The Frequency Dependence of the Surface Sensitivity of Resonator Biosensors

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A study of the sensitivity decrease of biosensors working at high frequencies is presented. With new technology such as film bulk acoustic resonators (FBAR), issues like the decay length is no longer irrelevant theory but may cause limitation in the system as well as it offers new detection possibilities.

To investigate the frequency response and sensitivity, layer-on-layer construction chemistry was used. A protocol involving activation with EDC/NHS and coupling chemistry with fibrinogen was optimized to ensure accurate thickness and uniformly distribution of each layer over the surface.

Surfaces were characterized using null ellipsometry and the protocol was tested in a traditional quartz crystal microbalance (QCM). Experiments with the FBAR were preformed at the Ångström laboratory in Uppsala were there is ongoing research and development in FBAR technology.

The results confirmed the theory of decreasing frequency and sensitivity further out from the surface. An experimental and estimated thickness was calculated which to some extent correlates to the theoretically calculated decay length.

A new terminology is suggested when the frequency levels off. It occurs approximately at twice the distance and thickness of the theoretically calculated decay length and is given the name; detection length. Beyond the detection length an inverted signal is observed which cannot yet be explained for.

Biosensor, QCM, FBAR, Protein kinetics
Abstract

A study of the sensitivity decrease of biosensors working at high frequencies is presented. With new technology such as film bulk acoustic resonators (FBAR), issues like the decay length is no longer irrelevant theory but may cause limitation in the system as well as it offers new detection possibilities.

To investigate the frequency response and sensitivity, layer-on-layer construction chemistry was used. A protocol involving activation with EDC/NHS and coupling chemistry with fibrinogen was optimized to ensure accurate thickness and uniformly distribution of each layer over the surface.

Surfaces were characterized using null ellipsometry and the protocol was tested in a traditional quartz crystal microbalance (QCM). Experiments with the FBAR were preformed at the Ångström laboratory in Uppsala were there is ongoing research and development in FBAR technology.

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Sammanfattning

En studie i hur känsligheten avtar från ytan hos biosensorer med höga frekvenser presenteras. Med ny teknologi som avancerade elektroakustiska tunnfilms komponenter, så kallade FBARs, blir tidigare outforskade områden som decay längden möjliga att studera.

För att undersöka hur frekvensvaret och känsligheten påverkas av interaktioner långt ut från en sensoryta används proteinkemi. Ett protokoll har optimerats innehållande aktivering med EDC/NHS och fibrinogen för att säkerställa en jämna tjocklek och fördelning av ett adsorberat proteinlager över en yta.

Dessa ytor kontrollerades först med hjälp av ellipsometri och sedan i ett QCM instrument. Alla experiment med de högfrekventa FBAR sensorerna utfördes vid Ångströmslaboratoriet i Uppsala där pågående forskning inom området finns.

Resultaten bekräftar teorin om en avtagande känslighet i och med ett ökat avstånd från ytan. En experimentell genomför och beräknad tjocklek för decay längden uppskattades som inte helt stämde överens med den teoretiskt beräknade.
En ny term föreslås då frekvenssvaret hos en biosensor planar ut. Detta är en effekt som sker vid dubbla tjockleken av den teoretisk beräknade tjockleken av decay längden och har fått namnet; detection length. Efter denna längd eller gräns observeras en inverterad signal som det än så länge inte finns någon förklaring till.
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Chapter 1

Introduction

1.1 Background

Since the development of the glucose sensor by Clark and Lyons in 1962, generally recognized as the first biosensor, the pursuit for new, more advanced, accurate and cheap sensor systems is always in progress [1]. Biosensors are analytical devices incorporating a biological or chemically derived material closely associated with or integrated within a physico-chemical transducer or transducing microsystem which may be optical, electrochemical, thermometric, piezoelectric or magnetic [2].

Biosensors have been implemented in a number of applications ranging from environmental pollutant detection to defense monitoring to the medical field [3]. Two types of piezoelectric transducers are of interest in this thesis; Quartz Crystal Microbalance (QCM) and Film Bulk Acoustic Resonator (FBAR). They are similar in that they both can utilize thickness shear mode (TSM) and able to operate at different frequencies. However, a typical FBAR resonates at around 1GHz, about 100 to 200 times higher in frequency then a QCM [4]. This means, according to theory, a greater mass sensitivity for the high frequency FBAR sensors since sensitivity increases with the square of the resonance frequency [5]. Sauerbrey was the first to show in 1959 how the frequency shift ($\Delta f$) is proportional to the change in mass ($\Delta m$) for thin films in gas or vacuum given by

$$\Delta f \propto \Delta m$$  \hspace{1cm} (1.1)

When TSM resonators such as QCM and FBAR operate in liquid it can measure either (i) the accumulation of mass onto the surface, and/or (ii) properties of the contacting liquid itself [6]. Factors, such as viscoelasticity or surface chemistry, will affect the response of these sensors in causing it to diverge from the linear relationship between frequency and adsorbed mass [5]. It is, nevertheless, a useful and reliable model.

Another interesting parameter, derived from equations related to TSM resonators in liquid is the decay length ($\delta$). The value of this variable is directly
related to the distance from the surface of which a propagating damped shear wave decays to zero in a fluid [5]. The decay length, depends on the angular frequency ($\omega$), liquid viscosity ($\eta$) and density ($\rho$) given by

$$\delta = \sqrt{\frac{2\eta}{\omega \rho}}$$  \hspace{1cm} (1.2)

The decay length theory is well established and accepted. However, there is no academic literature or published articles to validate the theory, much due to the fact that there are practical difficulties of reaching the decay length with oscillating sensors operating within the MHz vicinity. With new technology such as the FBAR the decay length is suddenly within the reach and of interest. The high frequency (GHz vicinity) may cause limitations such as high sensitivity to noise but may also provide new detection possibilities.

Molecular interaction studies are becoming increasingly important; something that has open the way for the biosensor industry. The quartz crystal microbalance (QCM) technology has been used for many years in various applications and there are a number of companies related to this technique. Attana AB uses a traditional QCM sensor while other companies have modified the technology. Biosensor Applications Sweden AB (BAAB) is one example which has developed a sensor system capable of detecting low concentrations of explosive substances such as trinitrotoluene (TNT) or chemical substances such as narcotics. The efforts at BAAB have resulted in their patented BIOSENS system. This analyzing device is highly selective and sensitive in detecting released molecules in the form of monoclonal antibodies.

Q-sense is another company with a commercialized QCM sensor which measures both the frequency and dissipation factor. They are today the leading supplier of acoustic resonator based instruments with their dissipation monitoring (QCM-D) technology.

### 1.2 Aims of the Master’s Thesis

The goal of the project was to evaluate the decay length and sensitivity of a biosensor with dependence on the resonance frequency and distance from the sensor surfaces. The investigation aimed to solve the problem using biochemistry with layer-on-layer adsorption. The main focus of this thesis have therefore been to find and evaluate a suitable biochemistry for such an experiment. A thorough search for relevant molecules were of importance as the design of a successful protocol for the technique. Estimation of mass deposition and film thickness was performed by ellipsometry as the primary method.

Chosen molecules were first tested on a traditional QCM system at Linköping University to verify its ability in a multilayer construction. A final study took place at the Ångström laboratory in Uppsala with the FBAR sensor. The result of the FBAR tests will hopefully shed some light over the decay length mystery and perhaps contribute to the ongoing research in FBAR technology.
Chapter 2

General Theory

2.1 Fundamental Principals of TSM Resonators

An acoustic wave device typically consists of a thin disk of an anisotropic solid, i.e. crystals such as AT-cut quartz, with electrodes patterned on both sides. Due to a phenomenon called piezoelectricity the crystal can be electrically excited in a number of resonant modes when an alternating voltage is applied across the electrodes. The result of these exited modes is deformation of the crystal plane. A Thickness shear mode (TMS) resonator is an acoustic wave device of shear deformation. [5]

There are other acoustic wave devices such as the surface acoustic wave (SAW) device. But these are not as suitable in liquid sensing as a TSM resonator. In the case of a SAW device the propagating wave will cause a vertical component deformation that in a liquid will be suppressed, i.e. a vast energy loss.

2.1.1 Piezoelectricity

The first material discovered to possess piezoelectric properties was quartz in 1880 by Pierre and Jacques Curie [7]. Piezoelectricity is a combination of mechanical and electrical properties, i.e. when the crystal is subject to mechanical stress, charge polarization will occur in the material and inversely when it is confronted to a voltage. [8]

At resonance, mechanical energy is transferred from kinetic energy stored in the moving mass of the crystal to potential stored energy in the elastic deformation. The frequency where these energy storage processes occur exactly out of phase the energy losses are minimized and the vibration amplitude reaches a maximum. This resonant frequency also depends on the coupling between the surface and the surrounding medium. [8]

To illustrate this in an equation, $F$ is the external applied force which sets the system in motion

$$ F = m\ddot{x} + kx - r\dot{x} \quad (2.1) $$
The first term represents kinetic energy storage, Newton’s second law. The second term, represents the energy stored in the spring, Hook’s law and the third term is the energy lost to friction which is essential when the surface is in connection with a liquid. [8]

2.1.2 Modelling of TSM Resonators

TSM resonators are based on the piezoelectric properties of a specific material. As mentioned earlier there are two models used to describe the behavior of the material; the mechanical and electrical. Neither describes the system in total but it is rather a combination of these two [7]. The electrical network implemented in a TSM system is illustrated with an equivalent electrical circuit (Fig 2.1 (a)). It is a modified Butterworth-VanDyke (MBVD) model and represents a one-port resonator with negligibly thin electrodes [4].

![MBVD circuit](image)

Figure 2.1. Modelling of TSM resonators.

The series resonance ($f_s$), also known as mechanical resonance, is measured when the electrical impedance is zero (ideally). The parallel resonance ($f_p$), is measured when the electrical impedance is set to infinite (ideally). The equations of these resonance frequencies are given by

\[
f_s = \frac{1}{2\pi \sqrt{L_m C_m}}
\]

(2.2)

\[
f_p = \frac{1}{2\pi \sqrt{\frac{C_0 + C_m}{L_mC_0C_m}}}
\]

(2.3)

where ($C$) and ($L$) are capacitance and inductance, respectively. It is important to understand that series resonance is less sensitive to resistance in series ($R_s$) due to the cancellation reactance by $L_mC_m$. In parallel resonance defined by $C_0$ in parallel with $L_m$ and $C_m$ the resonance frequency will be sensitive to the conductivity and dielectric permeability of the liquid. [4, 7]

The impedance magnitude of the series and parallel frequencies is illustrated in figure 2.1 (b).
2.1.3 The Sauerbrey Equation

The crystal can be electrically excited into several resonance modes corresponding to a unique standing wave pattern with displacement maxima occurring at the crystal face [6]. The resonance frequency in an AT-cut plate is given by

\[ f = n \frac{v_q}{2d_q} \] (2.4)

where \((v_q)\) is the wave velocity in the plate and \((d_q)\) is the thickness of the quartz plate. The resonance frequency for \((n=1)\) is called the fundamental resonance frequency and where \((n)\) equals an odd integer and is called the \(n\)th overtone. [6]

Sauerbrey made the assumption that the mass change of the crystal due to adsorption of some molecules onto the crystal surface can be treated as an equivalent mass change of the crystal itself. This means that a slight perturbation of the surface density \((\rho_s)\) caused by a uniformly distributed mass adsorption \((\delta m)\) on the surface area \((A)\) will cause an increase in kinetic energy while the potential energy remains unaltered. The wave velocity is thereby reduced, which in turn must results in a reduction of frequency as the first law of thermodynamics states that the total energy most remain constant [4, 6]. Thus, the equation known as the Sauerbrey equation, given by

\[ \Delta f = -\frac{2f_0^2}{\rho_q v_q A} \cdot \Delta m = -\frac{2f_0^2}{(\rho_q v_q)^{1/2}} \cdot \rho_s \] (2.5)

where \((f_0)\), \((\rho_s)\) are the resonance frequency, and the mass density of the accumulated layer. Further parameters are \((\rho_q)\), \((c_q)\), and \((v_q)\) corresponding to the piezoelectric layer mass density, stiffness constant, and wave velocity, respectively.

The Saurebrey equation is, however, valid only as long as the mass of the adlayer is i) small compared to the mass of the crystal, ii) rigidly attached to the surface, and iii) evenly distributed over the active area of the crystal. [7]

2.1.4 The Fluid Interaction

The liquid and the crystal are coupled through a boundary condition based on friction at the surface. The most commonly used condition is the assumption of no slip at the solid/liquid interface, i.e. particle velocity on the liquid side matches that on the solid side. A semi-infinite Newtonian fluid will couple to the sensing surface where the induced wave will be damped due to the viscous properties in the liquid. The velocity field generated in the liquid by the oscillating crystal is described by equation 2.6 and represents a critical damped shear wave radiated into the fluid by the oscillating surface [5, 9]

\[ v_x(y, t) = v_{x0}e^{-y/\delta} \cos \left( \frac{y}{\delta} - \omega t \right) \] (2.6)

where \((v_x)\) is the in-plane fluid velocity, \((v_{x0})\) is the surface particle velocity, \((y)\) is the distance from the surface, and
\( \omega = 2\pi f, \quad \delta = \sqrt{\frac{2\eta}{\omega \rho}} \)  

(2.7)

where \((f)\) is the resonance frequency and \((\delta)\) the decay length.

As a consequence of this damping (Fig. 2.2), the frequency shift and thus the mass change during oscillation in liquid may not correspond to that predicted by Sauerbrey in equation 2.5. In fact, for films that are non-rigid, the shear motion imposed at the interface can undergo a significant phase lag across the film, which causes elastic energy storage and dissipation in the film [6, 9]. Recently theoretical models have been proposed to describe a response for non-rigid adlayers of a given thickness and density [10].

![Cross-sectional view of a TSM resonator contacted by a fluid. The in-plane oscillation of the surface radiates a damped shear wave into the fluid. [5]](image)

Figure 2.2. Cross-sectional view of a TSM resonator contacted by a fluid. The in-plane oscillation of the surface radiates a damped shear wave into the fluid. [5]

### 2.1.5 The Decay Length

A boundary condition states that the fluid motion decays with distance from the surface, vanishing at infinite distance [8]. In practice this means that the energy transferred from the oscillating surface is dissipated by the viscosity of the liquid. The decay length can be defined as the distance from the surface where the amplitude of vibration has decreased to \(1/e\) of the original value. Something that may also be interpreted in surface velocity, i.e. when \(y = \delta\) in equation 2.6 the velocity \((v_x)\) has fallen with a factor \(1/e\) compared to the velocity at the surface \((v_{x0})\). An important parameter when predicting the decay length in equation 2.7 is the resonance frequency \((f)\). It should be noted that a higher frequency results in a smaller decay length and inversely. The consequence of reaching the decay length and beyond in a biosensor is not fully investigated and the response is unclear. A parameter most likely affected by interactions far out from the surface is the sensitivity which will be discussed further on.
Example 2.1: An example of a calculated decay length

The FBAR resonates at about 1GHz. If the sensor surface is in contact with water the viscosity will have a value of $8.90 \times 10^{-4} \text{kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$, and density the value of $998.2 \text{kg} \cdot \text{m}^{-3}$. Equation 2.7 gives the solution

$$\delta = \sqrt{\frac{2\eta}{\omega \rho}} = \sqrt{\frac{2 \times 8.9 \times 10^{-4}}{2\pi \times 1 \times 10^9 \times 998.2}} \cdot \frac{\text{kg} \cdot \text{s} \cdot \text{m}^3}{\text{m} \cdot \text{s} \cdot \text{kg}} \approx 17 \cdot 10^{-9} \text{m} = 170 \AA \quad (2.8)$$

The decay length of a QCM resonating at 10MHz under the same circumstances is approximately $1700 \AA$, i.e. 10 times longer.

2.1.6 The Energy Dissipation Factor, D

The most obvious quantity to measure, in addition to the resonance frequency, is the damping of the crystal oscillation, i.e. its dissipation factor, D, which is inversely proportional to the Q factor. [11]

The Q-factor is associated with acoustic and electrical losses within the system caused by the intrinsic physical properties of the material as well as by external effects. Such as imperfections and defects caused during processing, defects in the electrode material, and atmospheric damping [6, 10]. The dissipation factor is defined by

$$D = \frac{1}{Q} = \frac{E_{dissipated}}{2\pi E_{stored}} \quad (2.9)$$

where $(Q)$ is the quality factor, $(E_{stored})$ the energy stored in the oscillating system and $(E_{dissipated})$ is the dissipated energy during one period of oscillation.

We know from Sauerbrey that an external load may be treated as a mass increase of the crystal itself. However, for larger molecules, which may be viscous at the interface, the load will not couple perfectly to the oscillating motion of the crystal. As a consequence, the frequency shift will be less than predicted but there will also be an increase in the energy dissipated within the system through frictional losses between i) the surface and the adlayer, ii) the adlayer and the surrounding liquid, and iii) the adlayer itself. Hence, if the dissipation is measured with sufficient accuracy, it can provide additional information about the viscous properties and friction of adsorbed layers on the surface.

2.2 The QCM System

The Quartz crystal microbalance (QCM) is a sensitive weighing device based on the piezoelectric properties of the crystalline form of SiO$_2$ i.e. quartz. With the
development of quartz crystal resonators (QCR) as frequency control elements in the 1920s and 30s the effect of added mass on the resonance frequency became known. The QCM has evolved to enable measurement in applications in analytical chemistry, electrochemistry, and biochemistry.

It is a practical tool for real-time measurements such as adsorption of polymers, vesicles, proteins, nucleic acids and cells. The advantage of the QCM technique has so far primarily been the simplicity and sensitivity (in the $ng \cdot cm^{-2}$). The AT-cut quartz crystal ($\theta \approx 35^\circ$) is the most common type of crystal used in QCM devices. The advantage with this type of crystal is that it has nearly zero frequency drift with temperature around room temperature. However, frequency drift may occur due to variations in the ambient environment [12, 6]. The principal underlying this technique is the Sauerbrey equation with the limitations mentioned earlier, such as measurements with a viscoelastic film. These factors, which at first sight may appear as undesirable complications of the QCM technique can become useful with Q-factor or dissipation (D) measurements (QCM-D). [10]

2.3 The FBAR System

2.3.1 Background

Thin film electroacoustic (TEA) technology is a terminology often used in the telecommunication industry. The use of TEA technology is primarily for wireless communication in mobile cell phones and base stations, more specific in application such as delay lines, oscillators, resonators, sensors, actuators, dispersive delay lines, acoustic microscopy as well as in specialized military radar systems. The research in thin film electroacoustics spans for over 40 years and the most common piezoelectric materials today are zinc oxide (ZnO), lead zirconium titanate (PZT) and aluminum nitride (AlN). The latter appears to be the best compromise between performance and manufacturability and is the most suitable for mass production. Film bulk acoustic resonators (FBAR) is TEA technology that uses electroacoustic resonance. [4]

2.3.2 Fabrication

A standard FBAR structure with a AlN film is fabricated using a reactive sputtering deposition. That is, aluminum atoms are sputtered from the Al metal target while nitrogen atoms are introduced in a mixture of argon and nitrogen resulting in a $2\mu m$ thick film after about 30 minutes in the reactive atmosphere. There are of course other parameters involved in the process which can be found in [13]. The
bottom and top Al electrodes were patterned with standard lithography and etching processes. The overlap of these electrodes define the active area of 300x300µm within which the acoustic wave is excited. Additional etching is required to define a cavity and the channels (see chapter 3), which form the system for the analyte to be transported to the bottom electrode, i.e. sensing area. Figure 2.4 shows a cross section view of the fabricated resonator.

![Figure 2.4. Schematic illustration of the shear wave FBAR resonator with a microfluidic system. [4]](image)

Measurements have been carried out with vertical mode FBARs in liquid with poor performances, and shear mode FBAR is to be preferred in biosensing applications [14]. To obtain shear mode FBARs, deposition of a tilted AlN film on a substrate should be used. A trivial way to accomplish this is to tilt the substrate or place the substrate off axis relative to the target. However, films deposited in this way generally suffer from poor thickness uniformity. To solve this problem a new approach is suggested in [14] where a two stage deposition process is described to obtain a high quality AlN film. [4]

### 2.4 FBAR vs. QCM

The fundamental difference between a QCM and an FBAR sensor is the frequency at which resonance occur. QCM has a relatively low resonant frequency, thus a relatively low mass sensitivity (mass sensitivity of a resonant mass sensor is generally inversely proportional to the resonant frequency of the sensor) [15]. Mass sensitivity of a piezoelectric mass sensor can be defined as

\[
S_m = \lim_{\delta m \to 0} \left( \frac{\delta f}{f} \right) \left( \frac{1}{\delta m} \right) \tag{2.10}
\]

where \((\delta m)\) is the mass added to the mass sensor per unit area. A more generalized equation is given by

\[
S_m = \frac{1}{\rho_p l_p} \tag{2.11}
\]

where \((\rho_p)\) and \((l_p)\) are density and thickness of the layer in the composite resonator, respectively.
To exemplify this; an FBAR with the thickness of 3.1\(\mu\text{m}\) (all layer included) resonates at about 900 MHz. The calculated mass sensitivity would then be 649\(\text{cm}^2 \cdot \text{g}^{-1}\), around 50 times that of a QCM operating at 6 MHz. However, this huge gain in sensitivity is to some extent of less value since the loss in stability is linearly proportional to frequency and hence the mass resolution. [15, 4]

Other significant differences are temperature stability and the Q-factor. The latter, plays a significant role in the FBAR device where the acoustic energy loss due to dissipation into water is reduced to approximately half of the quality factor measured in air. This is unfortunate considering the value is only about 300 in air. The same reduction occurs in a QCM where only 10% remains of the Q-factor. On the other hand, the starting value in air for a QCM is approximately 20,000. The FBAR is superior in temperature stability. [14]

It might not be clear who the winner is between the well-established QCM and the relatively new FBAR. Commercially, FBAR sensors can be manufactured in high numbers of low cost, and make way for the possibility of create bio-analytic tools on a single chip [4]. This is perhaps the greatest advantage with new FBAR sensors. There is, however, a great deal of development to come before a commercialized FBAR product is available on the market.

### 2.5 Null Ellipsometry

Ellipsometry is an optical method used to determine surface properties like layer thickness or density. The method is based on the change upon substrate adsorption of the state of polarization of elliptically polarized light reflected at a planar surface. From the change in ellipsometric angels (\(\Psi\) and \(\Delta\)), the reflected index and thickness of the film can be obtained. The main advantages with this method is the quick and relatively easy procedure, applicable in both air and liquid, and that no additional chemicals or marker are needed. It is also proven to be a reliable technique. The disadvantages include the necessity of a reflecting surface, and the rather complex theory in cases when extremely detailed information is required [16]. Fig 2.5 shows the experimental setup for ellipsometry.

![Figure 2.5. Principle of ellipsometry. [7]](image)
2.5 Null Ellipsometry

2.5.1 Math Behind Ellipsometry

The obtained parameters when performing ellipsometry measurements are the ellipsometrical angles $\Psi$ and $\Delta$. The angular positions of the polarizer and analyzer should not be mistaken for $\Psi$ and $\Delta$ in equation 2.12. \(\tan \Psi\) represents the ratio of the difference in amplitude before and after reflection of the electric field in the plane of incidence (denoted $s$). Correspondingly, $\Delta$ represents the difference between the phase changes, $\delta$, of the s and p components that occur upon reflection [17, 18]. The change in polarization at the reflection of light ($r$) can be defined as

$$\rho = \frac{r_p}{r_s} = \left| \frac{r_p}{r_s} \right| \cdot e^{i(\delta_p - \delta_s)} = \tan \Psi \cdot e^{i\Delta} \quad (2.12)$$

Upon substrate adsorption onto the surface, the phase and amplitude of the reflected light changes. These changes are different from the s- and p-components of the incident light and hence translated into new values for $\Psi$ and $\Delta$. To estimate the thickness $d$ of an organic film, using the values of $\Psi$ and $\Delta$, a three phase model is used (see Figure 2.6). [17, 18]

![Figure 2.6. Illustration of the three phase model used to calculate the thickness of an adsorbed film. [18]](image)

In this model the complex reflections coefficients are given by:

$$r_p = \frac{r_{01p} + r_{12p} \cdot e^{-i2\beta}}{1 + r_{01p} \cdot e^{-i2\beta}} \quad (2.13)$$
$$r_s = \frac{r_{01s} + r_{12s} \cdot e^{-i2\beta}}{1 + r_{01s} \cdot e^{-i2\beta}} \quad (2.14)$$

the Fresnel coefficient for s- and p-polarized light are denoted $r_{xys}$ and $r_{xwp}$, respectively. The subscripts 01 and 12 denote the ambient/film interface and film/substrate interface, respectively.
The light will experience a phase shift (\(\beta\)) due to the thin film, called the phase thickness, given by

\[
\beta = \frac{2\pi d}{\lambda} \cdot N_1 \cdot \cos \phi_1
\]  

(2.15)

where \((\phi_1)\) is the refraction angle of the film, \((N_1)\) is the refraction index, \((\lambda)\) is the wavelength of the light and \((d)\) is the thickness of the film. Together equations 2.12, 2.13, 2.14 and 2.15 results in an expression for the film thickness which can be calculated using the McCrackin algorithm. [17, 18]

### 2.6 Layer-on-Layer

The idea of chemically creating layer upon layer of a substrate may seem simple. In fact, it is a rather easy process under the right conditions such as concentrations and pH. However, to control each layer, building a specific thickness each time and form this uniformly distributed over a surface is a different challenge.

There are several ways of building a multilayer and they all involve atomic interaction. Firstly, there are the three fundamental noncovalent bonds. i) Hydrogen bonds. Which are relatively weak, nonetheless crucial for macromolecules such as DNA. ii) Electrostatic interactions, where the strength highly depends on the electric charges on atoms and the presence of water. iii) Van der Waals interactions. These interactions are associated with small energies. [19]

The strongest bond present in biochemistry is a covalent bond or preferably a cross-linked bond. A typical carbon-carbon bond has bond length of 1.54 Å and a bond energy of 85 kcal \(\cdot\) mol\(^{-1}\); almost 100 times stronger then the noncovalent bonds. [19]

#### 2.6.1 Self Assembled Monolayer

Self-assembled monolayers (SAMs) are orderly assembled molecules, spontaneously formed when active molecules adsorb to a surface from a solution. The type of SAM that holds a great deal of interest are funcionalized alkanethiols, mainly because they are available at different lengths and may be synthesized with a variety of functional groups. They are also the most studied SAMs to this date [20]. The structure of a typical SAM consists of the head group, a thiol that readily adsorbs on a gold surface. The alkane chain points outwards from the plane with a tail of a functional group at the end (HS-(CH\(_2\))\(_N\)-X). Even though a self-assembled monolayer forms rapidly, it is necessary to use adsorption times of 15h or more and only at low concentrations (1-2 mM) in order to obtain well-ordered and defect-free SAMs.

#### 2.6.2 Activation and Coupling Chemistry

Crosslinked multilayers can be achieved by the immobilization of ligands using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The role of EDC is simply
to facilitate the formation of amide bonds between a carboxylate group and an amine [21]. The coupling chemistry of EDC is illustrated in Figure 2.7.

![Figure 2.7. Water soluble EDC reacts with carboxylate groups to form an active ester intermediate.](image)

A alternative path for the activated ester intermediate is to react with a sulfhydryl group and generate a thiol ester linkage, or with a water molecule and hydrolyze back to the original carboxyl group, option 2 and 3 (Fig. 2.7) respectively. The rate of hydrolysis is very rapid, so for practical purposes, the activated species must react with a ligand as soon as possible. [21]

Although EDC-mediated coupling reactions are quite efficient there is a procedure using N-hydroxy-succinimide (NHS) as an addition to EDC which makes the intermediate ester more stable. The intermediate ester, in this case NHS, reacts with the same amine functionality, see figure 2.8. The advantage in this process is the increase of half-life of the NHS-ester which subsequently increases coupling of amide bond formation. Since EDC and NHS mediates the formation of amide linkage without leaving a spacer molecule it is called a "zero-length" cross-linker. [21]
2.6.3 Fibrinogen

Fibrinogen is an extracellular human plasma protein (340 kDa) well studied because of its biological importance in the blood coagulation cascade. It is synthesized in the liver and circulating in the blood at a concentration of 2.6 mg/ml. When tissue damage occurs fibrinogen adsorbed to the wound is cleaved by another coagulation protein into an insoluble clot of fibrin. [22]

The most accepted model of fibrinogen was proposed by Hall and Slayter [23] in 1959 and consist of an elongated trinodular structure with one E domain and two D domains. Within the D domains are two sets of three polypeptide chains (alfa, beta, gamma) and the total length of the molecule is about 47.5nm. The fibrinogen molecule is illustrated in Figure 2.9 [24].

When activating proteins, including fibrinogen, the intermediate complex is either formed from the C-terminal of the peptid chains or in the side-chains of
the amino acids like aspartate (Asp) and glutamate (Glu) (Fig. 2.10) . As they contain a terminal carboxylic acid they are usually negatively charged at neutral pH. Out of the 20 different amino acids only a few have the possibility to react with the active ester intermediate. In particular lysine (Lys) and arginine (Arg) due to their relatively long side-chains that terminate with groups that are positively charged at neutral pH. Lysine is capped by a primary amino group and arginine by a guanidinium group, both shown in figure 2.10. [22, 24]

An important aspect of fibrinogen is the characteristics of being a "sticky" protein with a strong tendency of adsorbing well to most surfaces. This suggests that there are a lot of parameters involved, such as concentration and surface chemistry upon adsorption. Fibrinogen will experience conformation and orientation changes and even a certain degree of denaturation when exposed to different surface materials. Studies like [25] and [24] have been made in order to investigate such changes and an important conclusion has been made that the protein adsorbs more likely to a hydrophobic surface then a hydrophilic. [22]
Chapter 3

Experimental Details

3.1 Coupling Chemistry

Commercially available bovine fibrinogen (Sigma, USA) was used as protein ad-
sorbed layer-on-layer dissolved in sodium phosphate buffer (PBS) at pH 7.4 with
a concentration of 1mg/ml. The activation compounds was prepared according to
protocol LP0010 by Attana sensor technologies by using 0.2M EDC (Sigma, USA)
with 0.05M NHS (Sigma, USA) dissolved in distilled water (Milli-Q quality). In
order to avoid protein clusters a 0.005 Tween 20 (Sigma Aldrich, USA) solution
was added to the protein solution.

3.2 Null Ellipsometry

The instrument used when performing the ellipsometric measurements was an
automatic Rudolph research AutoEL III ellipsometer equipped with a He-Ne laser
at wavelength 632.8 nm set at an angle of incidence of 70 degrees. The refraction
index was set to N=1.5 for organic films when applying the three phase model.
The surfaces was prepared by cutting a square (2x2 cm) from a silicon dioxide
wafer with vaporized gold and cleaned in a basic solution with 5:1:1 parts of
distilled water (Milli-Q quality), hydrogen peroxide (\(H_2O_2\), 30%, Merck, USA),
and ammonia (\(NH_3\), 25%, Merck, USA), respectively, at 80 degrees during 5 min.
This step is by far the most efficient way of removing organic contamination and
will later be referred to as TL-1 wash.

When performing in situ ellipsometry a cuvette was used. To calibrate the
instrument the cuvette was filled with a PBS buffer used as a reference prior
to the measurements of the adsorbed layers. All in situ experiments was made
under static circumstances, the possibility of a flow cell was not available. The
thickness of the adsorbed layers was determined with a software program using
the McCrackin algorithm.
3.3 QCM

The Attana 100 system was used as QCM equipment. To optimize the liquid interaction in the experiments, deairation of the liquids was used and a flow rate set to approximately $50 \mu L \cdot min^{-1}$. The sensor chip was purchased from Attana and has a gold surface area of $0.159 cm^2$. Before measurements the chip was thoroughly rinsed in ethanol (99%) and incubated with a self assembled monolayer (SAM), mercaptopropionic acid ($C_3H_6O_2S$) at a concentration of 2mM for 24 hours. The chip has a resonance frequency of 10Mhz and measures the series frequency [7], see section 2.1.2. The measurement chamber volume is < $5 \mu l$.

3.4 FBAR

The FBAR sensors used in the experiments had been fabricated at Uppsala University according to the description in 2.3.2 with the addition of thin layers of Ti and Au thermally evaporated onto the bottom electrode. The active surface area is $0.0009 cm^2$ and the cavity below this area is a channel with a height of $300 \mu m$ and a width of $600 \mu m$. The flow rate was also here initially set to approximately $50 \mu L \cdot min^{-1}$. The operating frequency of the FBAR tested within the scope of this thesis was between 800-1.2GHz, i.e. an AlN thickness between 4$\mu m$-2$\mu m$.

The electrical measurements were performed using a HP 8720D network analyzer, which measures the characterizations of the resonator passively by varying the frequency. The instrument is limited to 1601 measurements points within a frequency sweep. To be able to improve the resolution for determining the resonance frequency a mathematical curve fit was made afterwards to the admittance and impedance responses from which the series and parallel resonance frequencies are derived respectively. Similarly, the series and parallel Q values are extracted from the phase slope at series and parallel resonance frequencies respectively.
Chapter 4

Results and Discussion

There were initially three fundamentally different methods examined in the search for a biochemical system able to form a multilayer. The first one involved polyethylene glycol (PEG), or more specific a PEG alkane thiol with a biotin conjugate. This molecule was of interest because of its high reactivity toward gold and its ability of forming self assembled monolayers (SAMs). The possibility of purchasing these PEGs in a variety of different lengths contributed to that interest. The theory was to test a set of sensor surfaces with biotinated PEGs of various length and see how they would differ in frequency response when streptavidin was introduced to the system. The advantage with this method is that i) biotin-streptavidin chemistry is well studied and ii) an accurate distance from the surface could be calculated. The disadvantage is that i) the sensor surface has to incubate in a PEG thiol solution prior to any test and ii) there were not enough length variation to choose from.

The second method involved electrostatic interactions. The theory was to use negatively charged proteins like human serum albumin (HSA) alternated with positively charged polyethyleneimine (PEI) in order to form a multilayer [7]. The advantage with this method is the simplicity and ability of creating very thick layers. However, layer thickness might vary and a similar test with electrostatic interactions had already been made at the Ångstöm laboratory in Uppsala with FBAR sensors, so a new approach was preferable.

The idea of cross-linking came from [26] where EDC/NHS was used to covalently bind proteins. There are of course many proteins to choose between when forming a multilayer and a few were tested, like HSA, catalase and fibrinogen. The latter was eventually chosen as the most promising due to the size and easy access but also the fact that it is a well studied protein. The theory of layer-on-layer adsorption could now be tested.
4.1 Ellipsometric Measurements

Test E1

In order to verify the theory of layer-on-layer adsorption by protein coupling a number of ellipsometric experiments was set out. The first and obvious was to see how proteins adsorb to a single gold surface. Fibrinogen was chosen early in the project, mainly because of its size which is favorable when building thick multilayers.

The parameters involved upon adsorption are the surface chemistry, protein concentration, and other factors such as pH and temperature.

Four surfaces was used in the test involving ellipsometry in situ, i.e in a cuvette, and dynamically measured for 30 minutes. They had all been washed using the TL-1 protocol and three had been incubated with 3-mercaptopropionic acid (MPA) for 24h. MPA is categorized as a Self assembled monolayer and is a relatively small alkanethiol with a 3-carbon tail and a carboxylic acid at the end. A surface covered in MPA will have hydrophilic properties and have a slight negative charge while an untreated surface quickly becomes contaminated with organics making it hydrophobic. The MPA treated surfaces were activated with EDC/NHS prior to the protein adsorption of fibrinogen in a physiological (neutral) pH of 7.4. Test E1 is shown in Figure 4.1.

![Figure 4.1](image)

Figure 4.1. A kinetic test in order to verify fibrinogen adsorption of different concentrations.

Test E2

To make sure that cross-linking connects proteins an ellipsometric test in air was performed. A gold surface coated with MPA was used and between the ellipsometric measurements the surface was incubated first in EDC/NHS for 30 min followed by fibrinogen (1mg/ml in PBS buffer with pH 5.5) for another 30 minutes. This alternation carried on for five cycles with a thorough washing procedure in distilled water and blow dried (N₂) before ever measurement. Test E2 is illustrated in Figure 4.2.
4.1 Ellipsometric Measurements

Test E3

To get an accurate and more realistic view of the thickness, in situ ellipsometry was performed. In this case the adsorbed proteins were only gently washed and always kept in liquid solution allowing the tertiary structure of fibrinogen to be undamaged, at least to some extent. However, this gentle treatment, in opposite of the E2 test, may result in unspecific interactions, i.e. proteins clustering together in electrostatic interactions instead of being cross-linked. An important aspect with this test is the pH, which was set to 5.5 in the protein solution. Test E3 is shown in Figure 4.3.

Test E4

A Final ellipsometric in situ test was made (Fig. 4.4) with an optimized protocol. The protocol is found in appendix C and will later be refered to as the FIB protocol. The purpose of this test besides testing the protocol, is to give an
approximative layer thickness value after fibrinogen injections which may be useful when comparing the ellipsometric result to later experiments with the QCM and FBAR. Average layer thickness was calculated to 28.2Å.

Figure 4.4. The final test was performed in order to verify the FIB protocol. The graph shows the ellipsometric measurement in situ with each cycle representing an absorbed fibrinogen layer.

4.1.1 Discussion and Conclusion

An acceptable protein coverage of the first layer should be in the region of 70% of the total surface area. A better cover is difficult to obtain due to repulsion forces between proteins. The first ellipsometric test (E1) showed that a protein concentration around 1mg/ml results in a fast adsorption rate followed by a stabilization at around approximately 40-45 Å. A similar behavior is observed at a concentration of 0.1mg/ml where the stabilized thickness reaches 35 Å. A protein solution less then 0.1mg/ml never reaches a maximum during the 30 minutes observed. The advantage of choosing a higher concentration is mainly the greater coverage of the surface. However, this may result in unspecific interaction that will favor a protein like fibrinogen to cluster and stick to each other. These non-covalently bond interactions can be reduced by adding a detergent like tween 20. In addition to a detergent a steady flow will also prevent proteins from sticking, something that should be taking into account in the ellipsometric graph which only shows experiments done in a static solution.

The effect of protein adsorption on a clean uncoated surface is also relevant to point out. The first test shows that a lower (0.1mg/ml) concentration of fibrinogen absorbs to the same extent on pure gold as a higher concentration (1mg/ml) on a MPA coated surface. The results confirm what O. Joshi et al. concluded with their test on heparinized, i.e. a hydrophilic and negatively charged surface, compared to an unheparinized [25]. Even though the interaction between the surface and the first layer of fibrinogen is not of covalent nature it is considered just as strong due to the strong interaction created by the high number of electrons surrounding the gold atoms and the fibrinogen molecule.
The importance of pH is clearly seen when comparing the E1 test (Fig. 4.1) with the E3 test (Fig. 4.3), with respect to the first adsorbed layer of fibrinogen. Both performed and measured in liquid with the same protein concentration and without a detergent. Since fibrinogen is dissolved in PBS the pH can easily be changed by adding hydrochloric acid (HCl). Using a PBS buffer with a pH at 5.5 (test E3) will have the effect that a higher number of amino acid side chains are positively charge knowing that the isolectric point (pI) for fibrinogen is approximately 5.5 [24]. This means that a surface coated with MPA (slightly negative) has a stronger attraction to such proteins. The result is more electrostatic adsorption on the first layer seen in the Figure 4.3 which is considered to be non desirable.

Another visible difference appears when comparing the ellipsometric measurements in air (Fig 4.2) and in situ (Fig 4.3). A much thinner first layer is observed in the E2 test, probably due to the fact that proteins denaturize and collapses on the surface after rough treatment including thorough washing and blow drying with N2 gas.

Test E4 is the successful result after using the FIB protocol. To sum up; i) SAM coated surface or not, the difference in protein adsorption is negligible, ii) a physiological pH at 7.4 is a better option to avoid any unspecific interactions, iii) a protein concentration of 1mg/ml with the addition of Tween 20 is most suitable.

4.2 QCM Measurements

The coupling chemistry with EDC/NHS is widely established and the ellispometric measurements have shown that layer-on-layer of fibrinogen is possible. However, applying the FIB protocol in the QCM system is quite different because it is a dynamic system with a continuous flow and on top of that a sensor surface oscillating at 10Mhz.

Test Q1

Figure 4.5 illustrates how a first layer of fibrinogen adsorbs to a clean gold surface. Even though a clear signal is visible the overall noise level is high and despite a long period of time (250 min) a stable baseline was never achieved. The test was then repeated with a MPA coated gold surface shown in figure 4.6. Most notable is the baseline stabilizing after about 90 min. This stabilization time varied from 90 to 120 min but did always occur when using MPA as a SAM prior to a QCM test. The somewhat loosely definition of a stable baseline in QCM is that the fluctuation, also known as drift, stays under 2Hz in 100 s.
Test Q2

Two of the more important parameters in the layer-on-layer technique were also examined with the QCM equipment. They are the buffer pH and the importance of a detergent. The result is shown in Figure 4.7 and resembles the thickness variations pointed out earlier in the ellipsometric test.

Two equivalent gold chips prepared in a MPA solution for 24 h were used. Both activated in freshly mixed EDC/NHS prior to the protein injection. One surface was allowed to interact with fibrinogen in a PBS buffer of pH 5.5. The other was interacted to fibrinogen in a PBS buffer of pH 7.4 with an additional 0.005 of Tween 20. Flow rate was set to 45 $\mu$L $\cdot$ min$^{-1}$ and the protein concentration was 1mg/ml. Not surprisingly, the latter showed a better result in the aspect of electrostatic and non specific interactions. The saturation after the first injection is confirmed when the second injection does not affect the resonance frequency markedly. The contrary is revealed in the other system where a combination of more positively charged regions on the protein and the lack of detergent enhances unspecific interactions upon each injection. Something which is clearly visible as a negative shift in frequency at each of the following two fibrinogen injections.
Figure 4.7. Two different protocols involving activation and fibrinogen. Left graph shows an unsaturated surface where additional protein injections adsorb to the surface. The right graph shows a surface where the first layer of fibrinogen gives a good saturation and no additional protein adsorption on the second injection which is desirable.

Test Q3

The characteristic appearance of the EDC/NHS injections was consistent during every test. However, variations in delta f were observed mainly between the first activations and the rest that followed.

As described earlier activation leads to an intermediate coupling molecule, which is relatively large. Expectably, this would increase the surface mass thus lower the resonance frequency, generally this was the case, but at the beginning a positive shift in frequency always occurred. Such a variation is shown in Fig 4.8. This may be due to hydration effects where water leaves the surface after a more hydrophobic environment was induced by the NHS ester. A similar explanation would account for a positive shift later on when activating in a multilayer fibrinogen matrix. Because every time a protein cross-links to another protein, inside or outside the matrix, a conformation change occurs making the protein or proteins more or less hydrophobic. Every conformation change after activation will result in the total ability of the surface to bind water, i.e. additional or subtracting mass.

Figure 4.8. The difference in resonance frequency after activation with EDC/NHS is shown. Most commonly is a decrease in frequency (left) but there is sometimes an increase in frequency (right).
Test Q4

A long run was finally made. Partly to confirm the technique with layer-on-layer and test the FIB protocol, but also to study any changes in sensitivity. To actually reach the decay length of the system was never considered (it would mean at least 40 alternation with EDC/NHS and fibrinogen). According to theory the sensitivity decays with distance from the surface so technically a tenth layer would give a less respond in delta $f$ then the first or second even though they correspond to the same mass. Due to the long run (over 10h) the data was cut in order to see the layer adsorptions more in detail. For example is the baseline stabilization period removed along with the first layer of adsorption and a small fraction in the middle. The test is illustrated in Figure 4.10 and was a great success. The whole test can be seen in appendix A but the best overall view is obtained in a bar chart in figure 4.9.

![Figure 4.9. A bar chart representing the Q4 test and figure 4.10.](image)

4.2.1 Conclusion

The theory of protein coupling tested in the ellipsometric study was proven to work just as well in a dynamic systems such as the QCM. The FIB protocol works well and the variation in added mass at each layer is not significant. Apart from the unstable baseline, fibrinogen adsorbs well to a pure gold surface, although a suitable SAM is to prefer in order get a hydrophilic surface and good wetting. The activation step showed that it can give the surface proteins different characteristics which may led to the surface ability to bind or release water. A change in sensitivity could not be detected with the twelve layers adsorbed in figure 4.9 or 4.10.
Figure 4.10. The final QCM measurement using the FIB protocol. The graph is cut in order to fit it on one page, the whole measurement can be seen in appendix A which also includes a description schedule of the different injections.
4.3 FBAR Measurements

The FBAR construction is in reference to the QCM system fairly similar. In fact, some parts of the equipment are identical to the Attana 100 system, such as the injection vault, the pump and plastic tubes. As mentioned before the fundamental difference is the frequency at which resonance occurs. Besides that, an FBAR can basically be described as a scaled model of a QCM. It was therefore surprising, at first, when the FIB protocol did not produce the same result in the FBAR system.

Test F1

Figure 4.11 illustrates how the system reacted when the FIB protocol was used. Prior to the test the surface was washed in ethanol (99%) followed by PBS as running buffer (pH 7.4) until the baseline was stable. The first and second fibrinogen injection (Fig 4.11 (a)), resembles the QCM test and confirms a saturated first layer. The activation step is also similar to the QCM. However, the third injection of fibrinogen is barely notable and does not correspond to the same response in frequency shift shown in the QCM data after an activation. Despite the indication of an unsuccessful activation the test continued (Fig 4.11 (b)) without improved results.

Besides the frequency, the flow rate should also be considered as different from the QCM system. Even though the flow rate is set to 45-50 µL·min\(^{-1}\) (same as the QCM) the actual velocity of the liquid passing the surface is more rapid due to smaller canals, a statement derived from Bernoulli’s principle (see appendix C). The explanation of the unsuccessful activation was however quite simple. While the QCM sensor is in a vertical position to the plane opposite to the flow, the FBAR sensor is positioned horizontally at the ceiling of the measuring chamber (see Fig 2.4 in 2.3.2). In such a system gravity will affect the molecule in a negative aspect making the mass transport less likely. The solution to this problem was simply to turning off the flow with EDC/NHS inside the measuring chamber allowing for diffusion and mass transportation to take place.

![Graph showing the unsuccessful result of activation with the first FBAR measurements.](image-url)
Test F2

A modification of the FIB protocol was done, now allowing EDC/NHS to incubate for 30 minutes in the measuring chamber, similar to the incubation in the ellipsometry measurements. A proper investigation was then set in progress lasting for two days. See figure 4.12 and 4.13.

![Figure 4.12](image1.png)

**Figure 4.12.** Day one with the new modified FIB protocol.

![Figure 4.13](image2.png)

**Figure 4.13.** Day two with the new modified FIB protocol.

The gold surface was first cleaned with ethanol running through the system followed by a first layer of adsorbed fibrinogen. Day one (Fig 4.12) shows the successful cross-linking between proteins with the new and modified FIB protocol.
Except for the longer activation step the graph is clearly similar to the QCM tests. However, day 2 (Fig 4.13) reveals something interesting. Somewhere around 180 to 220 minutes and 15 to 17 injections of fibrinogen, depending on the curve (parallel or series), the frequency reaches a minimum. The strange part is yet the following injections, which give rise to a positive frequency shift, something better illustrated in the bar chart (Fig 4.14). The complete graph including the parallel and series frequency of day one and two merged together and the injection schedule can be seen in appendix B.

![Figure 4.14. Bar chart of the F2 test.](image)

It is not the first time an inverted signal like this appears in an FBAR test. An unpublished and recent investigation by the thin film group at the Uppsala University have shown similar behavior in the FBAR when building multilayer by alternating streptavidin and biotin molecules. There is also an article by Siemens [27] in 2003 that reports positive frequency shifts in thick polymer films with measurements in gas.

To better understand the coupling chemistry two selected sections are illustrated in figure 4.15. The interesting part is the observation of how the frequency response is different upon activation early in the measurement compared to later. Figure 4.15 (a) illustrates the beginning of the test and shows a dip in frequency when EDC/NHS (E) is introduced into the system. After 30 minutes the running buffer of PBS (P) enters and give rise to a slight positive response followed by another frequency decrease at the fibrinogen (F) injection. This pattern eventually changes to a positive frequency shift upon EDC/NHS injections, a shift that increases with time, seen in Figure 4.15 (b).
4.3 FBAR Measurements

Figure 4.15. Graph shows the different frequency response at the activation step. When EDC/NHS (E) is injected at the beginning of the test a negative shift occurs, Fig 4.15 (a). As the measurement continues the response to EDC/NHS changes to a positive shift, Fig 4.15 (b), the PBS (P) is not shown here.

An explanation for this behavior may be the following. In the beginning the layers of fibrinogen are close to the surface and even though an injected EDC/NHS solution has a lower viscosity a negative frequency shift is to expect. This due to the fact that fibrinogen is more likely to bind water in an EDC/NHS solution than in a more salty solution such as the running PBS buffer. Further away from the surface, however, means less significance to such interaction. Instead the positive shifts visible after a few layers are actually viscosity changes, occuring when the salty PSB solution is changed to EDC/NHS.

Another interesting aspect in the EDC/NHS step is the overall negative shift during the activation which is consistent during the entire measurement. The decrease in frequency is a result of cross-linking inside the protein matrix. Each cross-linking strengthens the layers giving it a more rigid structure which corresponds to a frequency decrease.

Turning the attention to the last part of the measurement (Fig 4.16). By this point over 15 alternating injections of EDC/NHS and fibrinogen had been made. The first thing to notice is how the parallel and series frequency curves diverge from each other at two occasions, first one after 577 minutes and the second after 688 minutes, both when fibrinogen was injected into the system. This is not unusual since they are both sensitive to different perturbation. The series resonance is more sensitive to physical vibrations. While, as mentioned in the theory chapter, the parallel frequency can be sensitive to conductivity in the liquid.

The second thing to notice occurs after 652 minutes when two fibrinogen injections give rise to a positive shift in frequency for both the parallel and series curve. This could well be considered as the decay length and/or detection length for this specific FBAR system. A few more alterations with EDC/NHS and fibrinogen were preformed and showed further positive frequency shifts upon fibrinogen injection.
Results and Discussion

Figure 4.16. The graph illustrates the last section of the F2 test. Top curve shows the parallel frequency and underneath the series.

Test F3

It is always desirable to have reproducible results. Hence, the protocol was tested in a different FBAR system consistent of a silicon dioxide ($SiO_2$) sensor surface. Besides the material change the resonance frequency was twice as high compared to gold electrodes used in the F2 test. The measurement was divided into 3 days (See appendix B) and shows the same behavior as the previous F2 test. The interesting part is the last section where an inverted signal again is found, see Figure 4.17. It is obvious what causes the positive shift, namely the fibrinogen injections, after 481, 531 and 576 minutes. Another thing to notice in this graph is relatively small positive frequency shift upon EDC/NHS injections, as they do not resemble the same pattern seen in the F2 test.

Figure 4.17. The graph illustrates the last section of the F3 test. Top curve shows the parallel frequency and underneath the series.
4.3 FBAR Measurements

4.3.1 The Q-values

As mentioned in the theory chapter the quality factor Q is associated with dissipation and acoustic and electrical losses within the system. Practically this means that adsorbed molecules can be monitored parallel to the frequency by looking at the Q-value, i.e. dissipation at the surface will result in a lower value of Q since they are inversely proportional.

Figure 4.18 illustrates the Q-value of the F2 test. A decrease in the Q factor is clearly visible, unfortunately it is only in the beginning where it is possible to distinguish where an adsorbed layer on protein occurs and shifts the Q-value downwards. At day two it is almost impossible to obtain any valuable data from the graph when the Q-factor drops below 20.

![Figure 4.18. Q-value of the F2 test.](image)

Looking at the last section of the F3 test and its corresponding Q-value provides better visible data. The Q-factor is still associated with adsorbed mass and it is apparent that the Q-value drops remarkably at each fibrinogen injection indicating an absorbed mass on the surface, Fig 4.19. The resonance frequency graph (Fig 4.17) at the same time gives a positive frequency shift indicating a loss of mass. Two observations that contradict each other.
4.3.2 Discussion and Conclusion

The first thing to conclude in the FBAR technology is something quite trivial yet very interesting and important. In contrast to QCM, the FBAR tests always showed a quick and stable baseline. The constant drifting observed at the beginning of every QCM test was never seen in Uppsala. This fact meant that an incubation of MPA was not needed, which also would have been difficult.

The F1 test showed a limitation with the presently used FBAR system. Activation failed due to the construction but the problem was solved by simply stopping the flow, allowing for a mass transport and diffusion to reach the sensor surface. This procedure was only necessary in the case of activation where the reaction rate is slower then in protein coupling reactions. A more permanent suggestion of solving the problem would either be to turn the present system upside down, using gravity as a pulling force, or a complete different design, perhaps one inspired by the QCM technology.

The FBAR experiments provided better understanding of the activation step. The consequently drop in frequency during the 30 minutes of activation is most likely due to the increasing stability of the protein matrix. However, variations in frequency response upon EDC/NHS injections were found. From a negative frequency shift at the start of the measurement to a positive frequency shift later on. The explanation for behavior was possibly the changing ability of the surface to bind or release water.

Frequency sensitivity should according to theory be reduced by surface distance, something clearly visible with the FBAR system in both the F2 and F3...
test. However, it is difficult to find a specific relation between these two or to say that the frequency shift decreases by a certain factor.

The inverted signal found in the F2 and F3 tests is still under investigation and can not yet be explained. Previous tests, such as those done with biotin and streptavidin, showed similar results but they were somewhat questionable. Mainly because the layers are of electrostatic nature and not particularly strong. However, with the FIB protocol the interactions are primarily covalent and to break such a bond would mean a force greater then an oscillating surface and a flow vessel. So clearly, an explanation, other then bond breakage, is needed.

It has been suggested that the properties of the layer itself may be the cause. In a Newtonian fluid, like water, the penetrating wave decays to zero (Fig. 2.2). However, in a viscoelastic layer, like fibrinogen, reflection of this wave might occur and the result might very well be an inverted signal seen in figure 4.16 and 4.17.

4.4 Thickness Calculations

It is important to start this discussion by clarifying the difference between the decay length and detection length. Both expressions have been used in context and even though they probably do not exclude each other there is in the authors view a difference. Having reached the decay length is not equivalent with reaching the detection length. To correlate this statement to the experiments done in this work it seems like the decay length will first be reached followed by the detection length and then the inverted signal. It should also be noted that the terminology used is not to be confused with detection limit; the lower detection limit, $x_{min}$, which is the minimum concentration which can be detected by a given biosensor.

4.4.1 The Decay Length

The biochemical properties in a realistic biosensor is to be considered to be somewhere in between a ridged mass and a viscous layer. That is, some damping of the amplitude of vibration when going further out from the resonator surface is to be expected. Since the sensitivity is proportional to the amplitude of vibration, it is relevant to suspect decrease in sensitivity when increasing the thickness of such visco-elastic layer as the protein layers here investigated. It is suspected that the decay of the oscillating amplitude is related to the decay length for the pure viscous case, defined in equation 2.6. The viscous properties of the fibrinogen layer are suspected to be close to that of water. It might then be relevant to compare the decay length experimentally by looking at the decrease in resonance frequency shift upon fibrinogen injections, i.e. finding the layer (noted decay length layer) where the frequency shift equals $1/e$ of the frequency shift compared to that observed in the beginning, see section 2.1.4. This highly approximated calculation and observation for the FBAR tests (F2 and F3) is illustrated in table 4.1.

The table section noted "The corresponding length" is an approximated calculation of the layer thickness derived from the elliposometric E4 test, which is considered extra accurate due to the long run and the use of the FIB protocol. Average layer thickness in the E4 test was 28.2 Å, see section 4.1.
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Results and Discussion

<table>
<thead>
<tr>
<th>Test</th>
<th>Resonance frequency</th>
<th>The decay length layer</th>
<th>The corresponding length</th>
<th>The calculated decay length</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2</td>
<td>743MHz</td>
<td>∼11~12</td>
<td>310~338Å</td>
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<td>1.28GHz</td>
<td>∼7</td>
<td>∼197.4Å</td>
<td>149Å</td>
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Table 4.1. Approximated calculations of the decay length

4.4.2 The Detection Length

A suggested definition of the detection length in this thesis is the length from the surface which corresponds to frequency leveling off. This occurs at approximately layer 17 and 9 for the F2 and F3 test, respectively. The corresponding length is calculated as above; by multiplying the layers with the average layer thickness derived from the E4 test. There are no equations to theoretically calculate this limit. See table 4.2.

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<td>∼226Å</td>
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Table 4.2. Approximated calculations of the detection length

4.4.3 Discussion and Conclusion

The different values of the theoretically calculated decay length and experimentally estimated decay length is not surprising since there were many approximations. Neither is it wrong to assume that the sensitivity decrease by distance from the surface, this is clearly visible in the bar chart (Fig. 4.14). However, it might not be proportional to the resonance frequency shift.

The result involving the detection length is more interesting because it reveals a relationship between the resonating frequency and the detection length. The product between resonance frequency and layer of reached detection length in the F2 and F3 test are close to proportional to each other.

What actually causes the system to give an inverted signal beyond the detection length is not understood. The theory of reflection at the protein matrix/fluid interaction and a signal node might be the explanation but further investigation is needed.
Chapter 5

Summary and Outlook

The results of this project were founded on a few fundamental corner stones that were set out in the beginning; i) to find a suitable molecule and a biochemical system enabling multilayer formation, ii) being able to estimate the thickness while building the layers, iii) optimizing and creating a unique protocol and iv) verifying the systems functionality in a traditional QCM equipment. These were all done accordingly and before heading to the Ångström laboratory to investigate the frequency dependence and sensitivity of the FBAR system.

The most important conclusions from the experiments performed in Uppsala were; i) the limitation of the present FBAR construction, ii) the decrease in sensitivity, iii) the effects of activation with EDC/NHS, and iv) reaching the detection length and/or decay length and verifying the inverted signal.

Unfortunately, the explanation of the inverted signal has not been satisfying and as mentioned earlier, further investigation will be necessary. An interesting experiment in the future would be to see if other TSM resonators show the same results when applying thick multilayers. The QCM system could in practice be tested if the measuring time was not an issue. To simplify such an experiment there are QCM systems now available with an automatic loader and injection mechanisms.

The FBARs potential to commercially compete with other biosensors on the market looks promising considering the low manufacturing cost and size. Measurement with a network analyzer is a powerful method and provides data information in frequency shift and damping. One of the main objects further on will be to minimize the surrounding equipment and to control the environmental disturbances such as vibration, humidity, dust and temperature fluctuations. Knowing these problems the author of this work is convinced that they will all be solved and that the future of the FBAR technology is bright!
Bibliography


Appendix A

QCM; data and figures

Figure A.1. The complete Q4 test
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Appendix B

FBAR; data and figures

Figure B.1. The complete F2 test. The top curve represents the series resonance frequency and bottom curve represents the parallel resonance frequency.
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Figure B.2. The complete Q-value of the F3 test

Figure B.3. The complete F3 test. The top curve represents the series resonance frequency and bottom curve represents the parallel resonance frequency
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</tr>
<tr>
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<td>8 x EDC/NHS á 100s</td>
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</table>
Appendix C

Miscellaneous

FIB protocol

- Fibrinogen, 1mg/ml in 0.005% Tween 20 with PBS buffer pH 7.4
- EDC/NHS, 40mg EDC in 6mg NHS with 1ml Milli-Q water
- PBS pH 7.4 as running buffer
- Flowrate $\sim 50\mu L \cdot min^{-1}$

Bernoulli’s principle

$$P_1 + \frac{1}{2}v_1^2 + \rho gh_1 = P_2 + \frac{1}{2}v_2^2 + \rho gh_2 v_2 > v_1 \quad (C.1)$$

$$v_2 > v_1 \quad (C.2)$$