Electromechanical Detection of Pathogens with Self-Assembled Nucleic Acid Biosensors

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ABSTRACT

Monitoring of water samples in remote locations and the developing world is crucial to protect people from infectious diseases primary caused by enteric pathogens. Routine pathogen detection, which is usually based on cell cultivation methods, is labor intense and time consuming. Some pathogens may cause diseases with rapid-evolution symptoms. Therefore, fast, sensitive and reliable detection of pathogen contamination is of great significance. Biosensor technologies are currently under intense investigation for detecting pathogens responsible for diseases in various fields. For point of sample applications, the detection platform should be cost-effective, fast, sensitive, easy to use, stable under a wide range of operating conditions, and portable. Nucleic acid-based biosensors show great potential for integration in a lab-on-a-chip (LOC) for selective and sensitive detection of target microorganisms. Especially schemes employing electromechanical signal transduction are simple and straightforward, *e.g.* quartz crystal microbalances (QCM). In this work, we show two different QCM-based detection schemes: a proof-of-concept study for indirect detection of *Escherichia coli* (*E. coli*) via matching of characteristic DNA sequences and a direct capture biosensor utilizing an *E. coli* specific aptamer.

Keywords: aptamer, biosensor, quartz crystal microbalance, pathogen detection

1 INTRODUCTION

Infectious diseases, primarily caused by enteric pathogens, remain a major cause of death, especially in remote locations of the developing world[1]. Routine monitoring of pathogen contaminations is therefore crucial. We seek to apply quartz crystal microbalances (QCM) functionalized with highly selective nucleic-acid capture probes to provide a cost-effective, fast, easy to use and portable biosensing platform. While traditional methods are usually based either on time-consuming bacterial culturing or laboratory-based assays, which require specialized equipment and trained staff[2], these nucleic-acid based QCM biosensors can enable sensitive and reliable real-time detection of various analytes associated with pathogen contaminations (*e.g.* secreted toxins, characteristic DNA sequences or whole bacteria cells). In combination with reusable substrates, implementation in microfluidic LOC devices and the flexibility for tailoring analytical assays, electromechanical biosensing via miniaturized QCM systems is highly suitable for point of care applications. These nucleic acid-based biosensors can provide further advantages, such as long shelf-life and reusability, when compared to assays employing more delicate capture probes, *e.g.* antibodies[3].

QCM systems have been routinely used for many bioanalytical questions in research and laboratory settings[4]. However, to the best of our knowledge, no QCM systems for specific biosensing assays are routinely used in the field or at the point of sample. Quartz crystal microbalances are based on a measurement of the characteristic crystal resonance frequency and are capable of measuring mass changes in the range of nanograms per cm^2, which cause a decrease in the crystal resonance frequency (see Figure 1). When equipped with a highly specific capture probe, such QCM biosensors can be used for various biosensing applications, wherein the resonance frequency change can be attributed to specic binding of the target molecule to the functionalized QCM biosensor.

Besides the well-known base pairing and hybridization capabilities of single stranded DNA for the detection of characteristic DNA sequences, a new class of nucleic acid capture probes, aptamers, can undergo functional folding into a 3D structure, allowing them to specically bind a target molecule. Aptamers are selected in vitro, based on their affinity towards a certain analyte (*e.g.* ions, small molecules, proteins, whole cells)[3, 5, 6]. They exhibit strong binding affinities - similar or even stronger than those of antibodies. Owing to their excellent stability, consistent quality, small size and broad possible target range, many assays can be designed utilizing aptamers as receptor molecules[3]. The performance of aptamers, *i.e.* characterized by the binding affinity for their targets, is crucial for the sensitivity of the developed biosensor[7].

Herein, we show two different QCM-based detection schemes: a proof-of-concept study for indirect detection of *Escherichia coli* via matching of characteristic DNA sequences and a direct capture biosensor utilizing an *E. coli* specific aptamer.

2 MATERIALS AND METHODS

2.1 Materials

DNA oligonucleotides were purchased from Integrated DNA Technologies with respective modifications as shown in Table 1. All buffers and solutions were prepared with deionized RNAse free water (18MΩ·cm resistivity) from a Millipore MilliQ system. Tris(2-carboxyethyl) phosphine, HCl (TCEP), 6-Mercaptohexanol (MCH), hydrogen peroxide, 30% (wt/wt) and all buffer salts were purchased from Sigma-Aldrich. Sulfuric acid 98% (wt/wt) was a Merck product.

Table 1. Oligonucleotide sequences used in this work.

Sequence Name	Sequence (5' to 3')	Modification
Capture oligo	CCGGACGCTTAT GCCTTGCCATCTACAG AGCAGGTGTGACGG	5' Thiol
Complementary	CCGTCACACCTGCTCT GT	5' Alexa488
Non-complementary	CCATGGTGTGACGGG CGGTGTGTACAAG	none
Aptamer[8]	ATCCGTCACACCTGCT CTGTCTGCGAGCGGG GCGCGGGCCCGGCGG GGGATGCGTGGTGTT GGCTCCCGTAT	5' Thiol

2.2 Crystal Cleaning and Functionalization

Before use, AT-cut gold coated quartz crystals with a diameter of 0.5 cm and a resonance frequency on the order of 10 MHz (Gamry Instruments) were rinsed excessively with water and isopropanol, then dried under a nitrogen stream. Next, chemical cleaning was performed with hot piranha solution consisting of 98% sulfuric acid and 30% hydrogen peroxide at a volume ratio of 3:1. To clean each gold layer, 15 µL sulfuric acid and 5 µL hydrogen peroxide were directly added to the surface to oxidize surface-bound contaminants for 2 minutes. Subsequently, the gold surface was rinsed with copious amounts of water and finally dried under nitrogen. This procedure was repeated twice for each side of the quartz crystal, followed by immediate mounting of the crystal into a custom-made QCM chamber.

For the two different systems – indirect and direct biosensing – different immobilization strategies were followed. Briefly, for immobilization of thiol-modified sequences, oligonucleotides diluted to the desired concentration were incubated with TCEP in 200-fold excess for 20 min. Subsequently, the solution was heated at 95°C for 5 min and then let cool to room temperature.

For immobilization of the capture oligo, 200 µL of 8 µM activated oligo solution was drop casted onto a clean gold crystal and incubated for 4 hours. Rinsing was performed with phosphate bufferd saline (PBS) supplemented with 1 mM $MgCl_2$ (PBS^+).

For aptamer-functionalization, a co-immobilization protocol was chosen[9, 10]. Herein, activated oligo solution (1 µM) was mixed with MCH (20 µM) befrore drop casting of 100 µL solution onto the gold crystal. After immobilization over night, an additional blocking step with 1 mM MCH was carried out for 15 min. Between the steps, the gold crystal was rinsed repeatedly with aptamer selection buffer (SB, 10 mM Tris-HCl, 500 mM NaCl, 1 mM $MgCl_2$).

2.3 Sample Preparation

For DNA hybridization measurements, dilutions of complementary and non-complementary DNA were prepared in PBS^+.

For direct capture experiments, cultures of *E. coli* (Crooks, ATCC 8739) or *Rhodopseudomonas palustris*

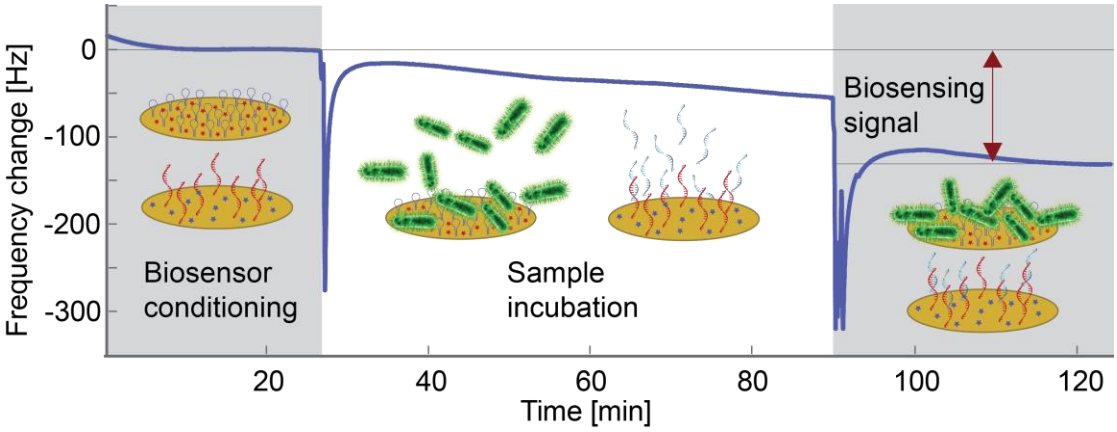

Figure 1: Schematics of a typical QCM biosensing experiment. After functionalization of the QCM crystal, a baseline of the resonance frequency is recorded. Subsequently, the sample solution is introduced and incubated on the QCM crystal while the resonance frequency is recorded in real-time. Finally, the biosensor surface is rinsed thorougly to remove unbound species and then the final biosensing signal is obtained.

(kindly provided by the Geobiology group at the Divison of Geological and Planetary Science, Caltech) were grown in nutrient broth or lysogeny broth, respectively, until an optical density of 0.5 (OD_{600}), which equals about 10^8 cells/mL. Samples of the cultures (1 mL) were spun down in a standard lab centrifuge (10 min at 5000·g), replacing the supernatant by 1 mL SB. Following the re-suspension of the cell pellet, the centrifugation and buffer replacement were repeated two more times before the final cell pellet was either resuspended in tap water or SB at an appropriate dilution.

2.4 Biosensing Experiments

For DNA detection via hybridization with a complementary sequence, the functionalized gold crystal was incubated for 1 min with heated PBS^+ prior to recording of a baseline in PBS^+ at room temperature. Subsequently, 200 µL of the respective DNA sample were casted on the gold surface and resonance frequency of the crystal was continuously measured for 2 h. Lastly, the surface was rinsed several times before recording the final resonance frequency in buffer after hybridization.

Biosensing experiments for the direct capture of pathogens were performed similarly: Before recording of the initial biosensor baseline, the gold surface was incubated with boiling water for 2 min and then rinsed with SB. 100 µL of *E. coli* suspension or other respective samples were exposed to the conditioned biosensor for 1 h, followed by rigorous rinsing with SB and recording of a final biosensing signal.

A typical signal evolution during the biosensing experiment is shown in Figure 1.

2.5 Data Analysis

Resonance frequencies during crystal functionalization and biosensing experiments were recorded using the Gamry Resonator Software. Results were expressed as change in resonance frequency (in Hz), comparing values during baseline establishment and after sample exposure. Therefore, frequency averages and standard deviations for all stages of the experiment were automatically calculated using a custom written Matlab code.

3 RESULTS AND DISCUSSION

3.1 Oligonucleotide Immobilization

Synthetic oligonucleotides can be modified with many different chemical functionalities during their synthesis. For subsequent immobilization on gold surfaces, thiol groups provide for the most simple strategies: self-assembled monolayers are obtained upon chemisorption of the activated thiol-oligonucleotides on the gold surface[11]. Mercaptohexanol can be used in a co-immobilization, filling in gaps and allowing for an ordered orientation of the DNA strands, which is expected to improve the functionality of the immobilized DNA probes[10].

In a QCM, DNA immobilization can be observed as a significant decrease in resonance frequency, depending on the employed aptamer concentration. Furthermore, electrochemical methods, such as cyclic voltammetry and colorimetric assays (*i.e.* Ellman's assay) were used in this study to show the formation of a continuous nucleic acid layer on the gold-coated quartz crystal (data not shown).

3.2 Biosensing Experiments

3.2.1 Biosensing of Complementary DNA

Characteristic DNA sequences are most commonly used as indicators for pathogen contamination in all assays based on polymerase chain reaction (PCR)[12]. While mostly fluorescence dyes are used to detect the PCR products, simple incubation with a DNA-functionalized quartz crystal allows for the quantification of amplified characteristic DNA. In the present study, gold-coated quartz crystals were functionalized with 8 µM DNA and exposed to different concentrations of complementary and non-complementary target DNA. While a resonance frequency decrease can be observed upon capture of complementary DNA strands (see Figure 2), the control with non-complementary DNA shows no change in the crystal frequency (data not shown). Hybridization of complementary DNA was also confirmed by fluorescence microscopy of the crystal, leveraging the fluorophore tag (Alexa488) of the complimentary sequence (data not shown). A detection limit of 10 nM target DNA was achieved.

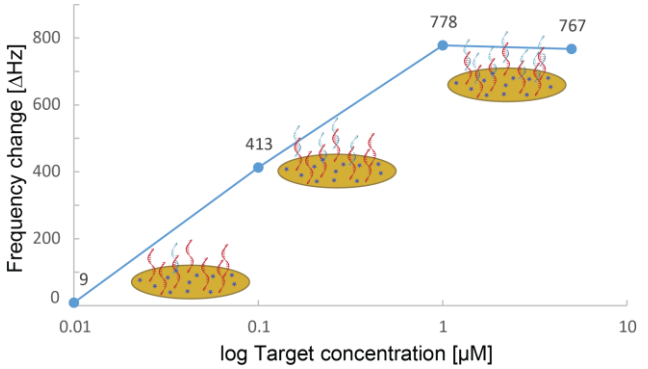

Figure 2: Frequency changes upon hybridization of complementary DNA strands at different concentrations and schematics thereof.

3.2.2 Aptamer-Based Direct Capture

Cell lysis, DNA purification and amplification are labor and instrumentation-intensive steps needed for PCR-based biosensing assays[2]. Herein, an aptamer selected to specically target the outer membrane proteins of *E. coli* Crook's strain[8] was employed to demonstrate the QCM's capability to bind and thus detect *E. coli* cells directly from a sample suspension without any pre-treatment. 1 µM

aptamer was co-immobilized on gold-coated QCM crystals and exposed to different samples. Results are presented in Figure 3, as resonance frequency decrease after removal of unbound species by thorough rinsing.

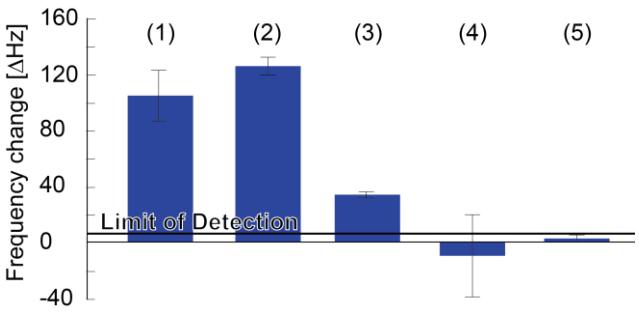

Figure 3: Results of direct capture biosensing experiments. (1) 10^8 cells/mL *E. coli* in buffer; (2) 10^8 cells/mL *E. coli* spiked into tap water; (3) 10^7 cells/mL *E. coli* in buffer; (4) 10^8 cells/mL *R. palustris* in buffer; (5) pure tap water (n≥3).

10^7 cells/mL *E. coli* could reliably be detected in an experiment with a duration of <2 hours, while negative controls with non-target organisms or unspiked tap water did not result in significant frequency changes.

4 CONCLUSION

This study shows the application of a simple and rapid biosensing assay, employing quartz crystal microbalances and nucleic acid receptor probes. The hybridization assay resulted in the sensitive detection of complementary DNA at concentrations as low as 10 nM. The direct detection of whole cells, on the other hand, required no previous sample preparation and pathogen detection could be achieved in less than 2 hours at a current detection limit of 10^6 cells. It is to note, that the demonstrated sensitivity is highly dependant on the utilized aptamer sequence and its target affinity. At the same time, a rapid adaption of the assay to employ different aptamer receptors and thus, capture of different pathogens, is easily achievable with the presented assay. Aptamer sequences have already been published *e.g.* for the binding of *Salmonella typhimirium*[13, 14] , *Mycobacterium tuberculosis*[15] or *Staphylococcus aureus*[16]. Our current research focuses on the implementation of supplemental amplification steps to lower the detection limit by using specific secondary binders. Also, a combination with pre-concentration steps could be easily implemented for increased sensitivity[17]. Furthermore, we are developing a low-cost miniature prototype to perform the entire biosensing experiments in an automated manner.

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REFERENCES

1. Yousef, A.E., Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems, 2008: p. 31-48.
2. Foudeh, A.M., et al., Lab on a Chip, 2012. **12**(18): p. 3249-3266.
3. Song, S., et al., Trends in Analytical Chemistry, 2008. **27**(2): p. 108-117.
4. Skládal, P., Trends in Analytical Chemistry, 2016. **79**: p. 127-133.
5. Tuerk, C. and L. Gold, Science, 1990. **249**(4968): p. 505-510.
6. Ellington, A.D. and J.W. Szostak, Nature, 1990. **346**(6287): p. 818-822.
7. Urmann, K., et al., in *BioNanoMaterials*. 2016.
8. Bruno, J.G., et al., J Fluoresc, 2010.
9. Reich, P., et al., International Journal of Molecular Sciences, 2017. **18**(11): p. 2484.
10. Keighley, S.D., et al., Biosens Bioelectron, 2008. **23**(8): p. 1291-7.
11. Doneux, T., et al., ChemElectroChem, 2014. **1**(1): p. 147-157.
12. Lazcka, O., F.J.D. Campo, and F.X. Muñoz, Biosensors and Bioelectronics, 2007. **22**(7): p. 1205-1217.
13. Dwivedi, H.P., R.D. Smiley, and L.A. Jaykus, Appl Microbiol Biotechnol, 2013. **97**(8): p. 3677-86.
14. Joshi, R., et al., Mol Cell Probes, 2009. **23**(1): p. 20-8.
15. Chen, F., et al., Biochem Biophys Res Commun, 2007. **357**(3): p. 743-8.
16. Cao, X., et al., Nucleic Acids Research, 2009. **37**(14): p. 4621-4628.
17. Xie, X., et al., Scientific Reports, 2016. **6**: p. 20516.