DESIGN AND DEVELOPMENT OF A QUARTZ CRYSTAL MICROBALANCE IMMUNOSENSOR FOR EXOSOMES

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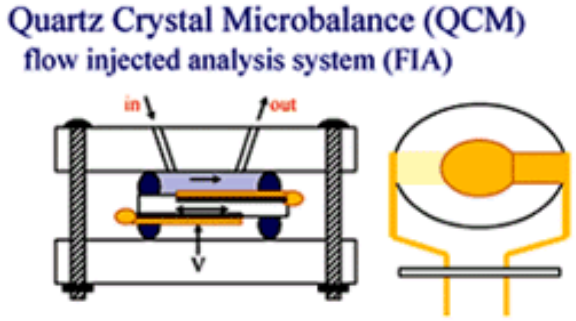
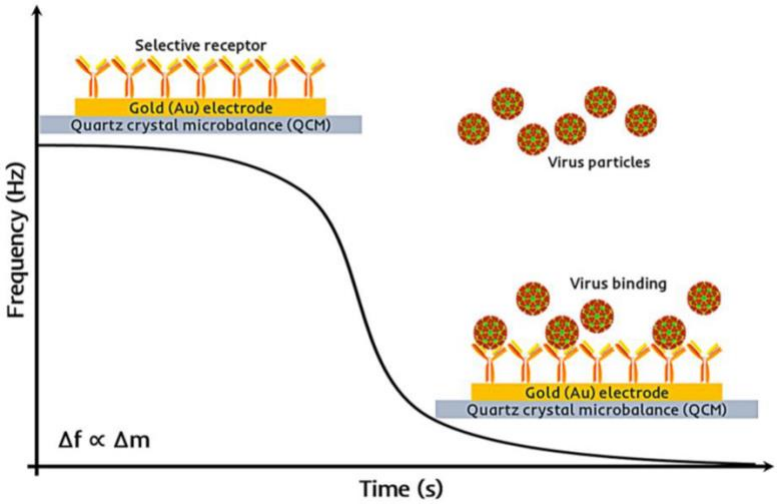
SPRING 2018

Background: Quartz crystal microbalances (QCMs) consist of a quartz crystal of known resonance frequency, the change in which can be measured and analyzed to determine the mass that is loaded onto the device.



A schematic diagram of a QCM, showing the layout of the inlet and outlet tubes, the crystal, and the gold electrode. Figure reproduced from ANT Technology Co., Ltd



A typical graph of frequency vs. time produced by a QCM in response to binding of analytes. Figure reproduced from *Chemosensors*[1]

The technology has existed for several decades, but uses are still being discovered and explored presently. In 1959, Dr. Günter Sauerbrey developed what is now known as the Sauerbrey equation. This achieved the mathematical description of the change in resonant frequency of a piezoelectric crystal with the mass deposited on it:

$$\Delta f = -\frac{2{f_0}^2 \Delta m}{A\sqrt{\rho_q \mu_q}}$$

Where Δf is the observed change in frequency, f_0 is the resonant frequency of the crystal, Δm is the mass deposited on the crystal, A is the active crystal area, ρ_q is the density of the quartz, and μ_q is the shear modulus of the quartz.

William H King, Jr. first wrote about the use of a QCM as a gas adsorption sensor in 1964[2]. Since then, QCMs have also been applied to analytes in vacuum[3] and in liquid media[4] to serve as tools for microgravimetry and viscosity testing. Since the sensitivity of the measurements made with a QCM is dependent only on the measurement of frequency, which is done easily with high accuracy and precision, QCMs were first used simply to measure extremely small masses. Applications were soon found in the field of biochemistry, however.

Chemists discovered that a QCM could be coated with certain materials that would make biomolecules of interest attach to the surface. If the analytes in question were in solution, the solution could be pumped over the crystal, depositing the biomolecules onto the surface and thus altering the resonant frequency. One early experiment used a crystal coated in polystyrene to detect the adsorption of insulin and related antibodies, which readily bound to the surface, while also showing that other molecules went undetected on the sensor[5]. The most common modification of QCMs for immunosensing is the addition of gold electrodes to the surface of the crystal. The gold electrode allows for the immobilization of several important groups in the field of biochemistry. A 2003 experiment formed a monolayer on the gold surface with 3-mercaptopropionic acid, resulting in a layer of carboxylic acid groups to interact with the analytes[6]. Schistosoma-japonicum molecular antigen was then attached to the carboxylic acid groups, and rabbit blood serum could be passed over the quartz crystal to determine if the blood was infected with the schistosomiasis parasite. The QCM is sensitive enough to detect the binding of molecules to the antigen, and thus a low-cost, small, portable infection detector had been created.

As stated before, the gold electrodes present on the surface of most QCMs allow for the immobilization of several groups of analytical interest. Thiol groups readily adsorb onto gold surfaces to form monolayers, allowing a straightforward mechanism for the immobilization of a wide range of synthetic and biological molecules. This strategy enables the immobilization of cysteine-containing proteins onto the surface of a QCM crystal. Protein G can be easily thiolated through the use of reagents such as 2-iminothiolane[7], leading to the easy formation of a Protein G monolayer. Antibodies will bind to protein G through their non-antigenic region, allowing for antigen in an analyte solution to bind the antibody and be detected by QCM. Protein A, another antibody-binding protein, can be immobilized onto gold surfaces, even without derivatization with thiols. Researchers have shown Protein A will form a monolayer on a gold electrode surface and allow for the immobilization of antibodies with more success than other methods involving multiple molecules[8]. The ease of immobilization for Protein A and Protein G makes the QCM a very valuable and intriguing tool for the analysis of biomolecules, since those proteins can bind a wide of antibodies and other compounds of interest.

The analytes at the center of this semester's work are exosomes. Exosomes are extracellular vesicles secreted by most types of human cells, but their secretion is not as well-studied or understood as other important biomolecules of the body. Their exterior consists of a lipid bilayer. The lipid bilayer encloses fluid that is protected from the outside environment. Often, the fluid will contain important regulatory biomolecules, such as enzymes or RNA. Intracellular secretory vesicles fuse with the cell membrane in order to release their contents

into extracellular space. Multivesicular bodies are secretory vesicles that contain additional vesicles; these secreted vesicles have the potential to fuse with another cell membrane, releasing their contents into a new cell. The vesicles secreted via multivesicular bodies are exosomes. Often, exosomes will travel to a cell far away from its origin to release what it carries. Because of this capability, exosomes are thought to be a potential major player in cell signaling. Even if exosomes are only a simple delivery mechanism between cells, the potential clinical applications for exosomes are very intriguing. It is certainly feasible that exosomes could be developed to serve as biomarkers for disease or vessels for delivery of medication in the future.

Unfortunately, as stated before, exosomes and their secretion dynamics are not well understood at this time. This is due to their miniscule size (typ. 50 – 100 nm diameter), which leads to great difficulty for their isolation and detection. The goal of the semester's research is to begin development of a QCM sensor specific for exosomes. The QCM's limit of detection, combined with the Sauerbrey equation, means changes in mass of at least 1.56 ng are reliably detectable. Since the mass of a typical exosome is $6.02 \bullet 10^{-7}$ ng, and the typical concentration of exosomes in biofluids is $1 \bullet 10^{11}$ exosomes/mL, a volume of 25.9 nL biofluids should be enough for reliable detection of exosomes. A major challenge is the selection and development of an appropriate system of molecules for immobilization on the gold electrode to attach exosomes, as well as a method of introducing the exosomes to the QCM to selectively immobilize exosomes for detection. If this goal can be met, it would enable new discovery in the fields of bioanalysis and medicine. Even better, if this can be done by a QCM alone, the method will be very cost-effective and relatively simple for a solution to a problem of this magnitude and importance.

Materials and Methods: Glycerol, insulin, bovine serum albumin, dithiothreitol, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $C_2H_3NaO_2$, and CH_3COOH were purchased from Sigma-Aldrich (St. Louis, MO). Protein A was purchased from ProSpec (East Brunswick, NJ). Phosphate-buffered saline (PBS) was prepared according to the following recipe:

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na_2HPO_4	10	1.42
KH_2PO_4	1.8	0.24

Unless otherwise noted, all solutions were made with PBS as the solvent.

Verification of Equilibration Time and Standard Deviations

The QCM arrived with a data sheet detailing the equilibration time and standard deviation of the results when analyzing static liquid and air in an open or closed environment. These numbers were verified in the laboratory. First, the QCM was left open to the air and allowed to equilibrate. Next, the QCM was put in the "closed" position by securing the lid on top, and once again allowed to equilibrate. Finally, milliQ water was placed onto the QCM crystal, and the device was placed in the closed position and allowed to equilibrate once more. The equilibration values and standard deviations were recorded.

Glycerol Viscosity Testing

Five glycerol standards were prepared ranging from 0% - 20% (w/w), as detailed in the table below:

% glycerol by mass	Volume glycerol (mL)	Volume milliQ water (mL)
0	0.000	5.000
5	0.200	4.800
10	0.405	4.595
15	0.614	4.386
20	0.828	4.172

The volumes were pipetted into vials for storage until they were analyzed with the QCM. Each standard was allowed to reach equilibrium, and then the next standard was pumped onto the QCM in ascending order by mass percent.

Thiol Immobilization and Insulin Testing

First, stock solutions of insulin and DTT were prepared. The insulin solution was prepared at a concentration of 10 mg/mL, which corresponds to 0.174 mM. The DTT stock solution was prepared at half the concentration of insulin, or 0.087 mM. These stock solutions were kept in storage at -20° C. At least 24 hours before each trial was run in this experiment, reaction mixtures would be prepared by mixing 1 mL insulin stock solution with 1 mL PBS, and also mixing 1 mL insulin stock solution with 1 mL DTT stock solution. In the first version of this experiment, the QCM was loaded with PBS and allowed to equilibrate. Next, the insulin/PBS solution was loaded and allowed to equilibrate. The QCM was then flushed with PBS and finally loaded with the insulin/DTT solution and allowed to equilibrate. In the second version of this experiment, the QCM was loaded with PBS and allowed to equilibrate. Next, the insulin/DTT solution was loaded and allowed to equilibrate. Finally, the QCM was flushed with PBS and allowed to come to equilibrium.

Thiol Immobilization and BSA Testing

First, a stock solution of BSA was prepared at a concentration of 0.174 mM. The second version of the procedure detailed above for thiol immobilization and insulin testing was repeated while substituting BSA for insulin.

Protein A Testing

First, a stock solution of Protein A was prepared at a concentration of 2.5 mg/mL. Next, a 0.1 M sodium acetate buffer was prepared and HCl was added dropwise to bring the pH to 4.69. The QCM was loaded with PBS, and the crystal's equilibration value was determined. Then, the crystal was removed, and 10 μL Protein A was pipetted onto the electrode surface, followed quickly by 10 μL sodium acetate buffer. The crystal was allowed to incubate at room temperature for two hours with the solutions, at which time it was analyzed in PBS with the QCM and compared to the prior resonant frequency in PBS.

Results/Discussion:

Verification of Equilibration Time and Standard Deviations

As described above, the initial testing of the crystal simply assured that it was working properly and to the standards desired for experimentation moving forward. Testing confirmed that when the crystal was running in air (closed or open) or static fluid, equilibrium was reached in under 30 minutes, and the standard deviation for the measurement was below 1 Hz for each scenario, corresponding to a relative standard deviation of less than 0.00001%. These results allow for the determination of a limit of detection (LOD) for the QCM, as well as a calculation of the amount of biofluids needed for the detection of exosomes. For the purposes of this work, LOD is defined as the minimum amount of analyte required to achieve a signal/noise ratio of 3. The standard deviation from static fluid determined above serves as the value for noise, meaning the minimum signal needed is a shift of 1.25 Hz. From the Sauerbrey equation, this corresponds to a mass of 1.56 ng. Exosomes are spherical macromolecules with a diameter of 100 nm and a density of 1.15 g/mL. This means that the mass of a single exosome is $6.02 \bullet 10^{-7}$ ng. Thus, a sample needs at least $2.59 \bullet 10^{6}$ exosomes for reliable detection. The concentration of exosomes in biofluids is known to be around $1 \bullet 10^{11}$ exosomes/mL. Using these numbers, it can be determined that a 25.9 nL sample of biofluid is the minimum requirement for the reliable detection of exosomes.

Glycerol Viscosity Testing

The Sauerbrey equation described above can be modified to correlate change in frequency with the viscosity of the material loaded onto the crystal. The aim of this set of experiments was to verify that the resonant frequency of the crystal shifts in response to viscosity changes as well as changes in mass. The initial mixtures of glycerol were found to be too viscous once 20% (w/w) glycerol was exceeded, as after this point the crystal immediately jumped to a resonant frequency reading of 16 MHz, which is the default when the crystal is overloaded. This was a valuable lesson to learn early on in the experimental process, as this problem was encountered frequently throughout the rest of the semester for various reasons. To fix the problem in this situation, a new set of standards were created with 20% (w/w) glycerol as the most dense mixture.

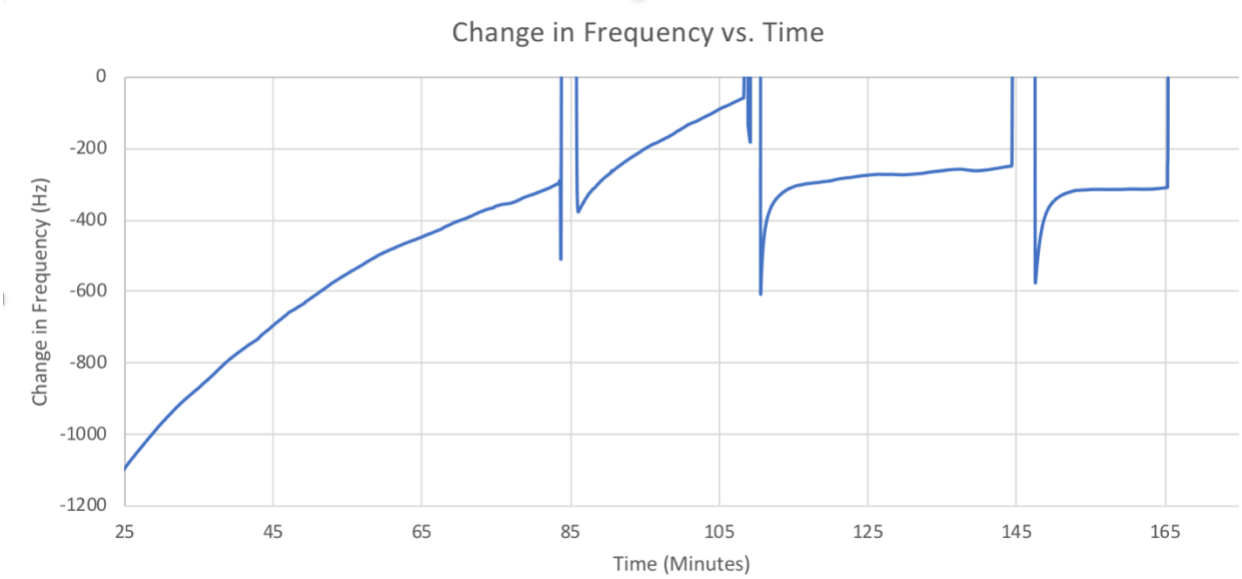


Figure 1: A plot of change in frequency (Hz) vs. time (s) for glycerol viscosity testing. The steady rise between 25 min - 80 min is the equilibration of 5% glycerol. The section from 85 min - 110 min is the equilibration of 10% glycerol. The section from 110 min – 145 min is 15% glycerol. The section from 150 min – 165 min is 20% glycerol.

In the figure above, we see the results of the glycerol viscosity testing. There are several points of interest that should be noted. First, the long, gradually flattening increase from time ~25 min – 80 min is the equilibration time. This is an example of very poor equilibration time. In fact, due to time constraints on this experiment, the first two samples had to be removed before they had a chance to fully equilibrate. In any case, the four relatively flat sections between the large spikes correspond to the four different standards that were analyzed (from left to right: 5% glycerol, 10% glycerol, 15% glycerol, and 20% glycerol). Theoretically, the equilibrium values should be highest with the leftmost sample and decrease incrementally as the graph continues to the right. It is unclear where the first sample would have equilibrated, but it is clear to see that the last three samples did follow the decreasing pattern that was expected, even if only two of them reached equilibrium. This experiment was replicated several times, but good performance and results were never achieved. This particular endeavor was eventually discontinued, as the ultimate goal of this work deals with change in mass, rather than change in viscosity. However, this work provided valuable initial experience in operating the QCM.

Thiol Immobilization and Insulin Testing

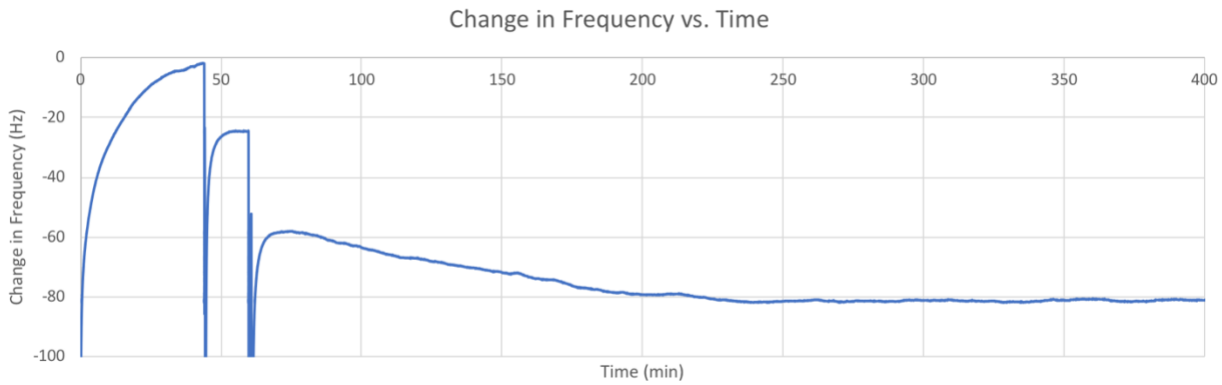


Figure 2: A plot of change in frequency (Hz) vs. time (min) for the thiol immobilization and insulin testing. The first section, from 0 min – 45 min, is the equilibration for pure PBS. The second section, from 45 min – 60 min, is the equilibration for the insulin/PBS mixture. The third section, from 60 min – 400 min, is the equilibration for the insulin/DTT mixture.

As stated above, thiols easily bind to gold surfaces and can form the basis of many immunoassay experiments. To test this process with the QCM in the laboratory, a series of tests were run involving Phosphate-buffered saline (PBS), insulin, and dithiothreitol (DTT). Insulin contains disulfide bonds, which are readily reduced by DTT to form thiol groups. As can be seen above, there are three phases to this specific experiment, again from left to right. The first is running PBS on the crystal and achieving equilibrium (due to scale it does not seem as though PBS equilibrated, although it did). Next, a mixture of insulin and PBS is analyzed by the QCM. As expected, this graph shows a frequency decrease from the first stage to the second. While, theoretically, no insulin is attaching to the crystal's electrode, the viscosity increase is significant enough to show a difference. The final stage is an insulin/DTT mixture that has been allowed to incubate overnight. It is with this stage that it is expected to observe insulin chemically attaching to the electrode after the breaking of its disulfide bonds by DTT, exposing free thiols for gold immobilization. This happens at a slow enough pace that it is actually observable on the graph above.

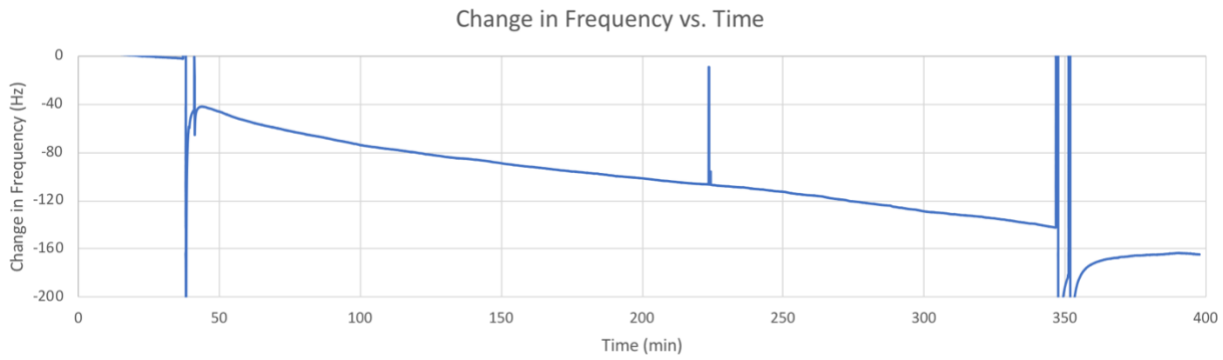


Figure 3: A plot of change in frequency (Hz) vs. time (min) for the thiol immobilization and insulin testing. The first section, from 0 min – 40 min, is the equilibration for PBS. The second section, from 40 min – 345 min, is the equilibration for the insulin/DTT mixture. The third section, from 350 min – 400 min, is the equilibration for the second run of PBS.

Shown above is the second part of the experiments involving thiol immobilization and insulin. After the first part of the experiment, detailed above, there was some slight concern that the decrease in resonant frequency observed when the insulin/DTT mixture was analyzed was a result of a change in viscosity, rather than a change in mass from insulin binding to the gold electrode. To rule out this possibility, the experiment represented above was performed. Again, from left to right, the first section is only PBS being analyzed by the device, just as the first experiment started out. The difference in this experiment is found in the second section, which is the insulin/DTT mixture instead of insulin/PBS. In this section once more the gradual decline in resonant frequency is observed as would be expected from insulin binding to the electrode. Finally, in the third section is only PBS once more. Since the crystal has been flushed with PBS but still shows a significant decrease from the original PBS equilibrium, it can be determined with confidence that the insulin has been immobilized on the crystal.

Thiol Immobilization and BSA Testing

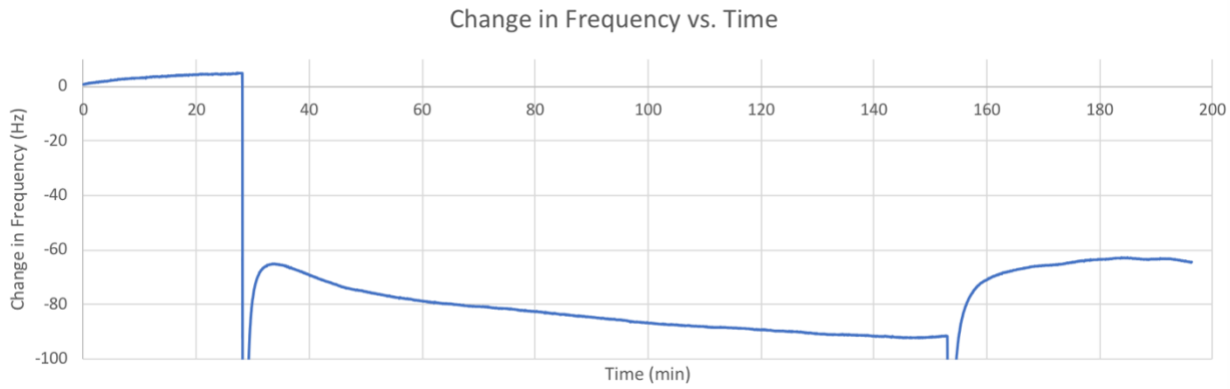


Figure 4: A plot of change in frequency (Hz) vs. time (min) for the thiol immobilization and BSA testing. The first section, from 0 min – 27 min, is the equilibration for PBS. The second section, from 28 min – 153 min, is the equilibration for the BSA/DTT mixture. The third section, from 154 min – 195 min, is the equilibration for the second run of PBS.

Bovine Serum Albumin (BSA) also contains disulfide bonds, and so the previous experiment was repeated with BSA in the place of insulin. BSA has a much higher molecular weight than insulin, and so the hope for this experiment was to see a much more drastic difference in the PBS baseline vs. the BSA frequency shift than was observed during the experiment with insulin. As can be seen above, the same rough trends were achieved with BSA that were observed with insulin, and there is definite evidence that BSA was immobilized onto the crystal. However, the decrease in frequency from the first equilibration of PBS to the equilibration of BSA/DTT is not as significant as what was seen in the insulin experiments. Since BSA has a much larger mass than insulin, this suggests that the BSA did not bind to the gold electrode nearly as readily as the insulin molecules did.

Protein A Testing

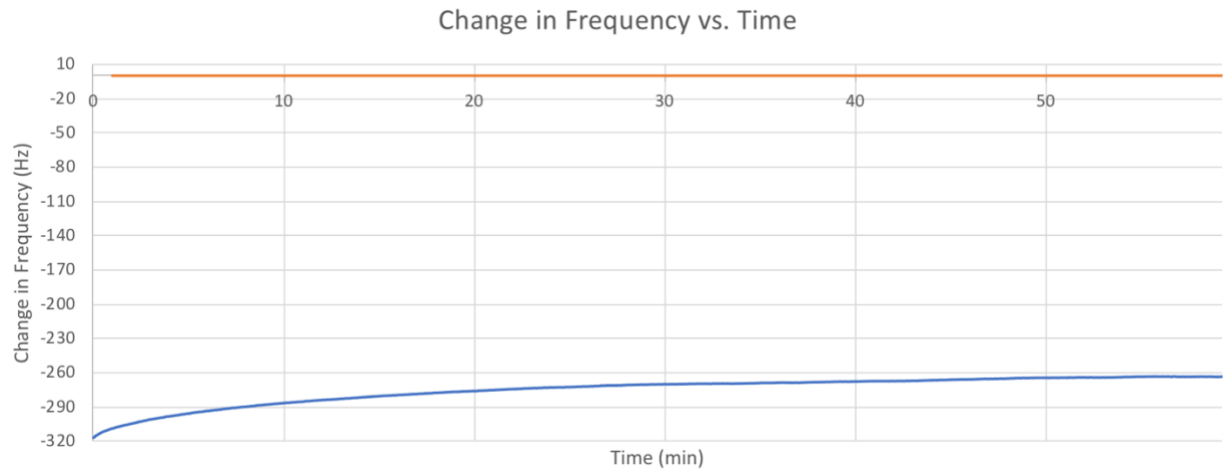


Figure 5: A plot of change in frequency vs. time for a crystal in just PBS (orange) and a crystal in just PBS after Protein A immobilization (blue).

Protein A was decided to be the agent of choice to help eventually immobilize exosomes onto the QCM. First, however, the method of immobilizing Protein A was tested, as set forth by Babacan et al. Protein A was deposited on the crystal, followed by sodium acetate buffer, and allowed to incubate for two hours at 22° C, then analyzed and compared to the crystal's resonant frequency in PBS, with the hope to see evidence of Protein A immobilization on the crystal.

As can be seen from the plot above, there was significant difference in the crystal's resonant frequency before and after the immobilization of Protein A, which is what is expected. There is no concern that the difference is due just to viscosity effects here, as both trials plotted above simply have the crystal in PBS. There is observed an approximately 260 Hz difference in the resonant frequency due to the immobilization of Protein A, which, from the Sauerbrey equation, corresponds to about 300 ng of Protein A that has attached to the crystal and is ready to bind antibodies or other biomolecules of interest.

A challenge that has been encountered while working with the QCM is proper storage and cleaning of the device and the crystals that go inside of it. These procedures had to be developed from existing literature as best as possible and applied while hoping for the best. Some problems seem to be had here, especially with the Protein A testing. These tests require the crystals to be stored at least one 24-hour period, if not more, and be ready to test again and give the same results. This has not yet been achieved to satisfaction, and is something that needs to be improved if these QCMs are to truly become effective devices for exosome detection.

Conclusion: The goal of this semester's work is the development of the QCM technology to generate a procedure for the detection of exosomes. When looking at the results of the experiments as a whole, one can see the progression from basic experiments to more complex and important immobilizations that lead to the possibility of detection of exosomes. So far, the culmination of the experiments has been the ability to immobilize Protein A on the QCM and detect it. At this point going forward, the next step is to explore and experiment with the use of various antibodies and similar molecules that have the ability to bind exosomes and similar analytes. A standard procedure would need to be developed, and the process would need to be able to selectively identify or detect exosomes among other similar molecules that are present in an exosome's normal environment. If this can be achieved, it would be a great breakthrough, that will not only contribute to the current knowledge in fields of biochemistry, physiology, etc., but will also provide a solution to the struggles of the valuable detection of these molecules at a very affordable cost compared to other modern immunoassay methods.

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