Nanoparticle-amplified immunobiosensor enables excellent sensitivity in rapid detection of viable *E. coli* O157:H7

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ABSTRACT

Escherichia coli O157:H7 is a significant pathogenic microorganism responsible for both food- and waterborne illnesses. However, time-consuming culture remains the routinely used method for detection. An immunoassay using the surface of quartz crystal microbalance (QCM) sensor as a platform was developed and optimized for rapid detection of viable *E. coli* O157:H7. QCM is a piezoelectric sensor able to sensitively detect the deposition of mass on its surface by changes in resonance frequency – in this case, the sequential mass increase due to antibody immobilization to the surface, bacterial capture, and the binding of a second antibody group conjugated with gold nanoparticles. The method achieved high sensitivity for rapid and specific detection of viable *E. coli* O157:H7, with a pre-enrichment detection limit of 3 log CFU/mL and post-enrichment detection limit of 0 log CFU/mL. The specificity of the method was demonstrated by a lack of significant frequency change when comparable concentrations of other bacteria were tested. Results are available within hours. The method has excellent potential for rapid coliform detection.

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KEYWORDS: quartz crystal microbalance, *E. coli* O157:H7, biosensor, nanoparticle

ABBREVIATIONS AND ACRONYMS:

QCM: quartz crystal microbalance

AuNPs: gold nanoparticles

BHI: Brain-Heart Infusion

BSA: bovine serum albumin

CFU/mL: colony forming units per milliliter

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BIOGRAPHY

I am a senior at Bangor High School in Bangor, ME. I have conducted research for the past two years. In addition to science, I enjoy playing the piano, creative writing, and a plethora of other activities. I hope to major in economics in the fall.

INTRODUCTION

Rapid detection of pathogens in both food and water has always been a key issue in safety. Due to increased media coverage of pathogen outbreaks, people have become more vigilant about pathogen contamination. Contamination by pathogenic bacteria can cause serious illness in humans, sometimes resulting in death. The US Centers for Disease Control and Prevention (CDC) estimates that more than 76 million cases of illness occur annually because of foodborne pathogens, and the Economic Research

Service estimated that costs associated with five major pathogens, including *Salmonella* and *Escherichia coli* amounted to about US$ 6.9 billion annually [15]. Needless to say, people have realized the importance of detection when it comes to pathogens.

Escherichia coli O157:H7 was first found in 1982 and is one of the most hazardous foodborne pathogens [8]. The US Centers for Disease Control and Prevention reported an estimated 73,000 cases of infection and 61 resultant deaths occur in the United States every year [1]. *E. coli* O157:H7 can easily contaminate several types of food and drinking water [6]. In drinking water, it poses a large threat to water safety: as a fecal coliform, *E. coli* in drinking water usually indicates sewage or animal waste contamination, usually resulting from agricultural runoff; in public water – such as pools – it indicates that the water may have been contaminated by human waste, and poses a large threat to human health – especially individuals with compromised immune systems [3]. As few as ten to one hundred *E. coli* O157:H7 cells can grow in the intestine and produce toxins that kill the cells of the intestinal lining, destroy the kidneys, cause blood clots in the brain, as well as cause seizures, paralysis, and respiratory failure [16].

Traditionally, testing bacteria is done with specific media to isolate and count the bacterial cells as observed on plates. The technique, while considered the traditional gold standard method, is labor-intensive and very time consuming, often requiring several days to obtain results [14]. For example, *E. coli* O157:H7 is conventionally plated on MacConkey Sorbitol Agar. This method takes 2-3 days for enrichment and detection, and up to 4 days for a final characterization [15]. While fairly accurate, it remains evident that the time-consuming step of waiting for cultures to grow detracts from the efficiency of the analysis process.

Thus, the development of a rapid detection method has become a very important topic in water safety today. Tests that can be completed within hours – or even minutes – would enable quick corrective action to be taken when contaminants are detected [10]. There is a variety of rapid methods proposed to detect microorganisms, including antibody-based methods, nucleic acid-based methods, enzymatic methods, and membrane filtration methods, to mention a few [17]. Modern biotechniques, such as real-time PCR [4], nanoparticles [8] and biosensing systems, or biosensors [8], have been developed for the detection of pathogens.

Biosensors can be an exciting alternative to traditional detection methods. They use a combination of biological receptor compounds (antibody, enzyme, nucleic acid, etc.) and the physical or

physicochemical transducer directing, in most cases, real-time observation of a specific biological event, such as antibody/antigen reactions [9]. The importance of biosensors relies on their high specificity and sensitivity, which allows for detection with minimal sample preparation. As a result, biosensor technology is gaining popularity. It has shown great potential for rapid, sensitive, and compact systems, but is not ready for a low detection limit and differentiation between live and dead cells [7].

Quartz Crystal Microbalance (QCM) has been widely used in chemical and biological sensing. The core of the QCM is a quartz plate that is excited to resonance and then monitored. Voltage is applied to the film electrodes, which are usually gold (Au), on the surface of the crystal, producing motion. The crystal is then induced to oscillate at a specific frequency. Any changes in the mass of surface material will alter the resonance frequency, resulting in an inversely proportional relationship between resonance frequency and mass on the QCM electrode at the nanogram level or less [18].

Immunosensors based on the detection of antibody-antigen binding [5] have been successfully employed for the detection of microorganisms [9]. One type of immunosensor uses a sandwich assay, in which interaction between immobilized antibodies, free antigens, and labeled antibodies "sandwiches" the antigen between the immobilized and the labeled antibodies [10].

Gold nanoparticles (AuNPs) have been previously used in biosensing methods. Because of their good biological compatibility, conducting capability, and high surface-to-volume ratio, AuNPs have become more and more widely used in electrochemical sensors [5]. They have been used in combination with a variety of techniques, such as electrochemical sensing [5], enzyme biosensing, and DNA sensing [2]. Antibodies can be directly immobilized onto AuNPs [12], and can therefore be used to improve the sensitivity of instruments using the sandwich assay. However, at the same time, nonspecific binding is introduced due to interactions between the nanoparticles and proteins [12]. Nevertheless, many studies have continued to use AuNPs without addressing the issue of nonspecific binding.

Brain Heart Infusion (BHI) is a nutritious, buffered culture medium that contains the nutrients necessary to support the growth of microorganisms. Although previously used in QCM work to create a biofilm on the surface of the chip to confirm bacterial growth [11], BHI has not yet been applied in viable pathogen detection in a biosensor setting. Thus far, methods for rapid pathogen detection have generally used nonviable bacteria [9] or bacterial DNA [2] in assay development; therefore, the methods developed detected both viable and nonviable bacteria. The number of viable bacteria is most closely

correlated with the seriousness of contamination, so a method that can specifically detect viable bacteria quantitatively is more desirable than one that detects indiscriminately.

In this study, a method for rapid detection of viable bacteria was developed and optimized, using a sandwich immunoassay on QCM platform (biosensor). AuNPs-antibody conjugate significantly amplified the signal of the biosensor; the coating of the AuNPs component of the conjugate with bovine serum albumin (BSA) to block the nonspecific binding of AuNPs to other substances also significantly improved the specificity of amplification. In addition, viability of the captured bacteria was confirmed by post-capture enrichment using BHI broth.

MATERIALS AND METHODS

2.1 Cultures

All pathogen species used were obtained from the American Type Culture Collection (ATCC; Manassas, VA.) including *Escherichia coli* O157:H7 (ATCC 35150), generic *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (Scot A ATCC 49594) and *Salmonella* Typhimurium (ATCC 6962). Cultures were grown in brain heart infusion (BHI, Acumedia manufacturers, Lansing, MI) broth at 37°C for 18~24 hours and diluted with phosphate buffered saline (PBS) before application to the QCM system.

2.2 Media

MacConkey Sorbitol Agar (MSA, Acumedia manufacturers), a selective medium for *E. coli* O157:H7 and generic *E. coli,* was used for plating to confirm bacterial counts. *E. coli* O157:H7 colonies appeared colorless, while generic *E. coli* appeared pink. Oxford medium (OX, Acumedia manufacturers) was used for enumeration of *L. monocytogenes*, which appeared black. XLD agar (Acumedia manufacturers) was used for enumeration of *S.* Typhimurium, which appeared black. BHI broth was used as nutrient for all bacteria and was filtered through a 0.22 μM aseptic filter to remove debris after autoclave and before use.

2.3 Chemicals

All chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). Phosphate buffered saline (PBS) at pH 7.4 was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 into distilled water to a final volume of 1 litter. After autoclaving, the buffer was filtered using a 0.22 μM aseptic filter (Millipore, Billerica, MA) before application to the system.

Phosphate buffer (PB) at pH 7.4 and 0.1 M concentration was used to dilute antibodies for conjugation with gold nanoparticles. PB was prepared by dissolving 3.1 g $NaH_2PO_4 \cdot H_2O$, 10.9 g Na_2HPO_4 (anhydrous) into distilled water to a final volume of 1 litter.

In order to further confirm bacterial capture, chips were stained with 0.1% acridine orange (AnaSpec Inc., San Jose, CA) and then examined using a motorized inverted microscope (Olympus IX81, Center Valley, PA).

Piranha solution, comprised of a 3:1 mixture of sulfuric acid (H_2SO_4, Fisher Scientific) and 30% hydrogen peroxide (H_2O_2, Fisher Scientific), and one percent of Polyethyleneimine (PEI, Sigma, St. Louis, MO) dissolved in methanol were used to pre-treat the chip surface before use. Glutaraldehyde (GA, Acros Organics, Morris Plains, NJ) was diluted with PBS to a working concentration of 2.5 %. Antibodies used were 55.6 µg/mL goat anti *E. coli* O157:H7 (Meridian Life Science, Saco, ME). Bovine serum albumin (BSA) was a product of Acros Organics.

Two and one half mM $HAuCl_4 \bullet 3H_2O$ (RICCA chemical company, Arlington, TX, USA.) and 38.8 mM sodium citrate (Fisher Scientific), and ddH_20, filtered to >18MΩ, using a Barnstead (MS, USA) Nanopure filtration system, were used for AuNP fabrication.

For gel electrophoresis, 1% agarose gel (Fisher Scientific) and 20% SDS (BioRad, Hercules, CA, USA) were used. A working 1x tris acetate (TAE) buffer (pH 8.3) was prepared by diluting the stock solution (50x TAE) with ddH_2O. TAE stock solution was prepared by adding 242 g Tris base (Fisher Scientific), 57.1 mL glacial acetic acid (Fisher Scientific) and 18.6 g ethylenediamine tetraacetic (EDTA, Fisher Scientific) to 900 mL ddH_2O; pH was adjusted to 7.6.

2.4 Equipment

The quartz crystal microbalance 200 Digital Controller (5 MHz) used in this study was purchased from Stanford Research System (SRS, Sunnyvale, CA). The piezoelectric quartz crystals (2.54 cm reaction area diameter), which consisted of a 5 MHZ AT cut quartz crystal slab with a layer of a gold electrode on each side, were also obtained from Stanford Research Systems, Inc. (Sunnyvale, CA). The system's five main components include a QCM25 Crystal oscillator, a QCM200 Quartz Crystal microbalance controller, a crystal holder with a axial flow cell, a circulating flow system (ISMATEC, Vernon Hills, IL) in a 37 ± 0.04 °C water bath (VWR, West Chester, PA), and a computer to chart the curve of frequency change (ΔF) in real time. The experimental data were analyzed by SRSQCM200 software in real time. The charts were made by R software. The sensor unit has the

following features: resolution: 1.0 Hz, gate times: 0.1 s, frequency scale: 200 Hz, temperature range: 0–40℃. The reaction cell was one sensor signal channel with 0.15 mL of reaction cell volume. The circulating-flow system consisted of a temperature controller, sample tubes, pipelines and one tubing pump with a flow rate of 130 μL/min, and a 1 mL sample volume. Tubing used for circulation was 0.44 mm in diameter (Ismatec, Glattbrugg, Switzerland).

A spectrophotometer DU Series 530 (Beckman Coulter, Fullerton, CA) was used to measure absorbance of the AuNPs colloid.

2.5 Antibody-conjugated Au Nanoparticles

Gold nanoparticles (AuNPs) with an average diameter of 13 nm were prepared by using 2.5 mM $HAuCl_4 \cdot 3H_2O$ (solution A) and 38.8 mM sodium citrate. A total of 15 mL ddH_20 was added to 10 mL $HAuCl_4 \cdot 3H_2O$ and heated with stirring in a glycerol bath. When the temperature of the glycerol reached 130°C, 3 mL sodium citrate was rapidly injected into the boiling solution A. Following injection, the solution was continuously stirred for approximately 10 min. Once the color turned red, the solution was removed from the hot plate. A spectrophotometer DU Series500 (CA, USA) was used to read the absorbance at 520 nm, the OD^{*}_{520nm} value of the colloid.

One μL 1.15 mg/mL antibodies and 49 μL 0.1 M PB were added to 950 μL AuNPs. The mixture was incubated at 4°C overnight (~12 hours) in a sealed test tube with periodic inversion. Before use, AuNPs-antibody conjugate were centrifuged for 10 min at 4°C and 514 g. The supernatant was taken and the aggregated precipitate discarded. The process was repeated twice before adding 10 μL of 1 mg/mL BSA to make a final concentration of BSA 10 μg/mL, and the colloid was incubated overnight (~12 hours) with periodic inversion.

One mL of the above BSA-coated AuNP-antibody conjugate colloid was centrifuged for 5 min at 15,557 g. The supernatant was discarded and the pellet then resuspended in 50 μL ddH_2O. The conjugation of antibodies and AuNPs was confirmed by subjecting 17 μL of the above suspension to 1% agarose gel electrophoresis in TAE buffer for 30 minutes under 125 volts. Gel electrophoresis samples were prepared by mixing 1 μL of 20% SDS, 2 μL of 60% glycerol, and 17 μL of colloid.

2.6 QCM Immunoassay

The Self-Assembled Monolayer (SAM) technique was used to prepare the QCM immunosensor (Fig. 1). The QCM chip was first covered with 1 ml of 2.5% glutaraldehyde (GA) solution for 30 min through circulation. One ml of 55.6 μg/ml anti-*E. coli* O157:H7 was then circulated for 30 min. The

QCM chip with immobilized antibody was then treated with 1 ml of 10 mg/ml BSA for 30 min. to block nonspecific sites on the chip surface. PBS was used to wash the system after each step to obtain a baseline for the next step.

Fresh *E. coli* O157:H7 was enriched in BHI broth for 18-24 hours. The bacteria were then centrifuged at 12,857 xg for 10 min at 4 °C, and the supernatant was discarded. PBS was used to resuspend the bacteria. The process was repeated twice. The resuspended bacteria were then diluted with PBS, and 1 mL of the diluted bacteria suspension was circulated in the QCM system for 1 hour. The concentration of the resuspended bacteria was enumerated by MSA plate.

Following the capture of bacteria by the immobilized first antibody, the second antibody (BSA-coated AuNPs-antibody conjugate colloid was circulated through the system for 30 min (Fig 1). The system was rinsed using PBS between steps to provide a frequency baseline for subsequent comparison.

After *E. coli* O157:H7 was captured by the immobilized antibodies, BHI broth was injected into the system to promote bacterial growth in the QCM chamber. Frequency and resistance were recorded by the QCM system for 18~24 hours during enrichment. In order to confirm bacterial capture and detection, chips were stained with 0.1% acridine orange and examined using a motorized inverted microscope.

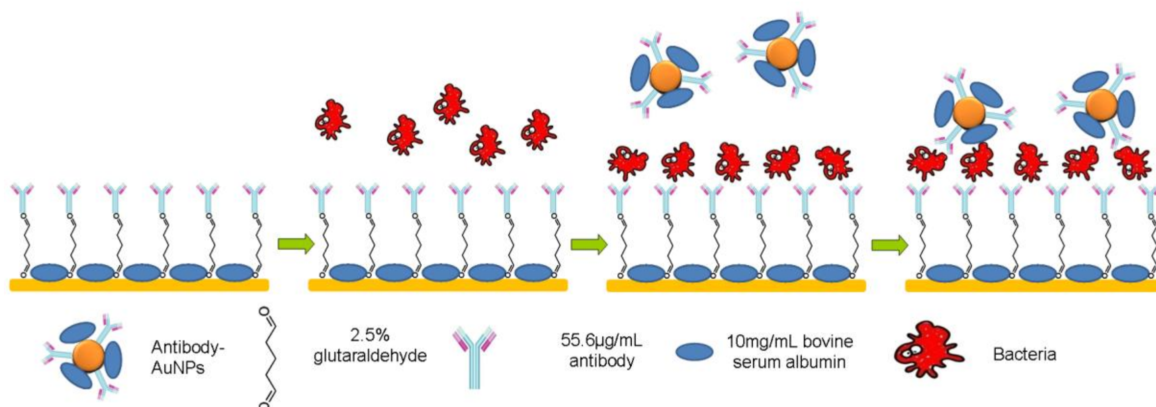

Fig. 1. Use of the antibody-conjugated nanoparticles for signal amplification in detection of *E. coli* O157:H7 with QCM immunosensor.

RESULTS AND DISCUSSION

3.1 Fabrication and optimization of BSA-coated AuNPs-antibody conjugate colloid

The fabrication of BSA-coated AuNP-antibody conjugate was optimized through three steps: preparation of AuNPs, preparation of AuNP-antibody conjugate, and coating of the conjugate with BSA.

AuNPs with average diameter of 13 nm were prepared as described in Materials and Methods. PB buffer was determined to be superior to PBS due to its ability to prevent aggregation and precipitation of AuNP, because of its lower ionic strength, which allows the negatively charged AuNPs to effectively repel each other.

In the subsequent conjugation of AuNPs with antibodies, antibody concentration was optimized to produce the most stable conjugate with least amount of aggregation. Five concentrations of antibodies were tested, including: 0.278 μg/mL, 0.556 μg/mL, 1.112 μg/mL, 2.224 μg/mL, and 4.448 μg/mL. It was noted that a 1.112 μg/mL concentration of antibody yielded the most stable product with minimal aggregation, as indicated by the color of the colloid. Aggregation turned the suspension into a dark purple-red color, whereas the stable non-aggregating conjugate remained the same dark red as the original colloidal solution.

The AuNPs-antibody conjugate was intended for specific binding to captured bacteria in the sandwich immunoassay; however, the AuNP component of the conjugate has a tendency to nonspecifically bind to any proteins or uncovered surfaces, including both bacterial surface proteins and the immobilized first antibody group, causing undesirable non-specific binding, regardless bacterial presence on the chip. To prevent this from happening, nonspecific binding sites on the surface of the AuNPs had to be masked. In order to do this, different concentrations of BSA were added into the prepared AuNP-antibody conjugate. The BSA-containing AuNP-antibody conjugate was then circulated through the system while using a QCM chip coated with BSA only, or a chip without immobilized antibodies or captured bacteria. In this case, any frequency changes were entirely due to nonspecific binding. As shown in Fig. 2, a BSA concentration of 10 μg/mL masked the nonspecific binding sites on AuNPs, causing no detectible frequency change. At lower concentrations of BSA, $\Delta F = 150$, indicating that the AuNPs were not fully blocked. However, at higher concentrations, BSA caused frequency changes due to nonspecific BSA binding.

Successful conjugation of antibodies to AuNPs was confirmed with SDS-Agarose gel electrophoresis. SDS-Agarose gel electrophoresis separates particles according to size. SDS is highly negatively charged. Because the binding to the antibodies and AuNPs overwhelms any charge difference the particles of interest may have, the resulting separation is based on size only. AuNPs coated with

BSA (BSA-AuNPs), and AuNP-antibody conjugate coated with BSA (BSA-AuNPs-antibody) were compared. BSA-AuNPs-antibody moved more slowly than BSA-AuNPs, which indicated larger size and consistent with successful conjugation between AuNPs and antibody.

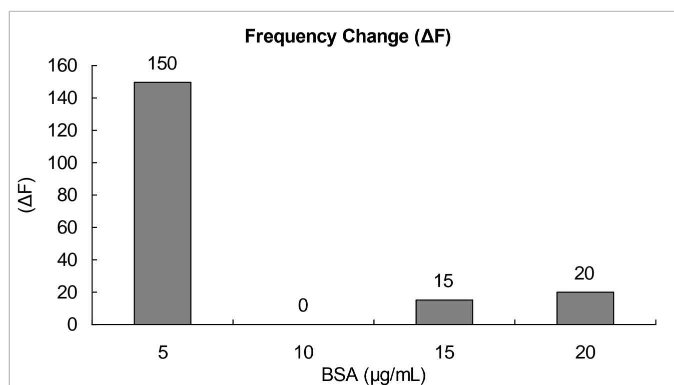

Fig. 2. Changes in frequency due to nonspecific binding of AuNPs-antibody to BSA-coated chip is dependent upon the concentration of BSA in the conjugate colloid.

3.2 Sensitive and specific detection of E. coli O157:H7 with sandwich immunoassay on QCM sensor

A representative tracing of changes in QCM frequency corresponding to the sandwich immunoassay on the surface of a QCM chip is shown in Fig. 3. The binding of *E. coli* O157:H7 at 6 log CFU/mL to the immobilized first antibody caused a change of frequency of 6 Hz, whereas the subsequent addition of BSA-coated AuNPs-antibody conjugate to the captured *E. coli* O157:H7 rendered a frequency change of much greater magnitude (95 Hz) (Fig. 3). The fifteen-fold increase in the magnitude of ΔF is highly desirable, showing this QCM biosensor to have improved sensitivity, or a lower detection limit.

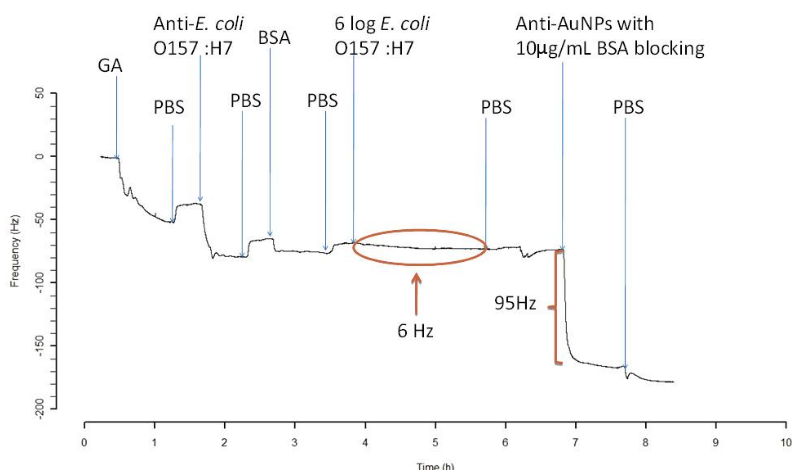

Fig. 3 Representative tracing of frequency changes of QCM.

Different concentrations of *E. coli* O157:H7 were tested using the above method. As shown in Fig. 4, as low as 3 log CFU/mL of bacteria produced a 5 Hz decrease in frequency. The magnitude of frequency change is positively related to the logarithmic concentration of *E. coli* O157:H7 between 3 and 6 logs, with a linear correlation (R^2 =0.9043) (Fig. 4).

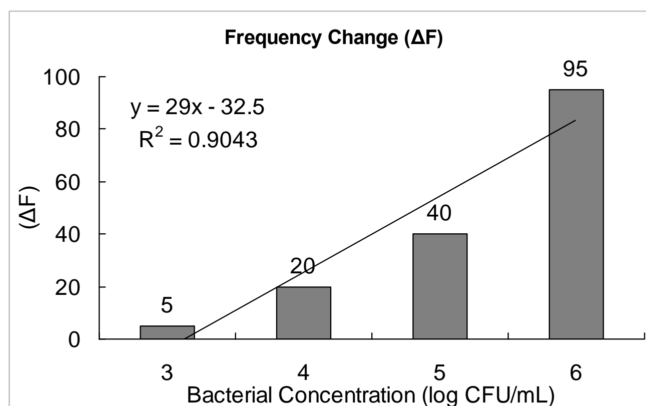

Fig. 4. Changes in frequency observed as a result of different concentrations of *E. coli* O157:H7.

Specificity was also tested. While testing of *E. coli* O157:H7 at 6 log CFU/mL resulted a magnitude of frequency change of 95 Hz, other bacteria, such as generic *E. coli*, *L. monocytogenes,* and *S.* Typhimurium, produced a changes of only 20 Hz at the same concentrations (Fig. 5). A control with no bacteria experienced a frequency increase of 10 Hz. Bacterial capture was confirmed by the use of a motorized inverted research microscope; the chip used to capture *E. coli* O157:H7 had much more bacteria on it than any of the other chips. The observed frequency changes (Fig. 5) therefore correlate with the amount of bacteria captured on the chip.

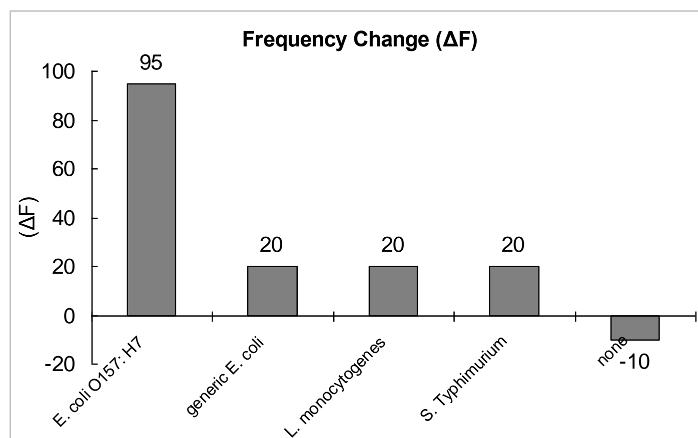

Fig. 5. Changes in frequency as observed with different genus of bacteria, all at 6 log CFU/mL.

3.3 Additional improvements in sensitivity through BHI enrichment on the QCM chip

As part of the assay process, BHI was introduced to the flow system and allowed to circulate overnight (Fig. 6). Notable frequency changes were observed with *E. coli* O157:H7, even with 0 log CFU/mL of bacteria initially present on the chip. This means that the enrichment assay can be used to determine whether or not target bacteria are present, even at very low initial concentrations. It was also noted that in generic *E. coli* showed no frequency changes throughout the same time period. The procedure serves as a qualitative assay to determine if viable specific target bacteria are present and further improves the sensitivity of the assay. Therefore, the assay can effectively detect the presence of *E. coli* O157:H7, even without an initial decrease in frequency.

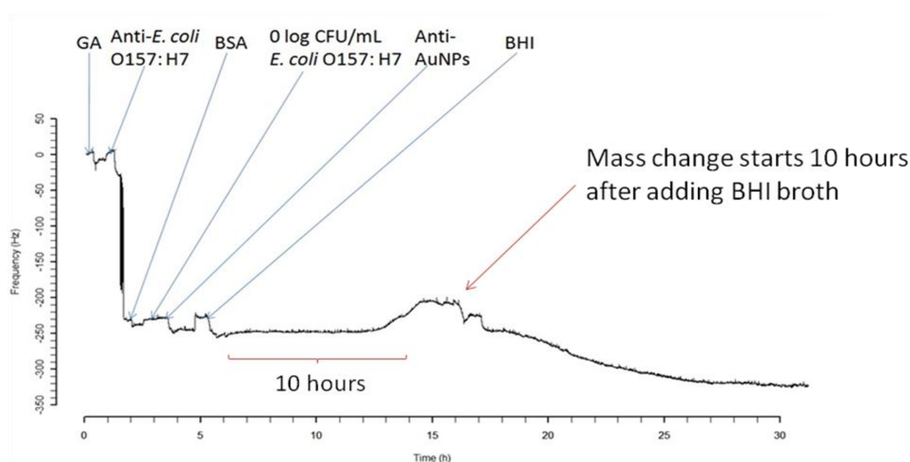

Fig. 6. Patterns in frequency as observed with 0 log *E. coli* O157:H7.

3.4 Using QCM assay to test water samples from Pushaw Lake & future work

Up to this point, the testing samples were diluted pure bacterial cultures. The performance of QCM assay was further assessed using water samples collected from Pushaw Lake. Samples were plated on Tryptic Soy Agar (TSA) and on MSA to determine viable log CFU/mL counts for all bacteria and for *E. coli*. TSA plates grow all bacteria, whereas MSA is a specific media for *E. coli.* While generic *E. coli* has no color, *E. coli* O157:H7 takes up the stain and appears a dark red. Preliminary results from plating indicated the presence of 1.2 logs CFU/mL of *E. coli* O157:H7 in the samples. Samples were also introduced to the QCM system and then enriched with BHI. While the pre-enrichment phase did not have a detectable frequency change, the post-enrichment phase shows frequency change, indicative of bacterial capture on the QCM chip. Therefore, the QCM immunoassay performs similarly with lake water samples as with bacterial cultures of *E. coli* O157:H7 of similar concentrations.

The results of this research were discussed with a member of the Maine Department of Environmental Protection, and the department has shown significant interest in the utilization of QCM technology for coliform detection in lake water. I am planning to do additional research work to further refine this technique and optimize it for coliform detection in lakes this summer.

CONCLUSIONS

1. A QCM immunosensor for the detection of viable *E. coli* O157:H7 was developed and optimized using AuNPs to lower the detection limit while still detecting specifically.
2. Nonspecific binding of AuNPs was addressed for the first time in sensing.
3. QCM can be used to specifically detect any microbe or coliform, given the specific antibody.
4. Preliminary data indicate that the QCM immunosensor is applicable in actual lake water testing.

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