TITLE:

Sample Preparation in Quartz Crystal Microbalance Measurements of Protein Adsorption and Polymer Mechanics

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SUMMARY:

The quartz crystal microbalance can provide accurate mass and viscoelastic properties for films in the micron or submicron range, which is relevant for investigations in biomedical and environmental sensing, coatings, and polymer science. The sample thickness influences which information can be obtained from the material in contact with the sensor.

ABSTRACT:

In this study, we present various examples of how thin film preparation for quartz crystal microbalance experiments informs the appropriate modeling of the data and determines which properties of the film can be quantified. The quartz crystal microbalance offers a uniquely sensitive platform for measuring fine changes in mass and/or mechanical properties of an applied film by observing the changes in mechanical resonance of a quartz crystal oscillating at high frequency. The advantages of this approach include its experimental versatility, ability to study changes in properties over a wide range of experimental time lengths, and the use of small sample sizes. We demonstrate that, based on the thickness and shear modulus of the layer deposited on the sensor, we can acquire different information from the material. Here, this concept is specifically exploited to display experimental parameters resulting in mass and viscoelastic calculations of adsorbed collagen on gold and polyelectrolyte complexes during swelling as a function of salt concentration.

INTRODUCTION:

The quartz crystal microbalance (QCM) leverages the piezoelectric effect of a quartz crystal to monitor its resonant frequency, which is dependent on the mass adhered to the surface. The technique compares the resonant frequency and bandwidth of an AT cut quartz crystal sensor (typically in the range of 5 MHz)[1] in air or a fluid to the frequency and bandwidth of the sensor after deposition of a film. There are several benefits for using QCM to study thin film properties and interfaces, including the high sensitivity to mass and potentially to viscoelastic property changes (depending on sample uniformity and thickness), the ability to perform studies in situ[2], and the ability to probe a much shorter rheological timescale than traditional shear rheology or dynamic mechanical analysis (DMA). Probing a short rheological timescale allows observation of how the response at this timescale changes both over extremely short (ms)[3] and long (years) durations[4]. This capability is beneficial for the study of a variety of kinetic processes and is also a useful extension of traditional rheometric techniques[5,6].

The high sensitivity of the QCM has also led to its heavy use in biological applications studying the fundamental interactions of extremely small biomolecules. An uncoated or functionalized sensor surface can be used to investigate protein adsorption; even further, biosensing through complex binding events between enzymes, antibodies, and aptamers can be examined based on changes in mass[7–9]. For instance, the technique has been used to understand the transformation of vesicles to a planar lipid bilayer as a two-phase process of adsorption of fluid-containing vesicles to a rigid structure by observing correlating changes in frequency and viscoelasticity[10]. In recent years, the QCM has additionally offered a robust platform to monitor drug delivery by vesicles or nanoparticles[11]. At the intersection of materials engineering and molecular and cellular biology, we can use the QCM to elucidate key interactions between materials and bioactive components like proteins, nucleic acids, liposomes, and cells. For example, protein adsorption to a biomaterial mediates downstream cellular responses such as inflammation and is often used as a positive indicator of biocompatibility, while in other instances extracellular protein attachment to coatings that interface with blood could induce dangerous clotting in vessels[12, 13]. The QCM can therefore be used as a tool to select candidates optimal for different needs.

Two common approaches for performing QCM experiments collect analogous data from the experiment: the first approach records the frequency shift and the half bandwidth (Γ) of the conductance peak. The second approach, QCM with dissipation (QCM-D), records the frequency shift and the dissipation factor, which is directly proportional to Γ through equation 1,[14]

$$D_n = \frac{2\Gamma_n}{f_n} \quad , \qquad (1)$$

where D is the dissipation factor and f is the frequency. Both D and Γ are related to the damping effect the film has on the sensor, which gives an indication of the stiffness of the film. The subscript n denotes the frequency overtone or harmonic, which are the odd resonant frequencies of the quartz sensor (n = 1, 3, 5, 7…). Further discussion of models using multiple harmonics to obtain the mass and viscoelastic properties of a film can be found in a review by Johannsmann[14] and previous papers from the Shull group[15–18].

One key consideration for preparing QCM samples is how to apply the thin film on the sensor surface. Some common methods include spin coating, dip coating, drop coating, or adsorption of the film onto the sensor surface during the experiment[19,20]. There are four regions for QCM samples: the Sauerbrey limit, the viscoelastic regime, the bulk regime, and the overdamped regime. For sufficiently thin films, the Sauerbrey limit applies, where the frequency shift (Δf) provides the surface mass density of the film. Within the Sauerbrey limit, the frequency shift scales linearly with the resonant harmonic, n, and changes in damping factor (D or Γ) are generally small. In this regime sufficient information is not available to uniquely determine the rheological properties of the layer without making additional assumptions. Data in this regime are used to calculate the surface mass density (or thickness if the density is known *a priori*) of the film. In the bulk regime where the medium in contact with the crystal is sufficiently thick, the evanescent shear wave propagates into the medium before being completely dampened. Here, no mass information can be obtained using Δf. However, in this region, the viscoelastic properties are reliably determined using the combination of Δf and $\Delta\Gamma$ [15, 18]. In the bulk regime, if the medium is too rigid, the film will damp out the resonance of the sensor, preventing the collection of any reliable data from QCM. The viscoelastic regime is the intermediate regime where the film is thin enough to have the shear wave fully propagate through the film as well as have reliable values for the damping factor. The damping factor and Δf can then be used to determine the viscoelastic properties of the film as well as its mass. Here, the viscoelastic properties are given by the product of the density and the magnitude of the complex shear modulus $|G^*|\rho$ and the phase angle given by $\phi = \arctan(G"/G')$. When films are prepared in the Sauerbrey limit, the mass per unit area can be directly calculated based on the Sauerbrey equation shown below[21],

$$\Delta f_n = \frac{-2nf_1^2}{Z_q}\frac{\Delta m}{A} \quad , \qquad (2)$$

where Δf_n is the change in the resonant frequency, n is the overtone of interest, f_1 is the resonant frequency of the sensor, $\Delta m/A$ is the mass per area of the film, and Z_q is the acoustic impedance of quartz, which for AT cut quartz is $Z_q = 8.84\,x\,10^6\,kg/m^2s$. The viscoelastic regime is most appropriate for the study of polymer films, and the bulk limit is useful for studying viscous polymer[22] or protein solutions[16]. The different regimes depend on the properties of the material of interest, with the optimum thickness for full viscoelastic and mass characterization generally increasing with the film stiffness. **Figure 1** describes the four regions with respect to the areal density of the film, complex shear modulus, and phase angle, where we have assumed a specific relationship between the phase angle and the film stiffness that has been shown to be relevant to materials of this type. Many films of practical interest are too thick for studying the viscoelastic properties with QCM, such as certain biofilms, where the thicknesses are on the order of tens to hundreds of microns[23]. Such thick films are generally not appropriate for studying using the QCM, but may be measured using much lower frequency resonators (such as torsional resonators)[23], allowing the shear wave to propagate further into the film.

To determine which regime is relevant for a given QCM sample, it is important to understand the

d/λ_n parameter, which is the ratio of the film thickness (d) to the shear wavelength of the mechanical oscillation of the quartz crystal sensor (λ_n)[15,16,18]. The ideal viscoelastic regime is $d/\lambda_n = 0.05 - 0.2$[18], where values below 0.05 are within the Sauerbrey limit and values above 0.2 approach the bulk regime. A more rigorous description of d/λ_n is provided elsewhere[15,18], but it is a quantitative parameter delineating the Sauerbrey limit and the viscoelastic limit. The analysis programs used below provide this parameter directly.

There are some additional limitations to analyzing thin films with the QCM. The Sauerbrey and viscoelastic calculations assume the film is homogeneous both throughout the film thickness and laterally across the electrode surface of the QCM. While this assumption makes it challenging to study films which have voids or fillers present, there have been some QCM investigations into films consisting of grafted nanoparticles[6]. If the heterogeneities are small compared to the overall film thickness, reliable viscoelastic properties of the composite system can still be obtained. For more heterogeneous systems, values obtained from a viscoelastic analysis should always be viewed with great caution. Ideally, results obtained from systems with unknown heterogeneity should be validated against systems which are known to be homogeneous. This is the approach we have taken in the example system described in this paper.

An important point that we illustrate in this paper is the exact correspondence between QCM measurements done in the frequency domain (where Γ is reported) and the time domain experiments (where D is reported). Results from two different QCM experiments, one time domain and one frequency domain, are described, each involving a different but conceptually related model system. The first system is a simple example of collagen attachment to the sensor to illustrate representative binding kinetics and equilibration of adsorption over time during a time domain (QCM-D) measurement. Collagen is the most abundant protein in the body, known for its versatility of binding behaviors and morphology. The collagen solution used here does not require additional functionalization of the sensor's gold surface to induce adsorption[9]. The second experimental system is a polyelectrolyte complex (PEC) composed of anionic polystyrene sulfonate (PSS) and cationic poly(diallylmethyl) chloride (PDADMA) prepared in the same fashion as Sadman et al.[22]. These materials swell and become soft in salt (KBr in this case) solutions, offering a simple platform for studying polymer mechanics using a frequency domain approach (QCM-Z). For each protocol, the process of preparing, taking, and analyzing a measurement is shown in **Figure 2**. The schematic shows that the main difference between the QCM-Z and QCM-D approaches is in the data collection step and the instrumentation used in the experiment. All the mentioned sample preparation techniques are compatible with both approaches, and each approach can analyze samples in the three regions depicted in **Figure 1.**

Our data demonstrate that the preparation of samples, whether by sensor coating before or during a measurement, dictates the ability to extract the viscoelastic properties of a system. By designing the early stages of an experiment appropriately, we can determine what information we can accurately gather during the analysis step.

PROTOCOL:

QCM-D Collagen Adsorption

1. Sample preparation and sensor pre-cleaning

1.1. Prepare 20 mL of 0.1 M acetate buffer, adjusting the pH with HCl and NaOH as necessary to achieve pH = 5.6.

1.2. Add rat tail collagen solution to the 20 mL of acetate buffer under sterile conditions to a final concentration of 10 μg/mL.

1.3. Clean the gold-coated quartz sensor to remove organic and biological material[25,26].

1.3.1. Place the sensor active side up in a UV/Ozone chamber and treat the surface for approximately 10 min.

1.3.2. Heat a 5:1:1 mixture of deionized water (dH_2O), ammonia (25%) and hydrogen peroxide (30%) to 75 °C. Place the sensor in the solution for 5 min.

1.3.3. Rinse the sensor with dH_2O and dry with a stream of nitrogen gas.

1.3.4. Place the sensor active side up in a UV/Ozone chamber and treat the surface for 10 min.

NOTE: The cleaning procedure should be immediately performed before a measurement to minimize environmental contamination on the sensor surface.

2. QCM-D measurement data acquisition

2.1. Turn on all necessary equipment to take a measurement including the pump, electronics unit, and computer software.

2.2. Remove the flow module from the chamber platform and unscrew the large thumb screws to open the module.

2.3. If the sensor has been left out after initial cleaning (steps 1.3.1-1.3.4), rinse the sensor with deionized water (dH_2O) and dry with a stream of nitrogen gas to ensure that there are no contaminants on the surface.

2.4. Mount the sensor in the flow module on the exposed O-ring, first drying the area with a stream of nitrogen gas and checking that the O-ring is lying flat. The sensor should be placed with the active surface side down and anchor-shaped electrode oriented toward the marker in the flow module.

2.5. Turn the thumb screws to seal the flow module and replace it on the chamber platform. Attach any necessary PTFE pump tubing to the flow module and external pump.

2.6. Using the appropriate computer software, set the temperature of the flow module to 37 °C. Monitor the changing temperature for 10-15 min to ensure that it equilibrates at the desired value.

2.7. Find the initial resonance frequencies of the sensor. If any resonance frequencies are not found by the software, check that the flow module is correctly positioned on the chamber platform or re-mount the sensor in the flow module to ensure that it is centered and making proper electrical contact.

2.8. Place the inlet pump tubing in the 1x phosphate-buffered saline (PBS) solution. Start the external pump flow at 25 µL/min and visually inspect the tubing to be sure that the fluid is flowing through the tube.

NOTE: Fluid flow may be easier to see by momentarily increasing the fluid flow rate to 100 µL/min or greater. If fluid does not appear to be moving through the tube, it is most likely that the two parts of the flow module are not creating a proper seal. Try tightening the thumb screws, tightening the connectors of the tubing to the inlet and outlet, or re-mounting the sensor to be sure that the O-ring is flat and centered.

2.9. Allow fluid flow of the 1x PBS through the flow module for at least 15 min to properly equilibrate.

2.10. Start the measurement in the computer software to begin data acquisition. Monitor the frequency and dissipation values for at least 5 min to ensure a stable baseline.

2.11. Stop the pump and move the inlet tubing to the collagen-acetate buffer solution, and resume fluid flow. Note the time of this event for later analysis.

2.12. Allow the new frequency and dissipation values to equilibrate to a stable value. Here, we expect this stabilization to occur after 8-12 h.

2.13. Stop the pump, move the inlet tubing back to the 1x PBS solution, and resume fluid flow. Note the time of this event for later analysis.

2.14. Allow the new frequency and dissipation values to equilibrate to a stable value. Here, this stabilization occurs after 30 min.

NOTE: Steps 2.13 and 2.14 can be repeated for each new period of fluid flow in more rigorous experiments with a greater number of stages.

2.15. End the data acquisition of the measurement and save the data.

2.16. Clean and dismantle the QCM equipment.

2.16.1. Increase the fluid flow rate of the external pump to 500 µL/min or greater and place the inlet tubing into a solution of 2% Hellmanex cleaning solution for at least 20 min.

NOTE: For other experiments, if further analysis of the sensor is desired, remove the sensor before step 2.16.1 and place another cleaning sensor in the module.

2.16.2. Stop the pump and move the inlet tubing to dH_2O, and resume fluid flow to further flush the system for at least 20 min.

2.16.3. Stop fluid flow and remove the sensor from the flow module. Dry the sensor and inside of the flow module with a stream of nitrogen gas. Turn off the computer software, electronics unit, and peristaltic pump.

NOTE: The gold-coated sensors can be properly cleaned, as detailed in steps 1.3.1-1.3.4, and reused for several measurements. Indications that a sensor can no longer be reused for reliable measurements may include but are not limited to large variability in initial resonance frequencies and significant drifts in baseline measurements with buffer flow. Data can be opened and analyzed in the preferred software, including those provided by companies that specialize in QCM-D equipment.

QCM Polyelectrolyte Complex Swelling

3. Sample preparation

NOTE: This experiment was performed using a MATLAB program developed within the Shull research group for data collection and analysis.

3.1. Collect a reference conductance spectrum for the bare quartz crystal sensor in air.

3.2. Submerge the sample holder in a lipless 100 mL beaker filled with distilled water and collect a reference conductance spectrum for the bare sensor in water.

3.3. Prepare a 0.5 M solution of potassium bromide (KBr).

3.3.1. Dissolve 1.79 g of KBr in 30 mL of distilled water. Shake until dissolved.

3.3.2. Insert a small silicon wafer into the KBr solution at an angle to create a slide for the quartz sensor during the annealing step to prevent the film from coming off the sensor.

3.4. Prepare the sensor for spin coating.

3.4.1. Set the spin coat parameters to 10,000 rpm, 8,000 acceleration, and 5 s.

3.4.2. Insert the sensor onto the spin coater and turn on the vacuum.

3.4.3. Cover the surface of the sensor with ethanol and run the spin coater to clean the sensor surface.

3.4.4. Add the PEC (PSS:PDADMA prepared in the same way as detailed in Sadman et al.[22]) to the surface of the sensor.

3.4.4.1. If the complex is in two phases (polymer rich and polymer poor), slowly insert the pipet into the solution. Evacuate the pipet by blowing bubbles while moving the pipet into the denser polymer rich phase.

3.4.4.2. After releasing a couple bubbles in the polymer rich phase, draw up 0.5-0.75 mL of the polymer rich solution into the pipet. Maintaining pressure on the pipet bulb to not allow the polymer poor phase to enter the pipet, draw the pipet out of the solution.

3.4.4.3. Wipe the outside of the pipet using a Kimwipe. Add enough solution dropwise onto the surface of the quartz sensor to completely cover the surface. Make sure there are no visible bubbles in the solution on the sensor surface.

3.5. Spin coat the PEC sample and immediately submerge the sensor in the 0.5 M KBr solution to prevent salt crystallization on the film.

NOTE: This step is sometimes difficult to coordinate. Release the sensor just above the KBr solution for best results.

3.6. Allow the film to anneal for at least 12 h.

NOTE: For ease of performing the experiment, prepare step 4 in the evening and allow the film to anneal overnight.

4. Measurement of the film in air and water

4.1. Transfer the sensor to a beaker filled with distilled water to remove the excess KBr from the film and back side of the sensor. Leave the sensor in the solution for 30-60 min.

4.2. Take a measurement of the film in air. Reference to the bare sensor in air. Allow the film data to equilibrate.

4.3. Insert dried calcium sulfate into a 100 mL lipless beaker and measure the completely dry film thickness. Remove calcium sulfate from the beaker and rinse the beaker with distilled water.

4.4. Fill the 100 mL lipless beaker with 30 mL of distilled water. Insert a stir bar to ensure the water is circulating around the film. Measure the film in water for about 30-45 min or until the

film data are equilibrated. Reference to the bare sensor in water.

4.5. Prepare a 15 mL solution of 5 M KBr in distilled water. Measure 5.35 g of KBr into a graduated cylinder and fill to 15 mL with distilled water. Swirl until dissolved.

4.6. Add the KBr solution to the beaker with distilled water in 0.1 M increments. **Table 1** outlines the 0.1 M increments in mL of 5 M KBr solution. Face the film away from where the KBr solution is being added to the water so that the film does not dissolve. Make sure the system has equilibrated before adding another addition of the KBr solution.

4.7. After all the data has been acquired, remove the film from the holder and place in a beaker of distilled water. Allow the salt to leave the film (30-60 min) and air dry the film.

4.8. To clean the PEC film from the sensor, add KBr to the beaker and gently swirl the solution. Allow to sit for 5-10 min. Repeat this process 2-3 times, then rinse the sensor with distilled water.

NOTE: The sensor can be cleaned and reused if the response from the sensor is still good. This can be checked by the sensor having small absolute bandwidth readings for the harmonics of interest (<100 Hz).

5. **Data analysis**

5.1. Open the QCM-D data analysis MATLAB GUI created by Sadman (https://github.com/sadmankazi/QCM-D-Analysis-GUI).[27] Open the film in air data file by selecting "Load QCM."

NOTE: The Shull group has developed a similar Python GUI for data collection and analysis for QCM (https://github.com/shullgroup/rheoQCM). A portion of the analysis code is provided in the supplementary information for both analyzing the data and generating the figures in this paper.

5.2. Select the desired calculation (either **3,5,3** or **3,5,5**), **gamma**, and **film in air** icons. Click **Plot QCM**.

5.3. Determine the thickness of the dry film using the most equilibrated data point (typically the last data point) from the experiment. Record this value.

5.4. Open the film in water data file. Select the same parameters as in Step 5.2, except for film in water instead of film in air.

5.5. After each equilibration step of the swelling experiment, determine the film thickness, complex shear modulus, and the viscoelastic phase angle. Record these values along with the ionic strength (ranging from 0-1 M in 0.1 M increments).

5.6. Determine the percent swelling as

$$swelling(\%) = \frac{d\rho - (d\rho)_{dry}}{(d\rho)_{dry}} * 100 \quad (3)$$

where $d\rho$ is the film thickness from the solution and $d\rho_{dry}$ is the dry film thickness.

REPRESENTATIVE RESULTS:

The changes in frequency with time during protein adsorption exhibit a characteristic curve and plateau shown in **Figure 3A-B**. The initial buffer wash of 1x PBS across the bare sensor surface induces only negligible changes in frequency, offering a steady baseline to act as a reference for future data points. The introduction of collagen solution causes protein adsorption to begin, observed as a steady decrease in frequency over time, until the density of adhered collagen plateaus at a stable baseline (**Figure 3A**). The exact frequency and mass values will be highly dependent on the purity and surface energy of the sensor. Given these parameters, the final buffer wash removes only a small amount of unadhered protein from the sensor surface, resulting in a slight increase in frequency. We should always expect only a slight decrease in mass during this period, demonstrating a stable amount of protein bound to the sensor (**Figure 3B**).

The importance of reaching a stable frequency measurement for each period cannot be overstated. Slight fluctuations in environmental variables like temperature, humidity, and solution concentration can lead to observable differences in the raw data. Therefore, altering these variables before at least 5-10 min of stable frequency and dissipation factor measurements can misrepresent the exact changes in frequency and dissipation. An example of a suboptimal dataset is shown in **Figure 3C-D**. Here, the same solution concentration and flow rate parameters are used as **Figure A-B**, but the instrument environment was not allowed to equilibrate before beginning the measurement. The natural settling of the sensor's oscillating frequency is occurring at the same time as a changing temperature and fluid concentration, disguising any potential baseline that will act as a reference (**Figure 3C**). We are instead forced to choose an average of the entire dynamic frequency range in the period to act as a reference. Finally, the collagen flow is not permitted to equilibrate at a stable mass before starting the final PBS wash, as seen by the still changing frequency shifts just before the PBS enters the system. This action does not impact the calculations of mass but does not fully characterize the adsorptive potential of the protein on the sensor (**Figure 3D**).

During the early stages of the collagen adsorption experiment, the film is in the Sauerbrey regime, indicated by values of $\Delta f/n$ that are independent of n ($t < 2$ h in **Figure 3**). As the experiment progresses the film moves into the viscoelastic regime, indicated by values of $\Delta f/n$ which no longer overlap ($t > 2.5$ h). Recognizing this change in behavior, the data obtained from the collagen experiment was analyzed to look at the areal mass and viscoelastic properties using two different methods. The first uses a Python script compiled by the Shull group. This script has the same mathematical underpinnings as the MATLAB data collection and analysis software used for the PEC experiment. It uses a power law model to account for property differences at adjacent harmonics[15] and is provided in the supplemental information. The second method uses values determined from a viscoelastic model in a commercial software package to calculate the areal

mass, complex shear modulus, and phase angle of the collagen film. The viscoelastic model from this software reports the thickness (d), elastic modulus (μ), and viscosity (η). The elastic modulus and viscosity are the elements of a Kelvin-Voigt model, and are converted to the magnitude and phase of the complex modulus via the following expressions:

$$|G_n^*| = \left(\sqrt{\mu^2 + (\eta\omega_n)^2}\right) \quad (4)$$

$$\phi_n = \tan^{-1}\left(\frac{\eta\omega_n}{\mu}\right) \quad (5)$$

where $\omega_n = 2\pi n f_1$ where f_1 is the fundamental frequency of the quartz sensor (5 MHz). **Figure 4** shows the viscoelastic properties determined for the collagen adsorption calculated from the Δf_n and ΔD_n values of the third and fifth harmonic. **Figure 5** compares the properties from **Figure 4** with the properties converted from the commercial software results. As can be seen in **Figure 5**, the commercial software values report the film to be softer than the Python script.

Figure 6 describes a relationship which has been observed in previous QCM experiments[3,22] showing a linear relationship between the viscoelastic phase angle and the logarithm of the magnitude of the complex shear modulus. The green line indicates this linear relationship, having end points of a Newtonian fluid such as water ($|G^*|\rho = 10^5\ Pa \cdot g/cm^3$ and $\phi = 90°$ at $f_3 = 15\ mHz$) and an elastic solid or glassy polymer ($|G^*|\rho = 10^9\ Pa \cdot g/cm^3$ and $\phi = 0°$). Many polymer materials studied using the QCM follow this general empirical trend, which was quantified using the PSS:PDADMA complex system[22]. As the PEC is subjected to solutions with higher salt concentrations, the sample transitions from being a rigid, glassy sample to being more viscous and fluid like; this spectrum of properties falls on the green line. For comparison purposes, the properties calculated using the Python script for the equilibrated collagen film are also plotted in **Figure 6**. The relationship between $|G^*|\rho$ and ϕ is expected to be the same for both systems, given that both systems are glassy polymers swollen with water. The water content of the film determines the specific point along the curve. Here, the PEC system with mechanical properties closest to the collagen system corresponds to a 20 wt% polymer solution. We infer from this comparison that the polymer concentration in the adsorbed collagen film is also close to 20 wt.%. This result is a very useful one, obtained in our case by the comparison of results obtained from two appropriately designed QCM experiments. One of these experiments was a time domain (QCM-D, collagen) experiment and the other was a frequency domain (QCM-Z, PEC) experiment, but these types of experiment are completely interchangeable, with either protocol sufficing in either case.

FIGURE AND TABLE LEGENDS:

Figure 1. Plot of the Sauerbrey, viscoelastic, bulk, and overdamped regimes. The plot shows regimes where different types of information can be obtained from QCM data, based on the sample areal mass (related to thickness) and the viscoelastic properties. Below the blue line is the Sauerbrey regime, where only the thickness of the sample is calculated. For the middle region, the mass and viscoelastic properties of the sample can be calculated. In the bulk regime at the upper left of the plot, viscoelastic information can be obtained but the experiments are no

longer sensitive to the sample thickness. In the upper right, the overdamped regime indicates the sample is too thick for a QCM measurement to be performed. In the plot, a linear relationship is assumed between the viscoelastic phase angle at the third harmonic and the log of the magnitude of complex shear modulus (green line in **Figure 6**). The bulk regime is defined as the region where the thickness is more than twice the decay length of the shear wave. The Sauerbrey regime is defined as the region where $\Delta f/3$ and $\Delta f/5$ differ by less than 10 Hz, and the overdamped regime is the regime where Γ_5 is larger than 20,000 Hz (D_5 > 1600 ppm).

Figure 2. Flow diagram of major steps within a QCM measurement. Schematic of a QCM-Z or QCM-D experiment. The diagram in the first step is a QCM sensor (gray) with the gold electrodes (gold) and film on top of the sensor (purple), with the different techniques used to apply a film to the sensor surface. The thickness of the film, d, is indicated. The second step highlights the data from the QCM-Z (top) and QCM-D (bottom) experimental protocols. The third step is where one determines the region where the sample can be analyzed. The fourth step shows the resulting data from the given analysis region.

Figure 3. "Good" and "Bad" QCM-D data for collagen adsorption. Plots of the frequency and dampening factors for the collagen adsorption experiment. **(A)** Equilibrated frequency shifts, **(B)** Equilibrated dampening factor shifts, **(C)** Non-equilibrated frequency shifts, and **(D)** Non-equilibrated dampening factor shifts. In **(B)** and **(D)**, the dampening factor shift is plotted as the dissipation factor, D, and the bandwidth, Γ, since the same parameter is measured by both shifts. The frequency and gamma shifts are normalized to their respective harmonics ($n = 3$ or 5).

Figure 4. Viscoelastic analysis of collagen using a power law model. The **(A)** areal mass, **(B)** complex shear modulus, and **(C)** viscoelastic phase angle for the collagen adsorption experiment. The first 10 h show the main adsorption stage of the collagen to the sensor surface, with the period between 10 and 20 showing the equilibration stage before the buffer wash performed at 20 h. The error bars represent uncertainties in the calculations for the thickness and viscoelastic properties, assuming an error in Δf and $\Delta\Gamma$ equal to 1% of Γ.

Figure 5. Viscoelastic analysis of collagen using a power law model and commercial software model. The **(A)** areal mass, **(B)** complex shear modulus, and **(C)** viscoelastic phase angle for the collagen adsorption experiment. The Γ values are determined with the Python script using the Δf and ΔD values from the experimental data while the D values are converted from the results of the viscoelastic model from the commercial software.

Figure 6. Modified Van Gurp-Palmen plot of the collagen and PSS:PDADMA data. A plot of the viscoelastic phase angle and the complex shear modulus over the general range of samples measurable using QCM. The green line indicates the linear relationship between the two properties which was assumed in the development of **Figure 1**. Data for the PSS:PDADMA polyelectrolyte complex (PEC) are reprinted with permission from Sadman *et al.*[22], copyright 2017 American Chemical Society.

Table 1. Molar increments for the PEC swelling experiment. The amount (in mL) of 5 M

potassium bromide solution necessary to increase the molarity of the water solution by 0.1 M for the swelling experiment.

Supplementary Files. Python Code

DISCUSSION:

The collagen adsorption results span the Sauerbrey and viscoelastic regimes. By plotting the frequency shifts normalized to the corresponding harmonic number, we observe that the Sauerbrey limit holds true for approximately the first 2 h of the measurement. With increasing mass adhering to the sensor, however, the normalized frequency shifts for the third and fifth harmonics begin to deviate from one another ($t > 2$ h), indicating an ability to determine viscoelastic properties of the adsorbed film.

A direct comparison between the viscoelastic modeling results from the software and the power law modeling from the Shull group indicate a noticeable difference in calculated material properties. Over the course of the measurement, the viscoelastic modelled data from commercial software represented a thicker, softer layer with a lower complex shear modulus (**Figure 5**). The differences in the viscoelastic properties between these models are due to the assumptions made in the calculations for each system. One difference concerns an assumption that needs to be made about the frequency dependence of the viscoelastic properties. Some assumption needs to be made because the frequency response at a given harmonic (n = 3, for example), depends on three parameters (ρd, $|G_3^*|\rho$, ϕ_3) but only two independent quantities (Δf_3 and $\Delta\Gamma_n \sim \Delta D_n$) are measured. Because of this discrepancy, we need to obtain at least one additional quantity (either the frequency shift or dissipation) from an additional harmonic without adding an additional unknown to the problem. The thickness and density obviously do not depend on the frequency, but the complex shear modulus does. The power law approach is based on the fact that over a small frequency range, we can assume that the phase angle is constant, with a rheological response equivalent to a material with a power-law behavior over a much larger range of frequencies[15,16,18]. The power law exponent, Λ, is not an adjustable parameter but is equal to $\phi/90°$, with ϕ in degrees. With the power law assumption, we have $\phi_3 = \phi_5$ and $|G_5^*| = |G_3^*| \left(\frac{n_5}{n_3}\right)^{\Lambda}$. For quantitative viscoelastic modeling, the power law model represents the best combination of accuracy and simplicity, giving more reliable results than other common approaches, including the Kelvin-Voigt model, where G' is assumed to be independent of n and G" is assumed to increase linearly with n.

Considering the experimental setup for the PSS:PDADMA data, experiments in the bulk and the viscoelastic regimes were performed for generating the data in **Figure 6**. The protocol details the sample preparation for the viscoelastic regime experiments, with the bulk experiments being performed by looking at the sensor response to a solution with the PEC, salt, and water present. In order to prepare the samples for the viscoelastic regime experiments, it is important to understand the target thickness range for remaining within the viscoelastic regime and avoid overdamping the response of the sensor. For the PSS:PDADMA system, this ideal range is $\sim 0.8 - 1.6\ \mu m$. Since the PEC initially increases in thickness by 45-50% when swelled in water, this

behavior had to be accounted in the initial film thicknesses, making a target range for the initial sample thickness of $\sim 0.45 - 0.65\ \mu m$. Having a good grasp of how the film will behave during the experiment is important for understanding the best target thickness range as well as the best method for sample preparation[18].

Regardless of the exact instrumental set-up, these procedures demonstrate the importance of considering sample preparation before beginning a QCM experiment. The thickness of the applied layer determines the information that can be extracted from the measured data. Before beginning any measurement, the researcher must consider which information is most needed from the experiment and understand the limitations of the technique. An understanding of the viscoelastic properties of the film is helpful when determining the correct sample thickness and preparation method. For appropriate samples, both time-domain and frequency domain QCM instruments can be expertly used to gather accurate data for a wide range of applications.

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DISCLOSURES:

The authors have nothing to disclose.

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