to a gold surface with or without a binding partner present).[3,4]

PEG modified SAM were used to minimize the nonspecific adsorption of proteins onto the surface. 90:10 combination of 1 mM of (1-Mercapto-11-undecyl) tri(ethylene glycol) and Carboxylic acid-capped tetra(ethylene glycol) undecanethiol were dissolved in alcohol (200 proof). Then freshly cleaned QCMs were immersed in the SAM solution for 24 hours.

After the 24-hours the QCM was washed in alcohol (200 proof) and dried in a stream of nitrogen. They were then placed in small dry containers having the stream of nitrogen gas blown into it to reduce the oxygen concentration, which could break down biological bonds. The containers were then wrapped with parafilm and kept at room temperature until use. For biotin-neutravidin linkage, 90:10 combination of 1 mM of (1-Mercapto-11-undecyl) hexa(ethylene glycol) and Biotin-terminated tri(ethylene glycol) hexadecanethiol were prepared the same way.[5]

For hybridization preparation, the QCM surface was activated by running 500mL of a NeutrAvidin (1 μ M) and buffer (in mM: NaCl 150, CaCl₂ 0.1, HEPES 10, pH 7.4) solution through the sensor chamber. Then the biotin linked probe DNA (either H1N1 or H5N1) was applied to the surface to link with the NeutraAvidin. 500mL of buffer with 500nM of probe DNA was used. The frequency change after NeutrAvidin was usually -40Hz, the change after the probe DNA was applied was about -15Hz. The matching, non-labeled DNA was applied at various concentrations (5 μ M, 1 μ M, 500nM, 100nM, 50nM, 10nM) over several trials in the running buffer.

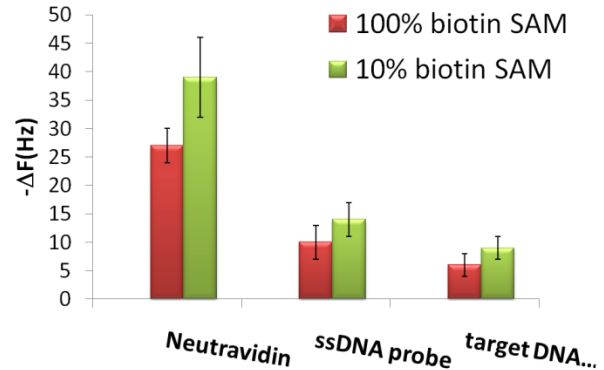


Figure 4. Frequency response comparison between 100% saturation of surface with biotin vs. only 10% saturation with biotin receptors

The control experiments with non-matching DNA strands were performed under the same conditions.

IV. RESULTS

Probe density optimization: Optimizing probe density of immobilized ss-DNA is important because, if ss-DNA is immobilized with high density, the DNA hybridization kinetics slow down due to the steric hindrance. The surface was modified by 100% or 10% biotin DNA density. At 10% probe density in the remaining area is covered by PEG as shown in Figure 3. PEG serves as a repellent for nonspecific (unwanted) biological binding. DNA strands that do not hybridize will not stick to the surface due to the PEG properties. The biotin terminations allow for the NeutrAvidin to bind, and the percent of biotin terminations relative to the surface of the sensor was studied. At 100% biotin saturation (no PEG terminations) the sensitivity of the sensor actually dropped, not only for the target DNA but for the NeutrAvidin and the DNA probe as well. At 10% biotin saturation (1:9 biotin to PEG ratio) the QCM-D sensing visibly improved (Figure 4) for both surface preparation and target DNA hybridization.

Effect of hybridization temperature: Temperature can be specified for the sensing chamber (equipment rated as working from 18°C to 45°C) at the initialization of the QCM-D measurement, and several trials were completed to determine the best sensing temperature. DNA hybridization ideally is optimal at higher temperatures, and yet the results in Figure 5 suggested little difference between 35°C and room temperature (25°C). The sensitivity did drop significantly when the temperature was lowered to 15°C. All subsequent experiments were run at 25°C since it is much easier to keep a controlled and steady environment (eliminating possible noise) when the temperature of the room matches the sensor chamber settings. The previous experiments were run at 25°C, but

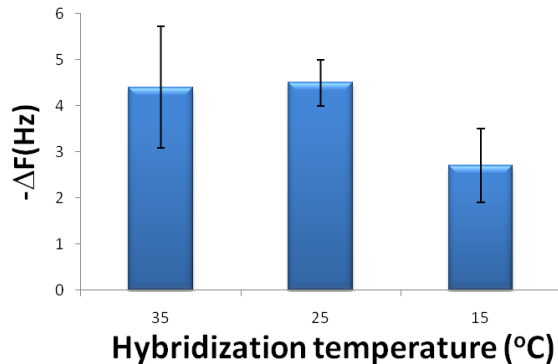


Figure 5. Effects of sensor chamber temperature on hybridization sensing

this temperature trial confirmed that it was an optimal sensing condition.

Effect of hybridization buffer: Kinetics of DNA hybridization is affected by the salt concentration in the buffer. Effects of salt concentration in the hybridization buffers were tested with matching probe and unlabeled target ss-DNA. At low salt concentrations (KCl 50 mM), the frequency showed no change at 1 uM of H1N1 DNA concentration, however, at 100 mM KCl (twice the salt concentration), the frequency change indicated that measurable binding had taken place (Figure 6). The high salt concentration buffer (Tris 10 mM, KCl 100 mM, EGTA 1mM at pH 8.0) was used in the subsequent experiments.

H1N1 DNA identification: With optimized sensing conditions, a typical sensorgram is shown in Figure 7.

The control experiment to ensure that there was no unspecific binding was to run a non-matching DNA strand over the surface. If the probe DNA was H5N1, then we could use both the H1N1 strand and a synthesized completely non-matching strand to check for any unwanted changes in frequency despite the lack of hybridization. The control experiment provided confidence that the sensor's surface indeed did not attract non-matching DNA strands. As can be seen in Figure 8, when using a H1N1 probe, the non-labeled H5N1 DNA strands did not cause any changes in frequency, whereas the hybridization of the matching H1N1 strands at the same concentration caused a measurable change.

Detection limit: Because the main reason behind these experiments was to determine the feasibility of implementing QCM-D technology as an alternative for seasonal virus detection, the main question was if the chosen system would be sensitive enough to detect polymerase chain reaction (PCR) amplified DNA. PCR is the common standard for amplifying concentrations of DNA gathered from a patient's fluid samples. It provides, on average, about 40nM concentrations of DNA. When running the trials at high concentrations, the surface was

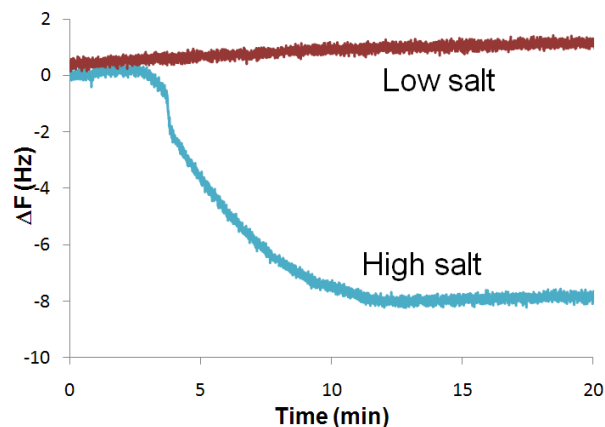


Figure 6. Comparison of frequency response before and after an increase in salt concentration

immediately saturated and showed no further change if additional DNA was added. At 10nM the effects of hybridization became indistinguishable from noise levels. As seen in Table 2, at 50nM, three or four samples could be run before saturating the surface of the crystal, thus having to switch it out for a new one. If the immobilized DNA level was 15 Hz, the detection limit of sensor reached 50 nM.

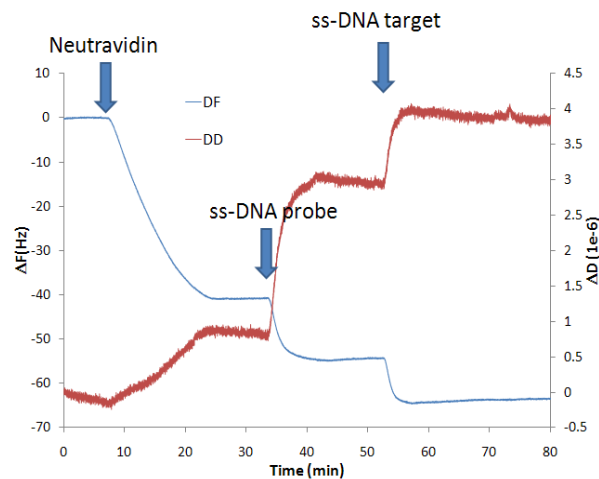


Figure 7. Example of Q-Sense plot of frequency and dissipation against time during binding as well as DNA hybridization

Target DNA concentration	Reusability	Immobilization Level	Detection Limit
500nM	1 time	10Hz	~100nM
50nM	3-4 times	15Hz	~50nM

Table 2. Reusability of surface in continuous trials at different concentrations

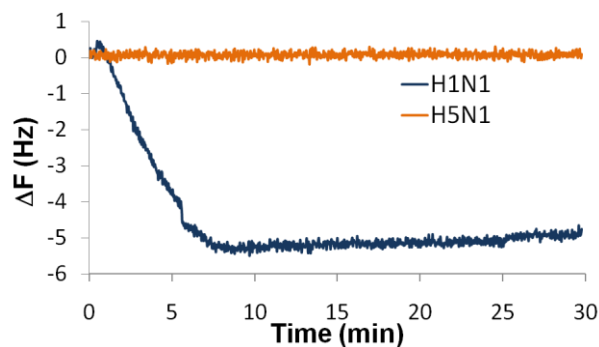


Figure 8. Hybridization results when using a surface prepared with the H1N1 probe

V. DISCUSSION

Current DNA hybridization can be done through micro arrays, with the advantage that they can test for a range of viruses (DNA strands) simultaneously, but have a high infrastructure and equipment costs. Gel electrophoresis is another technique that uses hybridization, but takes more than 18 hours before results can be analyzed. The method proposed in this paper is limited to one strand of DNA, but it is useful in the case of seasonal epidemics and outbreaks that need to be controlled as quickly as possible, and are difficult to diagnose properly despite the widespread infection.

Future work includes attempting to distinguish a perfectly matching strand's hybridization from one that has a single base which is mismatching. Nearing the end of our research, an Electrochemical Infrared Spectroscopy (EIS) system was set up in an attempt to detect smaller differences using a higher sensitivity sensing method.[7] The combined QCM-D and EIS measurement will allow distinguishing the difference between a single or couple of mismatches in target DNA sequences.

The trials described in this paper gave positive indication that the QCM-D system could be used to quickly diagnose patients for specific virus DNA strands. If the crystal surface and DNA amplification is prepared beforehand, setup takes about 30 minutes, with each trial plotting results in a matter of 10 minutes (up to four trials can be run on the same surface).

VI. ACKNOWLEDGEMENT

This project was funded by Andrews FRG (Dr. Kwon) and URG (Prieto).

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