Formation of protein self-assembled monolayer monitored by Quartz Crystal Microbalance

Yinan Liu, Recep Tolga Atalay
Department of Physics, Freie Universität Berlin

Abstract
Quartz crystal microbalance (QCM) is a sensitive balance can detect mass variations in nanogram sensitivity by measuring the change in frequency of its quartz resonator. QCMs are being used in biological systems to determine molecular affinities to various surfaces. It can also be utilized with electrochemistry to measure electroactive molecules bound to QCM. In this experiment we successfully confirmed the monolayer formation on QCM's surface and the number of proteins bound to modified surface's recognition sites. These results then compared to electrochemical QCM measurements and discrepancies are investigated.

Theoretical Background

Piezo-Electric Effect

Jacques Curie and Pierre Curie discovered mechanical stress on quartz crystal generates electric charge on the surface of quartz crystal in 1880. This phenomenon of charge generation due to mechanical stress is referred as piezoelectricity. Soon after discovery of piezoelectricity, reverse piezoelectricity is also discovered, in which a piezoelectric material deformed by an external electric field.
Deformation of piezoelectric material gives rise to surface charge on the material due to formation of electric dipoles. The linear relation between deformation $\vec{x}$ and polarization $\vec{P}$ for small deformations is described by the following equation:

$$\vec{P} = \underline{e}\vec{x} \tag{1}$$

Where $\underline{e}$ denotes piezoelectric coefficient.
Similarly, the ratio of deformation $\vec{x}$ of piezoelectric material under external electric field $\vec{E}$ is described by coefficient of piezoelectric expansion $\underline{d}$.

$$\vec{x} = \underline{d}\vec{E} \tag{2}$$

Resonator

Quartz wafers used in Quartz crystal microbalance are cut sections of a bigger quartz. The angle to the optical z-axis which wafer is cut affects the characteristics of quartz crystal such as the resonance frequency and temperature coefficient. In this experiment QCM consists of AT-cut quartz crystal and vapor deposited gold electrodes is used. Electrodes are used to apply signal

to quartz crystal and measure electronic oscillations. AT-cut crystals have very low temperature coefficients near room temperatures and undergoes shear deformation under external electric field.
AT-Cut quartz disk oscillates in thickness-shear-mode and results in a standing transversal wave in the disk.

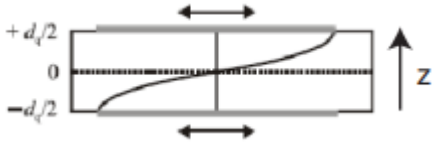

Figure 1. Transversal wave in quartz crystal.

The resonant frequency f_0 of TSM is related to quartz crystal's thickness (d_q) and velocity of transversal wave (c_{tr}) by equation 3.

$$f_0 = \frac{c_{tr}}{2} \cdot d_q \tag{3}$$

Equation 3 can be written as Equation 4 by the introduction of crystal specific frequency constant (K_R) and n to calculate higher overtone vibrations.

$$f_0 = \frac{nK_R}{d_q} \tag{4}$$

The frequency constant K_R depends on angle of cutting in the case of AT-Cut crystal $K_R = 1664 ms^{-1}$ [1]. Since only odd overtones result in surface charges, only odd values of n are allowed.

Electrical and Mechanical Description of Quartz Vibration
Oscillatory motion of quartz crystal can be modelled analogous to one dimensional damped harmonic oscillator. It is shown by Butterworth and Van Dyke, one dimensional vibrating system driven by an electric field can be modelled as a circuit of capacitance, resistance, and inductance. [2] The Butterworth-van-Dyke equivalent circuit of quartz crystal is shown in Figure 2.

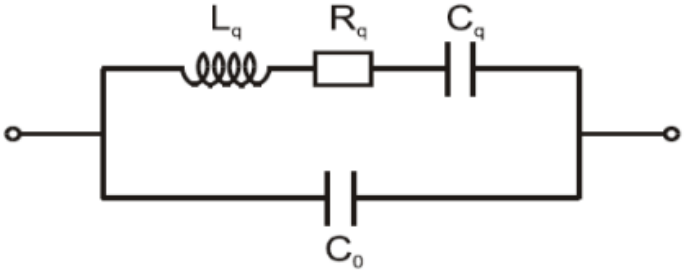

Figure 2. Butterworth-van-Dyke equivalent circuit of quartz crystal. [1]

The solution to RLC differential equation is then relates to mechanical analogous system with resonance frequency $f_0 = \frac{1}{2\pi\sqrt{LC}}$.

Deposited Mass

Increasing the thickness of quartz crystal by Δd results in change in frequency Δf as a result of equation 3.

$$\frac{\Delta f}{f} = -\frac{\Delta d}{d} \tag{5}$$

By rewriting Δd in terms of density and surface area we obtain:

$$\frac{\Delta f}{f} = -\frac{\Delta m_q}{\rho_q d_q A} = -\frac{\Delta m}{\rho_q d_q A} \tag{6}$$

Any mass m bound to the crystal results in same frequency change. By rearranging we obtain Sauerbrey equation [3]:

$$\Delta f = -\frac{f_0}{\rho_q d_q}\frac{\Delta \mathrm{m}}{A} = \frac{S_m \Delta \mathrm{m}}{A} \tag{7}$$

Sauerbrey equation describes the change in frequency of oscillating crystals with density ρ_q and thickness d_q and surface area A, in respect to rigid mass Δm deposited on crystal.

One of the shortcomings of Sauerbrey equation is it can only describe the change in frequency of a quartz crystal in air or vacuum. The change in frequency of a crystal in liquid environments are described by Kanazawa and Gordon [4] in 1985, by solving of Navier-Stokes (Equation 8) equation.

$$\frac{d^2 v_r}{dZ^2} = \frac{\rho_L}{\mu_L}\frac{dv_r}{dz} \tag{8}$$

Change in frequency of quartz resonator in liquid as described by Kanazawa and Gordon:

$$\Delta f = f_0^{3/2}\sqrt{\frac{\eta_L \rho_L}{\pi \mu_q \rho_q}} \tag{9}$$

Where η, μ and ρ are the viscosity, shear modulus and density respectively.

Thiol based self-assembly on metal surface

Self-assembly is a phenomenon that describes the formation of systems without external guidance other than the intrinsic environmental characteristics in which the process takes place. Self-assembled monolayers (SAMs) are usually two-dimensional molecular structures, which complex surface properties can be tuned by functional groups on the assembling molecules. In typical SAM systems, van der Waals forces among the molecules provide intermolecular stability and introduce long-range or short-range ordering while molecule to substrate adhesion is typically achieved through a reactive head and a strong molecule-substrate link. In terms of thiol based self-assembly on metal surfaces, some of the general mechanisms are well established. The S-H bond of the thiol would easily dissociate at room temperature due to sulfur's superb ability to stabilize a negative charge. It then forms a strong thiolate metal bond (Rs-Me) at the metal surface, and the H atom produced from dissociation of the SH bond can be eliminated from the surface through the formation of H_2. It was well documented that the self-assembly of thiol on gold has a characteristic angle of roughly 30 degrees. The formation of a monolayer of thiolates on metal surfaces would result in a decrease of the work function in comparison with the clean metal. Precise details of the thiol-metal interaction still demand further research, but it is currently being modeled with thiyl radicals. In the S-Au bonding of an SAM thiol-metal system, at low bias voltage, the bonding between S head and Au substrate is responsible for it electrical conduction, and at high bias voltage, the alkyl chains contribute to the conducting states of the surface. [4]

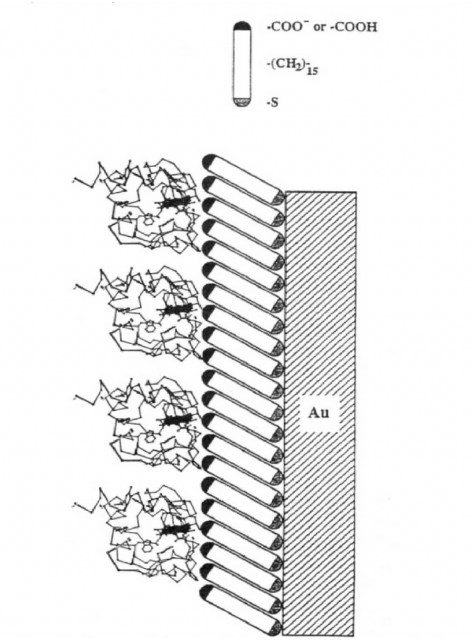

Figure 3. Illustration depicting an ideally ordered and oriented cytochrome c/16-MDHA/Au composite. [5]

Cytochrome c

The Cytochrome c protein used in this experiment is harvested from horse heart. It is water soluble, and has an atomic weight of 12kDa. It is an essential component in the mitochondrial electron transport chain of eukaryotes and its oxidation-reduction potentials are pH dependent.[6] Cytochrome C is structurally bound to a heme prosthetic group in a single polypeptide chain that consists of 104 amino acids. It's special sequence, Cys-Xaa-Xaa-Cys-His, mediates the binding of itself to the heme prosthetic group, which consists of a porphyrin ring and a central iron in the Fe+2 or Fe+3 oxidation state.[1]

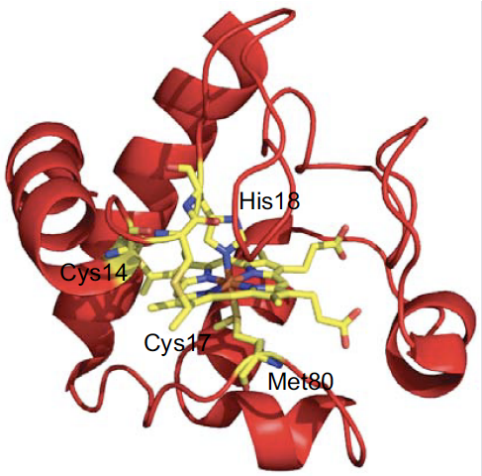

Figure 4. Model 3D structure of the cytochrome C protein. Heme prosthetic group are reentered in blue and yellow sticks, amino acids of the binding sites are indicated. The rest of the protein are shown in secondary structure in red.[1]

Cytochrome C from horse heart is covalently bound to the heme prosthetic group through cystine 14 and cystine 17 group. Histidin 18 and methione 80 form a complex with the central iron.

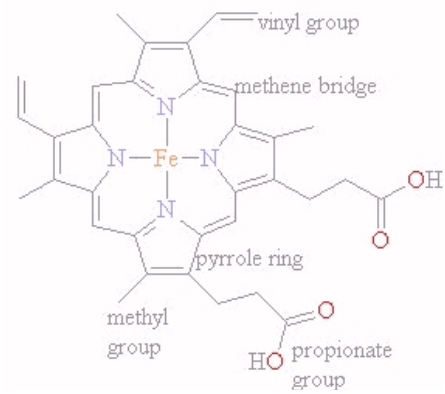

Figure 5. Structure of a heme prosthetic group.[7]

This important structure makes cytochrome c an electron carrier in the reparatory chain, particularly in the q cycle.[8] This process begins with the electron transfer from ubiquinone QH2 to cytochrome c, which is catalyzed through the binding of cytochrome c oxidase (complex

IV) to cytochrome bc1 complex (complex III). During this process, the central iron ion on the cytochrome c is reduced from Fe^{3+} to Fe^{2+}, and ubiquinone QH2 is oxidized to Q, releasing 2 protons that are pumped by complex III to mitochondrion's intermembrane space.

$$QH_2 + 2\,Cyt\,c_{ox} + 2\,H^+_{matrix} \rightleftharpoons Q + 2\,Cyt\,c_{red} + 4\,H^+_{\text{intermembrane space}}$$

Subsequently, cytochrome c oxidase then oxidizes the reduced cytochrome c. Electrons that are released during this process are used to reduce oxygen to water.

$$O_2 + 4\,Cyt\,c_{red} + 8\,H^+_{matrix} \rightleftharpoons 2\,H_2O + 4\,Cyt\,c_{ox} + 4\,H^+_{\text{intermembrane space}}$$

Experimental Set up

Schematics

The major components of a Quartz Crystal Microbalance (QCM) setup are a Teflon holder for the quartz crystal, a frequency meter, and a computer terminal for saving the data. The sample of interest is bound to the QCM, and the gold-plated crystal itself is used as a working electrode in a three-electrode cyclic voltammetry set up along with a platinum plated counter electrode, and an Ag/AgCl bounded reference electrode.

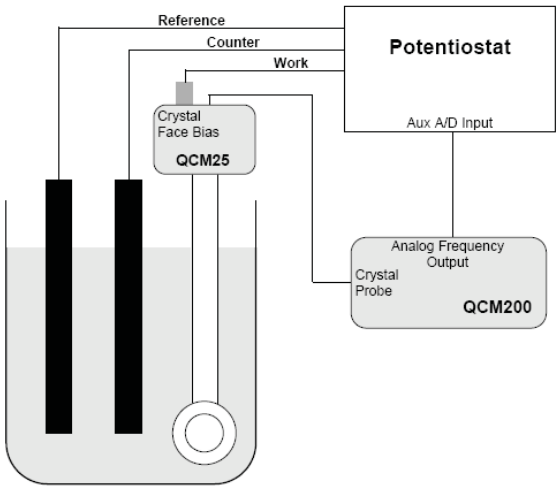

Figure 6. Schematics of the QCM cyclic voltammetry system with three electrodes, a potentiostat, and a frequency meter

The quartz crystal is inserted into the crystal cavity in the crystal holder head, held by two o-rings, a retainer ring, and a retainer cover. The crystal is tightened in such an orientation that only the side of the crystal that carries the working electrode is exposed to the liquid. The solution is also tempered in a water bath since the quartz crystal is temperature sensitive.

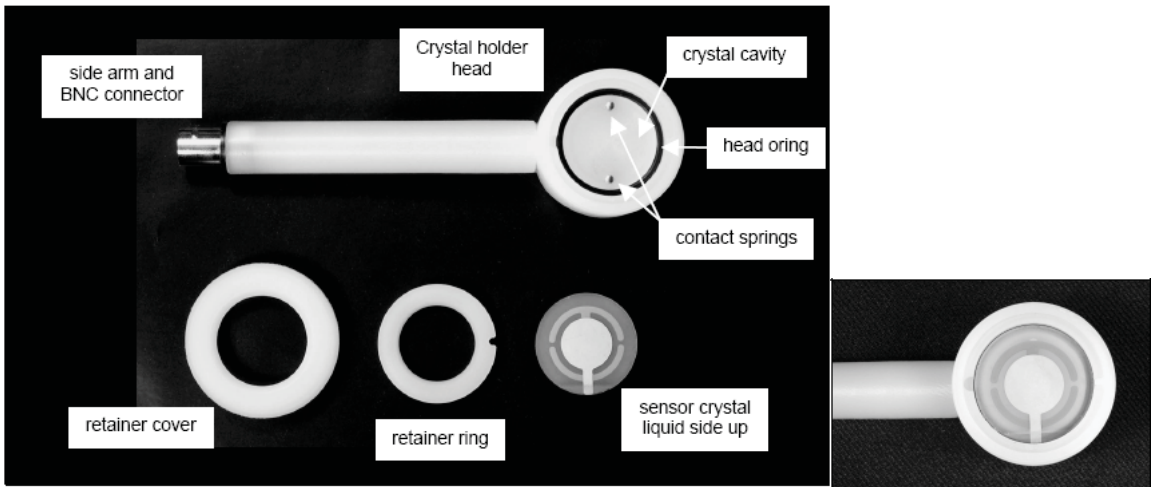

Figure 7. Left: components of the Teflon holder. Right: alignment of the crystal within the Teflon holder to ensure connection of the gold surface is connected to the potentiostat as a working electrode.

The QCM is connected to a PC terminal via the program *SrsQcm20.* Before measuring in liquids, an initial capacitance zeroing is performed. first set switch of the QCM200 controller to *ADJUST*, then increase or reduce the dial until both Null LEDs are on. After this procedure is complete, return switch to *HOLD*.

The program that controls the potentiostat is called *PowerSuite* on the computer terminal. To take a cyclic voltammogram measurement, *PowerCV* and then *cyclic voltammetry (Ramp) is selected.*

Experimental Procedure

We began this experiment by measuring the background of the equipment. First, eigen frequency of the quartz in air was taken. The gold-plated crystal was already incubated and rinsed. Next, QCM was directly submerged in ethanol without tempering in a water bath to measure the eigen frequency in ethanol after the frequency meter becomes stable.

After these background measurements, we proceeded to surface modification. A 50μM solution of 11-Mercaptundecanic acid (MUA) in ethanol was prepared. We then submerged the QCM in this solution to form a monolayer of MUA on the gold surface of the crystal. This set up was left in the laboratory for around an hour in order to let the frequency stabilize.

Next, we took the QCM out of this set up, rinsed the crystal with distilled water, and start to prepare the cytochrome c buffer. First, cytochrome c was dissolved in 10 mM KH_2PO_4/K_2HPO_4, pH 7.5 buffer. The buffer solution was then added into the beaker so that final concentration in the beaker should become 5μM. The head of the QCM was placed back into the solution and was left undisturbed until the frequency stabilize.

Finally, 5 cycles of cyclic voltammogram were taken using the three-electrode set up to check if cytochrome c is active at the surface and to infer the number of molecules adhered to the crystal surface.

Results

The measured eigenfrequency for the quartz crystal in air was 4997961.1Hz, using the equation 4 the thickness of the quartz crystal is calculated to be $332.93\mu m$. After submerging the QCM in ethanol, there is an almost vertical drop in the frequency with a measured change in frequency of -688.0 Hz taken from the difference of the two stabilized sections of the frequency meter.

According to the Kanazawa-Gordon Equation, with the viscosity of ethanol measured at $1.2040mPas$, and the density of ethanol as $0.7893gcm^{-3}$ at room temperature[9,10], the sheer modulus of the crystal at $2.947 \cdot 10^{11}gcm^{-1}s^{-2}$, and the density of the crystal given by the manufacturer at$2.648cm^{-3}$[1], the predicted frequency change is -695.5Hz.

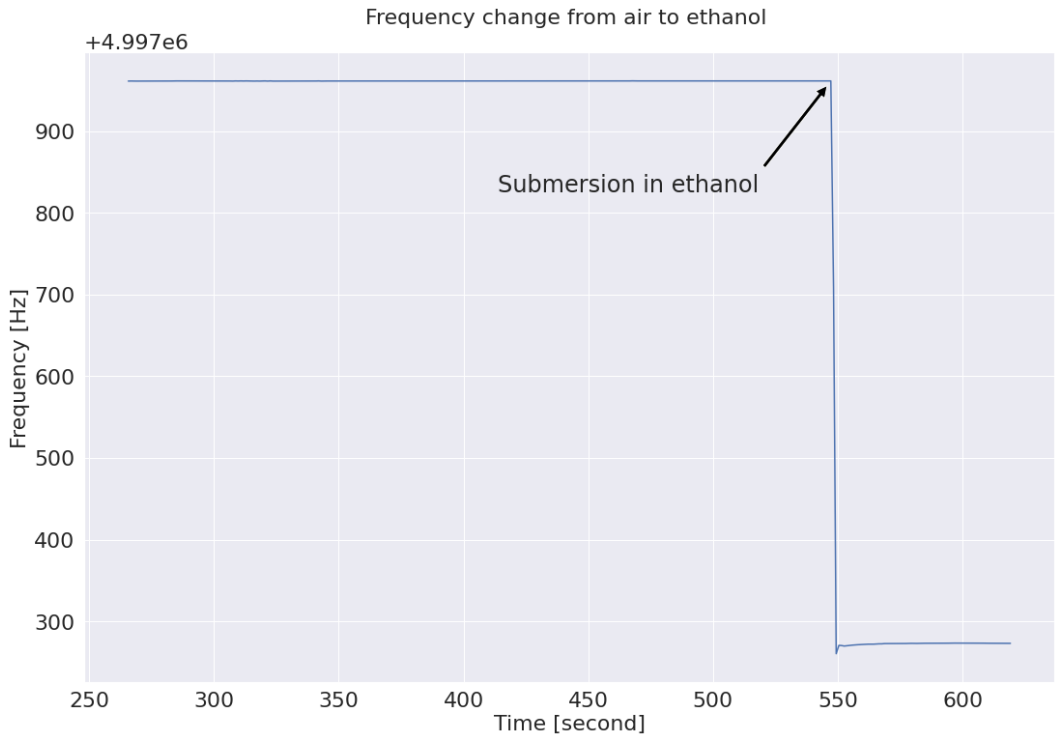

Figure 8. Frequency change of QCM from air to ethanol

The eigenfrequency for the QCM in is measured to be 4997273.82Hz before the addition of 11-MUA. After waiting for frequency to stabilize for an hour, the mean frequency of the QCM measured as 4997270.06Hz resulting in a change in frequency of 3.76Hz.

Using Sauerbrey equation, deposited mass on quartz crystal is determined to be 83.992ng. Which corresponds to 384.65pmol of 11-MUA and $303.65 pmol/cm^2$ of 11-MUA density on gold electrode.

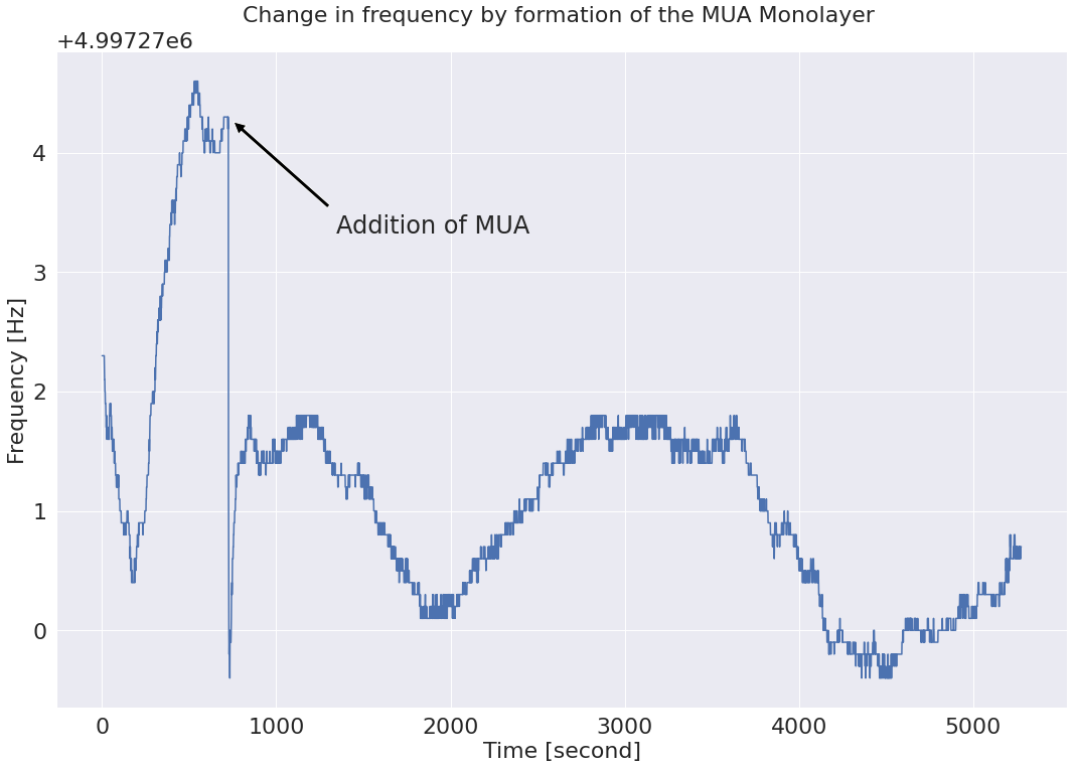

Figure 9. Frequency change of QCM as 11-MUA monolayer forms.

After the frequency meter fluctuates around one value and becomes relatively stable,

Resonant frequency of QCM in buffer solution is measured to be 4997254.37Hz. Cytochrome-C introduced into solution twice in successive intervals, sharp increase in frequency followed by decrease observed following addition of Cytochrome-C solution. After 15 minutes of stabilization period, mean frequency is calculated to be 4997248.43Hz, resulting in 5.94Hz decrease in frequency.

Using Sauerbrey equation, the mass of Cytochrome-C bound to 11-MUA monolayer is calculated to be 132.84ng. Which corresponds to $11.06 pmol$ of Cytochrome-C molecules and $8.74 pmol/cm^2$ of Cytochrome-C density.

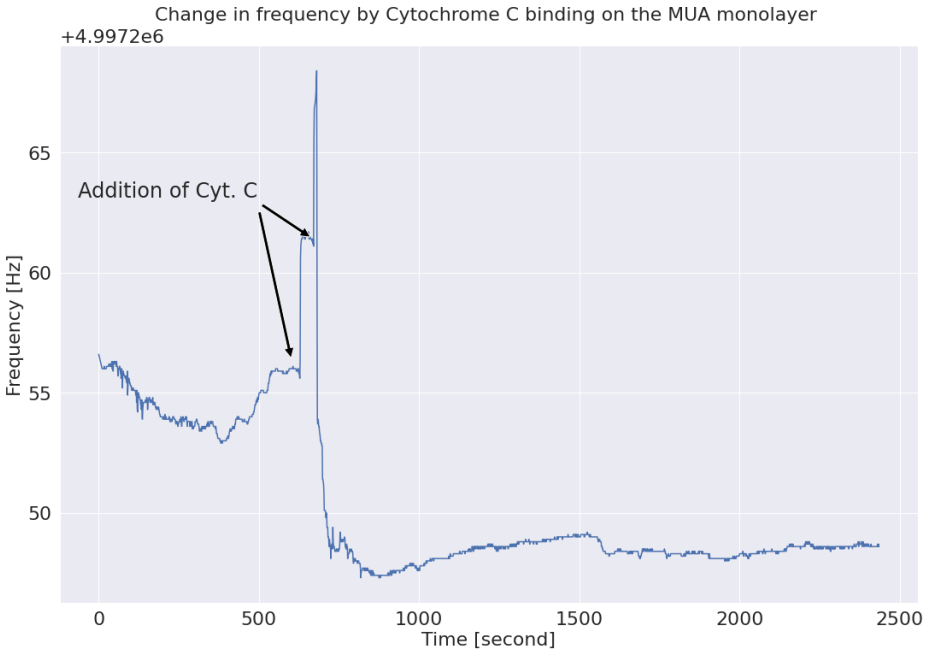

Figure 10. Frequency change of QCM during Cytochrome-C binding on 11-MUA.

Using Origin software the shaded area in upper half of cyclic voltammogram is calculated to be $2.67459 \cdot 10^{-9}\, VA$, total charge transferred is calculated by dividing area by scan rate: $Q = 5.3592 \cdot 10^{-8}\, C$. Corresponding number of electrons then calculated to be $\#e = 3.339 \cdot 10\text{^}\{11\}$. Number of electrons took place in electron transfer corresponds to number of Cytochrome-C as only single electron transfer takes place in redox reaction in Cytochrome-C. Then number of moles bound to MUA calculated to be $0.55 pmol$ which corresponds to surface density of $0.44 pmol\, cm^{-2}$, which is 20 times lower than the density calculated with QCM method.

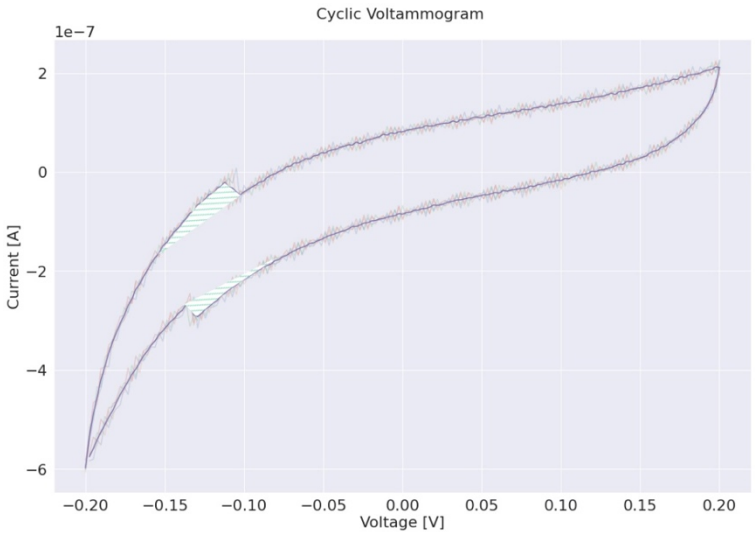

Figure 11. Measured Cyclic Voltammogram of Cytochrome-C.

Discussion

The measured eigen frequency of the crystal in air was in good agreement with the crystal's data from the manufacture. The frequency change from air to ethanol measured in our experiment, -688.0 Hz, has a 1.07% relative error with the result predicted by the Kanazawa-Gordon equation, which gives a value of -695.5Hz. Our experimental result is in good agreement with this model in ethanol. The small error could be a result of the temperature dependence of viscosity and density of the liquid, and the fact that our experimental set up was not tempered in a water bath with stable temperature.

The surface coverage of MUA on gold was calculated to be $3.03 * 10^{-10}\ mol/cm^2$. To further interpret this result, we used the lattice constant of pure crystalline gold at 0.407nm and estimated the density of gold atom at the surface to be $2.73 * 10^{-9}\ mol/cm^2$. This roughly gives the bonding of one MUA per 10 gold atoms on the surface. Literature value of nearest thiol-thiol neighbor has a distance of 0.499nm, which is slightly larger than the lattice constant of gold.[4] Therefore, the maximum theoretical density that MUA is able to achieve on gold service cannot exceed the density of gold atoms on the surface. Experimental full coverage has been reported at a surface density of $8.0 * 10^{-10}\ mol/cm^2$ with an immersion time longer than 12 h.[11] Depending on thiol self-assembled monolayer's packing structure and configurations, vacancies can occur as a result of the thiols attaching to the surface at a not optimized manner, or to free thiols being trapped in the self-assembled monolayer horizontal to the metal surface.

Another interesting aspect of the MUA self-assembly is that we observed time independent fluctuation of the frequency meter reading that is centered around roughly the same value. We would like to attribute this behavior to the dynamic equilibrium nature of the self-assembly kinetics. It is well described that the self-assembly process is not a static one, but under constant surface reconstruction as a result of the competitive chemical forces that lead to the self-assembled structure. The reading from the frequency meter suggests that the mass that is attached to the gold surface fluctuates over time as the thiols detach from the metal and attaches again or goes into an orientation that cannot make it be described as a rigid body with the crystal quartz, which is one of the assumptions of the Sauerbrey equation.

The number of Cytochrome C proteins bound on 11-MUA monolayer is calculated to be 11.06pmol, which corresponds to approximately 1 Cytochrome C for every 35 MUA molecules. Using the dimensions of Cytochrome-C [14] full coverage on surface would result in density of $14.4 pmol/cm^2$. Comparing it to our result of $8.74 pmol/cm^2$, we determine approximately 61% of the surface is covered by Cytochrome C proteins.

For the cyclic voltammetry, we were unable to observe a distinct characteristic cyclic voltammetry graph as reported in literature.[12] This could be a result of the reference electrode used, different buffer, as well as different gold surface modification with the literature set up.

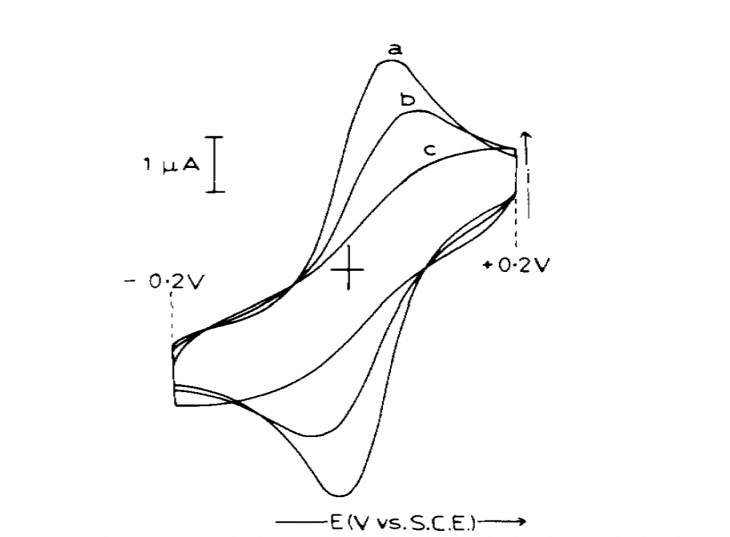

Figure 12. DC cyclic voltammogram of cytochrome C, 0.4 mM in 0.1 M NaClO4, 0.02M phosphate buffer with poly-L-lysine modification of the reference electrode at 3 different concentration.[12]

In our calculations, we attempted to integrate the two minor peaks shown in the graph, and resulted in an amount of cytochrome c molecule that is less than two orders when compared to the results obtained by QCM. We would like to conclude that these peaks are result of noise rather than active cytochrome c proteins. Other supporting evidence to this conclusion include the fact that the redox potential shown on our cyclic voltammogram is not characteristic of active cytochrome c with Ag/AgCl as reference electrode, which is commonly around 0.0V.[13] In a compelling study done by Sardari et al, cytochrome c was immersed in increasing concentration of urea as a denaturant to study urea's effect on the structure of cytochrome c. We can see here in figure 13 that increasing concentration of urea decreases current peaks of the cyclic voltammogram, and our experimentally obtained cyclic voltammetry actually resembles the voltammetry cycle of the denatured cytochrome c protein the most. The graph itself is also not symmetric, typical of classic redox cyclic voltammograms, but rather skewed towards the left at -0.10 V.

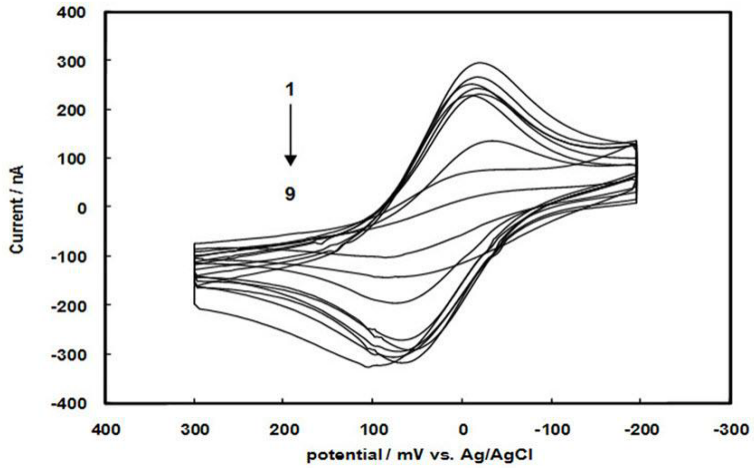

Figure 13. Cyclic Voltammogram of cytochrome C in increasing concentration of denaturant urea with 20mM phosphate buffer and 100mV/s probe speed. [13]

Overall, we would like to suggest that QCM could be a more reliable and accurate way of measuring the mass of the electro-active molecules modified on the surface in comparison to cyclic voltammetry. Although being a powerful tool that provides plenty of essential information in electrochemistry, it highly depends on the number of active molecules that is able to participate in the redox reaction. Since the Heme group that is mainly responsible for the electron transfer process is shielded in the center by the surrounding protein's secondary structure, it requires an optimal orientation for the central iron to conduct electron transfer, and for it to be subsequently picked up on the cyclic voltammetry. The proteins themselves could also be denatured and unable to complete the redox reaction. Therefore, cyclic voltammetry's high dependence on the number of active proteins makes it a not so reliable way to interpret the number of molecules adhered to the gold surface. It will only suggest, through calculations, number of active molecules at the surface. On the other hand, QCM is able to pick up the total mass of all the cytochrome c molecules, regardless of their electroactivity, as long as they are bound to the metal surface as rigid bodies.

Conclusion:

We were able to successfully execute the evaluation of eigen frequency of the quartz crystal in air and in ethanol. Our experimental data confirms that the Kanazawa and Gorden equation provides a reliable way to describe the frequency change of QCM in liquid environment. The surface modification of MUA was successful, and the self-assembly kinetics in dynamic equilibrium was observed. The surface coverage of MUA calculated through the QCM is in good agreement with literature values. We were also able to detect the surface adhesion of cytochrome c to the crystal surface by observing its frequency change. However, subsequent confirmation of active cytochrome c electron transfer process is not conclusive from five cycles of cyclic voltammetry, and this could be due to non-optimized molecular orientation, shielding or charges, or denatured protein. Overall, the quartz crystal microbalance is shown to be a reliable way to detect mass change in the nanogram range in this experiment.

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