A novel biotinylated surface designed for QCM-D applications

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Preface

This master’s thesis work was performed at the Division of Biological Physics at the Department of Applied Physics at Chalmers University of Technology, Göteborg during Jan – Jun 2009. The project was proposed and done in close collaboration with Q-Sense, Göteborg with the aim of reaching a commercial biotinylated surface for QCM-D applications. During the project, the master student Erik Nilebäck also participated at the 8th Q-Sense User Meeting 24-25 Feb at Chalmers University of technology presenting a poster and taking part in lectures and other activities.

Contacts with several possible test costumers, both in Sweden and abroad were established both at the mentioned Q-Sense User Meeting and with continued mail contact. A case study using biotinylated plasminogen was performed in collaboration with Johanna Deinum at AstraZeneca R&D.
Abstract
Control of protein immobilization at sensor surfaces is of great interest within various scientific fields, since it enables studies of specific biomolecular interactions. To achieve this, one must be able to immobilize proteins with retained native structure, while minimizing non-specific protein binding. The high affinity interaction between streptavidin (SA) and biotin is extensively used as a linker between a surface, where SA is immobilized, and the (biotinylated) molecule of interest. Self-assembled monolayers (SAMs) of poly- and oligoethylene glycol (PEG and OEG) derivatives have been proven in literature to minimize non-specific protein binding, and biotin-exposing SAMs have been shown efficient for immobilization of SA.

The aim of this master’s thesis project was to develop biotinylated gold surfaces for quartz crystal microbalance with dissipation monitoring (QCM-D) applications through the self-assembly of mixed monolayers of thiolated OEG (or PEG) derivatives with or without a terminal biotin head group. For this, different thiol compounds were to be compared and evaluated. For the systems under study, the required biotin density for maximum specific SA immobilization was to be established, while keeping the non-specific serum adsorption at a minimum. Model experiments with biotinylated proteins immobilized to the SA-functionalized surfaces were to be performed to evaluate the possibilities for commercialization.

A protocol for the preparation of a novel biotinylated surface was developed based on the immersion of gold substrates in an ethanolic incubation solution of dithiols with OEG chains (SS-OEG and SS-OEG-biotin, 99:1) and found to give reproducible results with respect to low non-specific protein binding and immobilization of a monolayer of SA. The modified surfaces allowed for subsequent immobilization of biotinylated bovine serum albumin (bBSA) and biotinylated plasminogen (bPLG). PLG was the subject of a challenging case study, using a combination of QCM-D and surface plasmon resonance (SPR), where the immobilized protein was subjected to low molecular weight ligands that were believed to induce conformational changes. The high control of the surface chemistry allowed for the interpretation of the increased dissipation shift upon ligand binding in terms of conformational changes.

An obstacle before commercialization of the described biotinylated surfaces is that they do not seem stable for storage > 7 days. The reasons for this have to be investigated further.
## Abbreviations and acronyms

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<th>Abbreviation</th>
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<td>AC</td>
<td></td>
<td>alternating current</td>
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<td>BSA, bBSA</td>
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<td>bovine serum albumin, biotinylated bovine serum albumin</td>
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<td>EACA</td>
<td></td>
<td>e-amino caproic acid</td>
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<td>EG</td>
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<td>ethylene glycol</td>
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<td>EIS</td>
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<td>electrochemical impedance spectroscopy</td>
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<td>FBS</td>
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<td>fetal bovine serum</td>
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<td>HBS-N</td>
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<td>hepes buffered saline</td>
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<td>OEG</td>
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<td>oligo (ethylene glycol)</td>
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<td>PBS</td>
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<td>phosphate buffered saline</td>
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<td>PEG</td>
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<td>poly (ethylene glycol)</td>
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<td>PLG, bPLG</td>
<td></td>
<td>plasminogen, biotinylated plasminogen</td>
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<td>POPC</td>
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<td>palmitoyl oleoyl phosphatidyl choline</td>
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<tr>
<td>QCM-D</td>
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<td>quartz crystal microbalance with dissipation monitoring</td>
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<td>SAM</td>
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<td>self-assembled monolayer</td>
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1 Introduction

Biomolecular interactions are the most fundamental and abundant events in the biological world. They govern all life-sustaining reactions in living organisms such as the photosynthesis and cell breathing, DNA-replication, complement activation, cell communication and a great many more. To be able to understand these interactions, cross-disciplinary fields have evolved such as that of biosensor technology. In biosensor technology, biological sensing elements are coupled to a transducer element to transform the biological response into a signal that is detectable [1]. The use of sensor technology for the study of biological systems enables quick and qualitative evaluation of protein interactions. This often calls for some kind of functionalization of the sensor surface to enable protein immobilization.

The affinity of the interaction between biotin and streptavidin (SA) is very high, \( K_a = 2.3 \times 10^{13} \text{ M}^{-1} \), which is the highest affinity interaction between a protein and ligand that has been observed in a living system. Since SA has four binding sites for biotin it can act as a linker between a biotinylated sensor surface and a biotinylated analyte [2]. This enables studies of the interaction between the captured biotinylated compound, often a protein, and additional biomolecules. One such interaction could be antibody-antigen interaction between a biotinylated antibody immobilized via SA on the sensor surface and an antigen that is subsequently added over the surface.

Q-Sense AB (Göteborg, Sweden) has developed sensor instruments based on quartz crystal microbalance with dissipation monitoring (QCM-D). QCM-D allows for real time adsorption studies of liquid or gaseous analytes to a sensor surface with respect to both adsorbed mass and viscoelastic properties [3]. This allows for biomolecular studies at the sensor surface where both the immobilization and interactions of the biomolecules can be studied [4].

In this master’s thesis, QCM-D and SA-biotin systems were integrated with the development of biotinylated gold coated crystals for possible commercialization by Q-Sense. This kind of biotinylated surfaces are not yet commercially available for QCM-D and would be of particular interest for researchers that want to focus on biomolecular interactions rather than on surface chemistry. Surface functionalization of gold substrates is conveniently performed...
by the well studied process of self-assembly of monolayers of thiolated compounds on gold, driven by the strong interaction between Au and sulfur [5]. Poly- or oligo (ethylene glycol) (PEG or OEG) chains have protein repellent properties and the combination of this technique with thiol-Au SAMs allows for the formation of highly ordered and protein resistant surface coatings [6]. Mixed SAMs of thiolated PEG chains on gold with and without end-attached biotin groups have been shown to work as a substrate for specific streptavidin immobilization with retained native structure of streptavidin. [7]

In this study, a novel variant of biotinylated thiol SAMs has been developed, designed for QCM-D applications. The novelty of the approach lies in the type of thiol compounds that have been used, namely SS-OEG/SS-OEG-biotin (fig. 1-1a). For a comparison, mixed SAMs prepared from SH-C11-OEG/SH-C11-OEG-biotin (fig. 1-1b) and SH-PEG/SH-PEG-biotin (fig. 1-1c) were included. These two types of SAMs and their application to SA immobilization have been described earlier in the literature. However, the performance and structure of a biotinylated SAM is highly dependent on what type of thiols that are used. Therefore this novel approach with SS-OEG was compared to the SH-PEG and SH-C11-OEG systems, both by structural and functional characterization, to evaluate the differences between the three different approaches. Also, sufficient rigidity was demanded of the SAMs to enable straight-forward interpretation of the results obtained with the QCM-D technique.

Figure 1-1 Molecular structures for the thiolated PEG and OEG (top) derivatives and their biotinylated version (bottom) that have been studied in this master’s thesis.

a) SS-OEG/SS-OEG-biotin

b) SH-PEG/SH-PEG-biotin, n=70-110[8]

c) SH-C11-OEG/SH-C11-biotin [9]
1.2 Aim

The aim for this master’s thesis was to develop biotinylated gold surfaces for the specific immobilization of biotinylated biomolecules, using streptavidin as a linker for use in QCM-D applications. Three different approaches based on the preparation of mixed SAMs of the PEG- or OEG thiol systems SS-OEG, SH-C11-OEG and SH-PEG (fig. 1-1) and their biotinylated derivatives were to be investigated regarding both structure and function. One important issue was to establish the optimal biotin density on the surface for maximized streptavidin immobilization and subsequent immobilization of additional biotinylated biomolecules. Issues such as storage stability and regeneration, e.g. the repeated use of a biotinylated crystal, were also to be addressed.

In addition, there was a desire to test the surfaces with different model systems to establish the performance of the system for possible commercialization.
2 Theory

2.1 Self-assembled monolayers

In nature many complex processes are controlled by self-assembly, that is the spontaneous formation of a complex of interacting molecular components formed when reaching thermodynamic equilibrium. Man has adopted these processes to develop systems that are based on the self-assembly of surface active molecules in a monolayer on a surface, that are named self-assembled monolayers, abbreviated SAMs. [5] One of the most studied kind SAMs is based on the adsorption of organosulfur derivatives from solution onto gold in the form of alkane thiols, SH-(CH$_2$)$_n$-X. There are mainly three reasons to why this kind of system has been so extensively studied: Firstly, gold has inert properties and does not oxidize easily. Secondly, the interaction between sulfur and gold is strong and highly specific which allows for the SAM molecules to contain other functional groups, X, without disabling the adsorption process. [10] Lastly, the long alkane chains in SH-(CH$_2$)$_n$-X form very well-defined monolayers on gold because of non covalent interactions between the alkane chains. Alkane thiols have also been determined to order in a tilted manner, see fig 2-1, with an angle of 20-30° to a normal from the surface. [11] As is indicated in fig. 2-1 the formation of a well ordered SAM, under commonly used conditions with ethanolic solution and millimolar concentrations, requires an incubation time of > 12 h at room temperature.

![Figure 2-1](image)

**Figure 2-1** Self assembly of SH-(CH$_2$)$_n$-X molecules from disordered in solution (left) to highly ordered with a 20-30° tilt (right). Black lines indicate the alkane chains and the yellow dot the SH-groups. Images not in scale.

To obtain a SAM with highly protein repellant properties OEG and PEG chains are often used as the functional group X in fig. 2-1. The origin for the protein repellence of PEG or OEG layers are likely dependent on the property of the ethylene glycol (EG) chain to form hydrogen bonds with water. This makes the surface more hydrophilic and also the bound
water acts as a steric barrier for proteins to adsorb to the hydrophobic gold. [12] For these systems, surface coverage of the EG chains has been determined to play a central role for the protein repellence, more than the length of the EG chain. [6] An interesting result from different protein repellence studies is that SAMs of SH-(CH₂)ₓ-EGₙ derivatives readily resist protein adsorption on gold but not on silver. This has been assumed to depend on the fact that SH-(CH₂)ₓ-X in a SAM on silver assume a close packed all-trans conformation in contrary to the more loosely packed helical conformation on gold. Since the all-trans conformation is more rigid and ordered than the helical conformation this disables water to be coupled to as high extent and the protein repellent properties is therefore lowered for SH-(CH₂)ₓ-X derivatives on silver substrates. [12, 13]

### 2.2 Streptavidin/biotin coupling

Streptavidin (SA) is the bacterial form of the egg white protein avidin, isolated from the bacterium *Streptomyces avidinii*. The biological function of SA is more or less unknown but it is widely used in biosciences due to its very strong binding to the vitamin biotin. The affinity between biotin and streptavidin has been described as the highest between a protein and ligand with an affinity constant, $K_a$, of $2.3 \times 10^{13}$ M⁻¹ which can be compared with $10^9$-$10^{12}$ M⁻¹ for a typical receptor-ligand complex. [2]. Streptavidin has a molecular weight of approximately 60 000 Da [14] and the dimensions have been determined by crystallography to 4.75 nm in length for each side suggesting a cubic conformation. [15] The reason to why SA is interesting in for example biosensing is that it is a tetrameric protein with four binding domains for biotin, see fig 2-2. This enables the adsorption of a monolayer of SA to a surface that in turn enables the specific immobilization of biotinylated molecules.

![Figure 2-2](image)

*Figure 2-2* A SH-(CH₂)ₓ-X SAM, described in section 2.1 with an X head group functionalized with a biotin (red). Streptavidin has been subsequently immobilized to the biotinylated SAM. Image not in scale.
2.3 Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) has been the main technique for this thesis work since the aim was to develop biotinylated surfaces for that sensing technique. Because of this, QCM-D, and its applications, will be given a more extensive theoretical description.

2.3.1 Brief historical background

QCM was first developed in the 1960ies to detect mass changes at surfaces in vacuum and gas phase after the work by Sauerbrey, linking the mass change of a vibrating piezoelectric quartz crystal to the associated frequency shift, the so-called Sauerbrey equation (see section 2.3.3). [16] The technique was further developed in the 1980ies when it was proven to work also in liquid phase, which enabled it to be used for biological systems that often are solely liquid based. [17] QCM-D, where $D$ stands for dissipation, was developed in the 1990ies at the Dept. of Applied Physics at Chalmers University of Technology. [3, 4] This enabled studies of the viscoelastic properties of the adlayer and opened up for studies of polymer layers, protein interactions, DNA hybridization and lipid bilayers to mention a few.[18-23] It also resulted in the company Q-Sense AB being established 1996 in Göteborg, Sweden. Today they are leading manufacturer of instruments for QCM-D measurements and have also developed several combined systems such as the electrochemical module (EQCM-D) that will be described in section 2.3.5.

2.3.2 Sensing crystal

A piezoelectric material is deformed when subjected to an electric potential [24]. In QCM, this is used to induce thickness-shear oscillations of an AT cut, thin quartz crystal disk by the application of an alternating voltage over the metal electrodes on either sides of the crystal, see fig. 2-3.

![Figure 2-3](image)

**Figure 2-3.** The image to the left shows a schematic illustration of a piezoelectric crystal with top and bottom electrodes in a broken circuit. The right image shows how the AT cut piezoelectric crystal oscillates in the thickness-shear mode when the circuit is closed and the alternating voltage is applied
An AT-cut crystal (cut at an angle of 35°15’ to the optic axis) oscillates very stably in the thickness-shear mode at temperatures 0-50 °C, which is the main reason for using this type of crystal in QCM-D. [24] The substrates used during this work have been gold coated QCM-D AT-cut crystals with a fundamental mode frequency of 5MHz (thickness 0.3 mm) obtained from Q-Sense. The 100 nm thick Au electrodes are deposited on the top and bottom faces via sputtering through a mask, using titanium as an adhesive layer. Note that the top electrode is wrapped around the crystal edge in order to allow for contacting on the backside, i.e. both the top and bottom electrodes are contacted from the backside. The dimensions are shown in fig. 2-4.

![Figure 2-4 QCM-D gold coated crystal with inserted dimensions](image_url)

The gold surface makes thiol chemistry suitable as a method for surface modification following the strong covalent binding that is formed between Au and SH – groups. This enables formation of SAMs of organosulfur derivatives. [10]

### 2.3.3 Measuring principle

When applying an alternating potential over the crystal, (fig. 2-3), the sensor crystal is forced into oscillation. At a certain frequency, when the wavelength is twice the thickness, $T_q$, of the crystal, the crystal will be in resonance. This resonance frequency, $f_i$, of a quartz disk is determined by:

$$f_i = i \frac{V_0}{2T_q} \quad \text{(Equation 1)}$$
where \( v_q \) is the speed of sound in quartz (3300 m/s) and \( i \) represents the overtone number. The overtone numbers are found at odd multiples of the fundamental frequency that corresponds to \( i = 1 \), e.g. overtones are found at \( i = 3, 5, 7, \ldots \) [25] A 5 MHz crystal has the fundamental resonance frequency, \( i=1 \), at 5 MHz.

When mass is added or removed, \( \Delta m \), from the sensor surface a frequency shift, \( \Delta f \), will occur. This is why QCM has been much used in sensor applications, and is described by the relation between \( \Delta m \) and \( \Delta f \) called the Sauerbrey equation where a negative frequency shift is related to mass increase at the surface [16]:

\[
\Delta m = -\frac{C \Delta f_i}{i}
\]

(Equation 2)

where \( C \) is the mass sensitivity constant that is dependant on crystal properties, and is typically equal to 17.7 ng/(cm\(^2\)*Hz) for a 5 MHz sensor. However, the Sauerbrey relation is only valid for rigid, uniform, thin films that do not experience any energy losses upon oscillation. A way to determine the validity of the Sauerbrey equation is to look at the viscous properties of the film through the dissipation, \( D \). [25]

### 2.3.4 Dissipation monitoring

During oscillation of the crystal in liquid, energy losses will occur because of:

1) coupling of the bulk liquid to the motion of the crystal surface
2) viscoelastic properties of the adsorbed film

This damping of the crystal oscillation is called dissipation and plays a very central and important part of a QCM-D measurement since it gives valuable information about the viscoelastic properties of the adsorbed layer. The dissipation is described by the dissipation factor, \( D \):

\[
D = \frac{1}{Q} = \frac{1}{2\pi} \times \frac{E_{\text{dissipated}}}{E_{\text{stored}}}
\]

(Equation 3)

where \( Q \) is the quality factor, \( E_{\text{stored}} \) and \( E_{\text{dissipated}} \) is the energy stored and lost, respectively, during one period of oscillation. [3]
To be able to measure the energy that is lost during each oscillation, and thus measure the dissipation, the crystal is excited to oscillate in a pulsed manner and the decay time, $\tau$, can be measured.

$$\tau_{\text{max}} \left( \frac{1}{e} \right) \text{max}$$

Figure 2-5 Showing the difference in decay time, $\tau$, of a QCM-D crystal with a rigid and viscoelastic film. Image modified with the permission of the copyright owner Q-Sense AB.

The decay time is the time it takes for the oscillation to go from maximum amplitude in oscillation to $(1/e) \times$ (maximum amplitude) schematically shown in fig. 2-5. The decay time can be related to the dissipation, $D$, through the following relation:

$$D = \frac{2}{\omega \tau}$$

(Equation 4)

where $\omega$ is the angular frequency of the oscillating curve [3], that can be obtained by fitting the recorded curve as a damped sinusoidal curve that is described by:

$$A(t) = A_0 e^{-\frac{t}{\tau}} \sin(\omega t + \phi) + C \; , \; t \geq 0$$

(Equation 5)

where $A(t)$ is the amplitude at time $t$, $\phi$ is the phase and $C$ is a constant that depends on the dc offset [3].

For more viscoelastic systems that show high dissipation shifts, i.e. for which the Sauerbrey relation is not valid, an alternate model for calculating adsorbed mass has been developed, based on a Voigt element. This model takes the density, thickness, shear viscosity and elastic shear modulus of the adsorbed layer into account. [26]
2.3.5 Combinations with QCM-D

As a stand-alone technique QCM-D allows for a lot of information to be extracted about different surface based systems, but even more can be done by combining it with other experimental techniques. For example QCM-D was recently combined with reflectometry.[22] The combination of QCM-D and an optical technique allows for determination of the amount of medium which is acoustically coupled to the adsorbed film.

In this master’s thesis a QCM-D module has been used that enables for electrochemical impedance spectroscopy (EIS) measurements to be conducted simultaneously with a QCM-D measurement under flow conditions. This module has been developed by Q-Sense and is commercially available. To enable electrochemical experiments, the electrode on top of the sensor crystal acts as working electrode and a platinum plate in the reaction chamber as counter electrode. A reference Ag/AgCl electrode with a stable redox potential is included to ensure that the correct potential is measured. The principles of impedance spectroscopy are explained in more detail in section 2.4.

2.4 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) measures the current/impedance through a measuring cell when applying an alternating current (AC) potential while changing the frequency. [27] This allows the determination of the electrical properties of a monolayer adsorbed to a metal surface. In SAM applications, the coverage, Θ, is the parameter of interest and can be modeled from obtained impedance spectra. [28] Before describing how this is done, some basics about impedance spectroscopy should be considered.

The impedance, Z, is the AC equivalent to resistance and describes the ability to resist flow of an alternating electrical current through a circuit element. It can be expressed analogous to Ohm’s Law using the following complex equation:

\[ Z(\omega) = \frac{E(\omega)}{I(\omega)} = Z_0(\cos\phi + j\sin\phi) \]

(Equation 6)

where \( \omega \) is the angular frequency, \( E(\omega) \) the AC potential (excitation signal), \( I(\omega) \) the AC current (response signal). Because of the complex nature of the impedance both the absolute number, \( |Z| = Z_0 \) and the phase shift in \( \Phi \) are usually plotted in the output signal against the
frequency (using a so called “Bode-plot”). In EIS the examined system is commonly modeled using an equivalent circuit describing the electrical behavior of the system. The system analyzed in the present thesis can be modeled by a series connection of a resistance $R$, representing the buffer, and a capacitance $C$, representing the SAM (fig 2-6). [23]

![Figure 2-6 An equivalent circuit for an adsorbed SAM in a liquid cell showing a resistor representing the buffer and a capacitor representing the SAM in series.](image)

The impedance of a resistor $R$ and a capacitance $C$ are given by the following equations: [27]

$$Z_{\text{capacitor}} = \frac{1}{j\omega C} \quad \text{(Equation 7)}$$

$$Z_{\text{buffer}} = R \quad \text{(Equation 8)}$$

The impedance of the equivalent circuit depicted in fig. 2-6 is:

$$Z = R + \frac{1}{j\omega C}, \text{ with} \quad \text{(Equation 9)}$$

$$|Z| = \sqrt{(R^2 + \frac{1}{\omega^2 C^2})} \quad \text{and} \quad \phi = \arctan\left(\frac{1}{\omega RC}\right) \quad \text{(Equation 10 and 11)}$$

From the capacitance values, extracted from the impedance spectra, the coverage ratio, $\Theta$, for SAMs prepared from thiols with terminal ethylene glycol chains, can be calculated using following relation: [28]

$$\Theta = \frac{C - C_{Au}}{C_{\text{theo}} - C_{Au}} \quad \text{(Equation 12)}$$

where $C$ is the measured capacitance, $C_{Au}$ is the capacitance for a clean gold surface and $C_{\text{theo}}$ is the calculated expected capacitance at full thiol coverage given by:

$$C_{\text{theo}} = \frac{\varepsilon \varepsilon_0}{d} \quad \text{(Equation 13)}$$

where $d$ is the thickness of the SAM, $\varepsilon$ the dielectric constant of the SAM and $\varepsilon_0$ the dielectric constant of the vacuum ($\varepsilon_{SH-C11-OEG} = 2.1[29]$, $\varepsilon_{SS-OEG} = 5.1 \ [30]$) and $\varepsilon_0 = 8.854*10^{-14}$ F/cm$^2$).
2.5 Surface plasmon resonance

Surface plasmon resonance (SPR) is one of the most commonly used techniques for real time, label-free studies of biomolecular interactions and was first presented for biosensing by Liedberg et al [31]. In collaboration with Pharmacia these findings led to the creation of the company BIAcore (today part of GE Healthcare) that is the main manufacturer of instruments for SPR studies of biological systems. [32]

The measuring principle is based on the excitation of a surface plasmon that is a charge density wave propagating along the surface of a metal, preferably Au, in connection to a dielectric medium. A surface plasmon can be described by its wave vector, \( k_{sp} \), which is described by the following relation

\[
k_{sp} = \frac{\omega}{c} \sqrt{\varepsilon(\omega)\varepsilon_a} \tag{Equation 14}
\]

where \( \varepsilon(\omega) \) is the dielectric function of the metal at a given angular frequency \( \omega \), \( \varepsilon_a \) is the dielectric constant for the ambient medium and \( c \) is the speed of light. When \( k_x = k_{sp} \) some of the reflected light penetrates the metal and creates the evanescent field that is what is studied in SPR.

To enable the excitation of the surface plasmons present on an Au surface, a Kretschmann, total internal reflection configuration is often used, see fig 2-7. [33]

![Figure 2-7](image_url)

**Figure 2-7** Schematic illustration of a Kretschmann configuration showing total reflection of incident light and the evanescent field that is produced when \( k_x = k_{sp} \).
Here $k$ is the wave vector for the incident light with frequency $\omega$, $\varepsilon$ is the dielectric constant for the prism glass, $\Theta$ is the angle of incidence and the blue field penetrating into the ambient is the evanescent field. The component of $k$ that is parallel to the surface, $k_x$, can be described by

$$k_x = \frac{\omega}{c} \sqrt{\varepsilon} \tag{Equation 15}$$

A Kretschmann configuration allows the angle of incidence to be altered so that total internal reflection can be obtained at angles larger than a certain critical angle $\Theta_c$. When $\Theta > \Theta_c$ most of the light is reflected but a small fraction of the light penetrates outside the glass into the metal film and the ambient medium and this produces the evanescent field. This allows for studies of the metal-ambient interface, as long as the metal film is thin enough. The aim for an SPR measurement is therefore to find the resonance angle $\Theta_r$ when $k_x = k_{sp}$ which indicates resonance and can be observed as a minimum in the reflected light, since the amount of incident light that is coupled to the evanescent field is increased at resonance. [33]

During an SPR measurement, $\Theta_r$ is highly affected by changes in the optical properties of the ambient medium since it interacts with the evanescent field. Changes in $\Theta_r$ can therefore be converted into changes in the optical properties of the metal-ambient interface. The changes in $\Theta_r$ that occur during an SPR measurement is the output signal converted to response units (RU) during a BIAcore measurement, $1^\circ \sim 10$ kRU. This can be converted into changes in the refractive index of the ambient which in turn can be coupled to the amount of adsorbed mass that caused the change in optical properties. [34] A rule of thumb is that 1000 RU has experimentally been observed to equal proteins being immobilized at a surface concentration of approx.100 ng/cm$^2$. [33] This allows for surface modification techniques such as SAMs to be used on a gold surface to enable studies of specific biomolecular interactions. A typical BIAcore chip has a gold film that is 50 nm thick and since the evanescent field only penetrates $\sim 150$ nm it is important that the adsorbed sensing layer is $< 100$ nm to obtain maximum signal. [34]
2.6 Contact angle goniometry

Contact angle goniometry measures the wetting properties of a surface by placing a liquid droplet on the surface and measuring the angle, $\Theta$, between the liquid droplet and the surface. These wetting properties can then be coupled to the surface energy of the system. The surface energy of a surface based system, such as SAMs, can provide valuable information for predicting and interpreting results from adsorption studies made at a surface-liquid interface. Information could also be obtained about the orientation of the thiols in the monolayer if several possible orientations are to be expected that alters the surface energy. [35] Surface energy is related to the contact angle through Young’s relation:

$$\cos \Theta = \frac{\gamma_{SV} - \gamma_{SL}}{\gamma_{LV}}$$  \hspace{1cm} (Equation 16)

where $\gamma_{SV}$, $\gamma_{SL}$ and $\gamma_{LV}$ is the interfacial free energy between the solid-vapour, solid-liquid and liquid-vapour interface respectively, see fig. 2-8. [36]

Figure 2-8 Sessile drop on a gold substrate with the energies $\gamma_{SV}$, $\gamma_{SL}$ and $\gamma_{LV}$ drawn with magnitudes of which are dependant on the contact angle $\Theta$, see eq 16.

When water is used as liquid, low energy is coupled to hydrophobicity and is indicated by high values of $\Theta$, while hydrophilicity is related to low angles and high surface energy. Generally, $\Theta < 90^\circ$ for hydrophilic surfaces and $> 90^\circ$ for hydrophobic. This can be intuitively understood by regarding hydrophilic surfaces as “water-loving” and thus allowing water to spread more readily over the surface resulting in low $\Theta$ values, and the opposite for hydrophobic being “water-hating”. [37] Measurements of the contact angle can be made both in static- and in dynamic mode. In static mode contact angles are measured as a mean value of the angles on the right and left side of a sessile drop while dynamic mode measurements are made while adding and withdrawing liquid, which produces an advancing and receding angle.
The values of the advancing and receding angles give information on how the droplet is spreading over the surface which can be related to surface roughness. [36]

### 2.7 Variable angle spectroscopic ellipsometry

Ellipsometry is a non-destructive technique that measures optical properties and thicknesses of thin layers proximal to the surface. Since the change in polarization upon reflection is dependent on the surface properties, thin adsorbed films, such as SAMs, can be evaluated with this technique. Ellipsometry is mainly used for thickness measurements of thin films on surfaces that can be modeled from the ellipsometric angles $\Psi$ and $\Delta$. [38] Polarized light incident towards a surface can be decomposed into two perpendicular components, $E_p$ and $E_s$ that are parallel and perpendicular to the surface respectively. In ellipsometry the ratios of the amplitude of the incoming and reflected light for $E_p$ and $E_s$ are denoted $r_p$ and $r_s$. The parameter that is measured in ellipsometry, $\rho$, is the ratio between these two reflectance ratios and is coupled to $\Psi$ and $\Delta$ through the following relation [39]:

$$\rho = \frac{r_s}{r_p} = \tan(\Psi) e^{i\Delta} \quad \text{(Equation 17)}$$

where $\tan(\Psi)$ is the amplitude ratio and $e^{i\Delta}$ is the phase shift obtained after reflection. To extract the thickness of a thin (< 50 nm) adsorbed layer from these measured data, the refractive indices of the layer has to be known or modeled. In spectroscopic ellipsometry measurements are done at several wavelengths. This allows the refractive index, $n$, to be modeled by a Cauchy model:

$$n = A_n + \frac{B_n}{\lambda^2} + \frac{C_n}{\lambda^4} \quad \text{(Equation 18)}$$

where typical values for the constants are $A_n = 1.45$, $B_n = 0.01$ and $C_n = 0$ for ethylene glycol based SAMs [40].

A schematic setup for an ellipsometer is described in fig. 2-9. Light emitted from a light source such as a laser or light bulb is polarized in more than one direction. In the ellipsometry setup in fig. 2-9 the light emitted from the light source is first linearly polarized and then phase shifted by a rotating compensator, producing elliptically polarized light that is reflected upon the surface. After reflection the polarization of the light has been altered and the phase
shift, $e^{\Delta}$, and change in amplitude, $\tan(\Psi)$, are detected at the detector after the light has been linearly polarized by the analyzer. [39]

The sensitivity of $\Psi$ and $\Delta$ may vary with $\Phi$. To always obtain data at maximum sensitivity different angles of incidence are used during variable angle spectroscopic ellipsometry measurements. [40]

**Figure 2-9** Schematic ellipsometry setup. Light is elliptically polarized and reflected on the sample surface where polarization changes occur that are monitored by the detector.
3 Materials

3.1 Substrates

All QCM-D measurements were made on 5 MHz AT-cut quartz crystals with a 100 nm thick Au layer sputtered onto a titanium adhesive layer obtained from Q-Sense, Sweden. SPR measurements were done with Au coated surfaces purchased with the SIA Kit Au from BIAcore, Sweden with unknown Au thickness on a glass slide.

3.2 Thiol compounds

Three different thiol compounds functionalized with PEG or OEG, derivatives (fig. 1-1) were used to create SAMs. All SAMs were prepared as mixed monolayers with different ratios of biotinylated and non-biotinylated thiol compounds, described below for all the different systems. No purity tests were performed to verify the content of the thiol source material.

3.2.1 SS-OEG

The disulfides with OEG chains of 7-8 residues, abbreviated SS-OEG/SS-OEG-biotin, were purchased from Polypure, Norway. Dilution was done in 100 % spectroscopy grade ethanol to a total thiol concentration of 0.5 mM. See fig. 1.1a for molecular structures of the non-biotinylated (MW: 771 Da) and biotinylated (MW: 1539.9) SS-OEG.

3.2.2 SH-PEG

Long chain PEG thiols, SH-PEG, were purchased from Rapp Polymere, Switzerland. The incubation solution was prepared according to an incubation protocol recently developed in the group to a total thiol concentration of 0.5 mM. See fig. 1.1b for molecular structures of the non-biotinylated (MW: 3.5 and 5 kDa) and biotinylated (MW: 5 kDa) SS-PEG.

3.2.3 SH-C11-OEG

Undecane thiols, SH-C11-OEG, functionalized with OEG chains with 4 (non-biotinylated) and 6 (biotinylated) EG units were purchased from Assemblon, USA and Prochimia, Poland respectively. Dilution was done in 100 % spectroscopy grade ethanol to a total thiol concentration of 0.5 mM. See fig. 1.1c for molecular structures of the non-biotinylated (MW: 349.6 Da) and biotinylated (MW: 694 Da) SS-C11-OEG.
SH-C11-OEG-biotin was prepared as suggested from the manufacturer, but after dilution in ethanol, precipitates or possible contaminations, were present in the solution that did not dissolve after several sonication and heating cycles. These solutions were used as prepared and this could have affect the results obtained from measurements done on SAMs containing SH-C11-OEG-biotin. No purity measurements were done to establish the source of the contamination.

### 3.3 General chemicals

All water was purified and deionized to a resistivity of 18.2 MΩ/cm with a Milli-Q system (MilliPore, France).

Phosphate buffered saline (PBS) with a composition of 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate, pH 7.4 and hepes buffered saline (HBS-N), 150 mM NaCl, 10 mM Hepes, pH 7.4, have been used as both running and diluting buffer. PBS was prepared by dissolving tablets from Sigma Aldrich, Sweden in water. HBS-N was prepared by diluting a 10x concentrated HBS-N solution from BIAcore in water. All solutions were readily degassed before QCM-D or SPR measurements since both these instrumental systems are sensitive to air bubbles.

All proteins were purchased from Sigma-Aldrich except for the biotinylated plasminogen that was obtained from Technoclone GmbH, Austria. Protein solutions were prepared by dilution in buffer and stored in freezer, at temp. < -20 °C prior to use.
4 Experimental Section

In this chapter the experimental setups and procedures underlying all the results in chapter 5 will be presented.

4.1 Sample preparation

Substrates used during this project have mainly been new Au coated QCM-D sensor crystals from Q-Sense, described in section 2.3.2; the only exception has been the SPR measurements where BIAcore Au coated sensor surfaces were used. Since SAMs were to be formed on gold surfaces there was a great need for clean substrates and all surfaces were therefore washed in a 5:1:1 solution of water, 25 % ammonia and 30 % hydrogen peroxide for 10 min at 80 °C prior to thiol incubation, to remove organic contaminants. After rinsing the surfaces repeatedly with water they were dried in nitrogen and placed in an ethanolic thiol solution of 0.5 mM for incubation > 15 h prior to use. For SH-PEG, buffer replaced ethanol in the incubation solution. To ensure that 0.5 mM would be sufficient for repeated incubations with the same solution calculations were made with respect to the excess of thiol groups in 5 ml solution for the dithiol SS-OEG, whose sulfur atoms are schematically shown in fig. 4-1. As reported by Ulman in 1996 the intermolecular distance, d, in a thiol lattice on gold is 4.97 Å and each sulfur atom occupies a surface area of 21.4 Å² which corresponds to the radius \( r = 2.59 \text{ Å} \). [5] By assuming that non-specific thiol adsorption occurs on the polystyrene container as well as the liquid-air interface by half the amount compared to the gold surface an excess of > 500 times was calculated. If only adsorption on the sensor surface was taken into account the excess was > 3500.

![Figure 4-1](image-url) Schematic view of the sulfur atoms in SS-OEG with intermolecular distance d and radius r.
After incubation, the surfaces were rinsed in ethanol and ultrasonicated for 3-5 min to remove any non-covalently bound thiols. Before mounting the surfaces into the QCM-D or SPR instrument they were rinsed in ethanol and dried in nitrogen gas.

Since the aim of this master’s thesis project was to prepare biotinylated sensor crystals, all created SAMs were mixed monolayers with different molar ratios of biotinylated and non-biotinylated, thiolated ethylene glycol derivatives presented in section 3.2. Incubation solutions were prepared to give a constant total concentration of 0.5 mM. The molar ratios that were tested for SS-OEG, SH-C11-OEG and SH-PEG are presented in 5.1.2 as the molar percentage of the biotinylated compound in the incubation solution.

4.2 Functional characterization

All QCM-D measurements were performed with an E4 Q-Sense instrument with four parallel flow modules that allowed for up to four simultaneous measurements, see fig 4-2 below.

![Figure 4-2 Q-Sense E4 instrument with the chamber open showing a sensor surface being placed into a flow module. This image is shown with the approval of the copyright owner Q-Sense AB.](image)

During the QCM-D and SPR measurements degassed hepes buffered saline (HBS-N) was used as running buffer and as diluting agent for protein/ligand solutions. The flow was controlled by an Ismatec IPC-N 4 peristaltic pump that produced stable flows in the range 50-500 µl/min. All measurements, except the temperature regeneration test, were performed
at controlled temperature 22 °C. For data collection and interpretation the softwares QSoft 401 and QTools 301 were used, both developed by Q-Sense.

4.2.1 Serum/vesicle adsorption

To test the surfaces for non-specific protein binding, crystals were exposed to fetal bovine serum (FBS) for 30-60 min under static conditions in QCM-D. The FBS had a total protein concentration of 30-45 mg/ml. After serum exposure the crystals were rinsed with HBS-N and the amount of adsorbed serum proteins was measured. Vesicle adsorption tests on SS-OEG were done with the same procedure except that FBS was exchanged to a vesicle solution of 0.2 mg/ml of palmitoyl oleoyl phosphatidyl choline (POPC) unilaminar vesicles (80-100 nm in diameter) in HBS-N, prepared by extrusion. [41]

4.2.2 Streptavidin and bBSA immobilization

All biotinylated SAMs were tested with QCM-D for specific immobilization of streptavidin (SA) and the subsequent binding of biotinylated bovine serum albumin (bBSA). SA at a concentration of 25 µg/ml in HBS-N, was flown over the biotinylated crystals until reaching saturation. After rinsing with HBS-N, the crystals were subjected to 100 µg/ml bBSA. To test the specificity of the SA layers, non-biotinylated BSA of 1 mg/ml was flown over the adsorbed SA layer on some crystals before subjecting them to bBSA.

4.2.3 Immobilization and subsequent ligand interaction of plasminogen

To enable biotinylated plasminogen (bPLG) to be immobilized on a crystal with a SAM of SS-OEG with 1 % biotin content, SA was first immobilized in the same way as in 4.2.2 in a QCM-D instrument. bPLG was then flown over the crystal in pulses to maximize the bPLG binding. The reason for this was that bPLG had shown a slower binding kinetics than bBSA and by reaching saturation in several steps the adsorption could be maximized while minimizing sample consumption. Once bPLG was immobilized on the surface, ligands known to induce conformational changes in plasminogen were presented to the immobilized bPLG. Two different ligands were used, the low-affinity ligand ε-amino caproic acid (EACA) (1, 10 and 100 µM) and a more high-affinity ligand with unknown structure denoted ligand-X (0.1, 1 and 10 µM).
Surface plasmon resonance (SPR) measurements were also conducted with the same procedures for SA, bBSA and bPLG immobilization as mentioned above. The only difference in experimental setup was that the ligand concentration was extended to 0.01, 0.1, 1, 10 and 100 µM for ligand-X and to 0.01, 0.1, 1, 10, 100 and 1000 µM for EACA.

4.2.4 Storage tests

Surfaces of 1 % biotinylated SS-OEG and 10 % biotinylated SH-C11-OEG were prepared according to 4.1 and stored in air or HBS-N buffer in a sealed petri dish for 7 days. Storage tests were also made on crystals that had been subjected to ellipsometry and contact angle measurements approximately 5 h after they had been collected from the incubation solution.

4.2.5 Regeneration studies

Regeneration studies were made by QCM-D with two separate methods, (i) heating to 50 °C in water and (ii) exposure to 8M Guanidine*HCl at pH 1.5. The heating procedure was conducted under constant flow of water at 50 °C for 1h, following the study made by Holmberg et al [42] for a clean Au coated crystal and 1 % biotinylated SS-OEG without bound SA as reference substrates. The test crystals were coated with SAMs of 1 % biotinylated SS-OEG, one with only SA immobilized and the other with subsequently bound bBSA following the procedure in 4.2.2.

For the incubation in 8M Guanidine*HCl at pH 1.5, both 1 % biotinylated SS-OEG and 10 % biotinylated SH-C11-OEG were used. To these crystals SA and bBSA were immobilized and subjected to the denaturing agent Guanidine*HCl solution for 1 h under constant flow, based on the study made by Kim et al. [43] After the denaturing procedure the crystals were reintroduced to SA and bBSA to test for reusability.
4.3 **Structural characterization**

Ellipsometry, contact angle goniometry and electrochemical impedance spectroscopy was performed to get data of the different SAMs for structural evaluation.

4.3.1 **Electrochemical impedance**

Electrochemical impedance measurements were performed with a Reference 600 potentiostat from Gamry Instruments Inc combined with the EQCM-D setup, see 2.3.5. Values for the impedance $|Z|(f)$ were collected for frequencies ranging from 0.1 to $10^5$ Hz with 10 data points per decade at an alternating voltage of 10 mV. PBS was used as running buffer. The collected data were recorded with Gamry Framework software and later analyzed using the equivalent circuit given in fig. 2-6. From the capacitance values determined, surface coverage was calculated according to equation 12.

4.3.2 **Ellipsometry**

Variable angle spectroscopic ellipsometry measurements were done with a rotating compensator ellipsometer of the type M2000-F™ (J.A. Woollam Co., Inc., Lincoln, Nebraska, USA) for wavelengths ranging from 245 to 1000 nm and for angles of incidence of 65°, 70° and 75°. To calculate the film thickness, $d$, of the polymer SAMs, a Cauchy model (see section 2.7) with $A_0 = 1.45$, $B_0 = 0.01$ and $C_0 = 0$ was used as an estimate for the refractive index of the polymer.

4.3.3 **Contact angle goniometry**

Wettability tests were made by static contact angle measurements with a DSA10 goniometer from Krüss for a 5 µl sessile drop of water. Data was collected with the software DSA1 Drop shape analysis. Dynamic measurements, where the contact angles is measured over time, were also tested but were discarded since they were cumbersome to perform and only differed with < 5 % in contact angle values from the static mode.
5 Results

5.1 Functional characterization

Presented here are the results from functional studies of the biotinylated thiol SAMs with respect to both specificity in biotinylated protein immobilization and the repellant properties for non-specific protein adsorption. Results are also presented for the case study with low molecular weight compounds interacting with plasminogen.

5.1.1 Serum/vesicle adsorption

Adsorption of FBS was tested by QCM-D according to section 4.2.1 to find the differences in protein repellant properties between the SS-OEG, SH-C11-OEG and SH-PEG SAMs on gold coated crystals. SS-OEG was also tested for adsorption of POPC vesicles. The results from these measurements are shown below in fig. 5-1 and table 5-1. All polymer SAMs adsorb substantially less serum proteins than the clean Au surface. The SS-OEG and SH-PEG SAMs even had slightly positive frequency shifts which suggest that material is rather lost from the surface. POPC vesicles adsorb readily on SS-OEG with a frequency shift in the magnitude of serum adsorption on bare gold.

![Graph showing frequency shifts associated with serum adsorption and POPC vesicle adsorption.](image)

*Figure 5-1* Blue bars show frequency shifts associated with serum adsorption as mean values of three separate measurements. The yellow bar represents the frequency shift obtained when SS-OEG was exposed to POPC vesicles (single measurement). All frequency values are normalized values of the 7th overtone.
Table 5-1 Dissipation and frequency shifts resulting from FBS adsorption to different surfaces. Mass values have been calculated with the Sauerbrey relation, eq. 2. All mean values are taken for three separate measurements and for the 7\textsuperscript{th} overtone. The frequency shifts are also represented in the bar diagram in fig. 5-1.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(\Delta f_{\text{n=7}}) (Hz)</th>
<th>(\Delta D_{\text{n=7}}) (10^{-6})</th>
<th>(\Delta m) (ng/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-OEG</td>
<td>0.71 ± 0.86</td>
<td>0.27 ± 0.16</td>
<td>-12.6 ± 15.2</td>
</tr>
<tr>
<td>SH-C11-OEG</td>
<td>-4.7 ± 1.09</td>
<td>1.2 ± 0.53</td>
<td>83.2 ± 19.3</td>
</tr>
<tr>
<td>SH-PEG</td>
<td>1.9 ± 1.05</td>
<td>-0.12 ± 0.11</td>
<td>-33.6 ± 18.6</td>
</tr>
<tr>
<td>Au</td>
<td>-52.5 ± 10.6</td>
<td>3.3 ± 0.42</td>
<td>929 ± 187</td>
</tr>
</tbody>
</table>

5.1.2 Streptavidin and bBSA immobilization

Since the specificity of the streptavidin/biotin interaction at the SAM interface was one of the major topics to be evaluated and optimized in this study, extensive studies were made with the SA/bBSA model system.

An important part of this study was to establish what concentration of biotinylated OEG/PEG thiol derivatives in the incubation solution that resulted in the immobilization of a monolayer of streptavidin. This was evaluated for all OEG/PEG thiol systems in this project and is presented in figures and tables below.

In figure 5-2a the SA immobilization can be seen to saturate, by QCM-D, at -23 Hz at 0.7-1 % of biotin content in the SS-OEG/SS-OEG-biotin SAMs. The subsequent bBSA immobilization has a maximum of \(\Delta f = -17\) Hz at 0.4-1 % of biotin content. The SA immobilization showed highly reproducible, independent values of \(\Delta f = -23\) Hz measured at different occasions with different crystals, represented by the cluster of data points in fig. 5-2a. Frequency shifts in this range have been reported several times earlier for SA immobilization in QCM-D applications and are suggested to correspond to the immobilization of one monolayer of SA. [20, 22, 23]

The corresponding bBSA value was clustered at \(\Delta f = -17\) Hz (fig. 5-2b) which is approx. 74 % of the corresponding frequency shift for immobilized SA.

By calculating the number SA proteins required for a SA monolayer, a surface concentration of 3 % of biotinylated SS-OEG was suggested to give maximum SA coverage, assuming a 1:1 relationship between SS-OEG-biotin and SA. Additional SA binding was assumed to be negligible. This could then be translated into the following model, assuming linearity between biotin concentration and SA binding:
F(b) = -7.5b, 0 ≤ b ≤ 3
F(b) = -22.5, b > 3

where F is the frequency shift obtained at biotin concentration b. This model is shown as a red line below in fig. 5-2a and deviates slightly from the measured values for SS-OEG, where maximum SA immobilization can be seen at b = 1%.

![Diagram](image)

Figure 5-2 Negative, normalized frequency values for the 7th overtone plotted against the solution concentration of biotinylated SS-OEG for SA (a) and bBSA (b) adsorption. Red line indicates the linear model from eq. 19.

For the SH-C11-OEG system saturation had not been reached at 1% biotin content as in the SS-OEG case but as can be seen in fig. 5-3a, the SA immobilization saturate at Δf = -23 Hz at 10% of SH-C11-OEG-biotin. The bBSA immobilization for the 10% biotinylated SH-C11-OEG (fig. 5-3b) is approx. -15 Hz which is 10% lower than the maximum amount of bBSA bound to the SS-OEG/SS-OEG-biotin surfaces and corresponds to 65% of the amount of immobilized SA.
Dissipation shifts upon adsorption of SA were small for both SS-OEG and SH-C11-OEG and are shown below in table 5-2 together with dissipation values for the bBSA immobilization.

Table 5-2 Dissipation shifts, ΔD, from QCM-D measurements of the subsequent SA and bBSA immobilization on gold surfaces coated with SS-OEG or SH-C11-OEG. All dissipation values are presented for the 7th overtone.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SA, $\Delta D_{n=7}$ ($10^{-6}$)</th>
<th>bBSA, $\Delta D_{n=7}$ ($10^{-6}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-OEG (0.01 % biotin)</td>
<td>0.26</td>
<td>0.1</td>
</tr>
<tr>
<td>SS-OEG (0.05 % biotin)</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>SS-OEG (0.4 % biotin)</td>
<td>0.11</td>
<td>0.57</td>
</tr>
<tr>
<td>SS-OEG (0.7 % biotin)</td>
<td>0.19 ± 0.05 (n=2)</td>
<td>0.49 ± 0.16 (n=2)</td>
</tr>
<tr>
<td>SS-OEG (1 % biotin)</td>
<td>0.163 ± 0.162 (n=10)</td>
<td>0.614 ± 0.070 (n=10)</td>
</tr>
<tr>
<td>SS-OEG (10 % biotin)</td>
<td>0.03 ± 0.028 (n=2)</td>
<td>0.53 ± 0.042 (n=2)</td>
</tr>
<tr>
<td>SH-C11-OEG (0.01 % biotin)</td>
<td>0.41</td>
<td>0.15</td>
</tr>
<tr>
<td>SH-C11-OEG (1 % biotin)</td>
<td>0.5</td>
<td>0.35</td>
</tr>
<tr>
<td>SH-C11-OEG (10 % biotin)</td>
<td>0.338 ± 0.255 (n=5)</td>
<td>0.642 ± 0.198 (n=5)</td>
</tr>
</tbody>
</table>
The SA/bBSA model system was also used to test the SH-PEG for specificity, though in a
briefer manner than for SH-C11-OEG and SS-OEG. Mostly due to time and material
limitations these measurements were only performed once and also the concentrations of
biotinylated PEG were limited to 1 and 10 percent in the incubation solution. As is described
in 4.2.2 two different chain lengths were tested of the non-biotinylated SH-PEG which are
characterized by their approximate molecular weights, 3 and 5 kDa. The biotinylated PEG
chains had a molecular weight of 5 kDa on both cases. Results from these studies are
presented in table 5-3:

Table 5-3 Negative frequency and dissipation shifts from the subsequent SA/bBSA adsorption on gold crystals
modified with SH-PEG with both 3 and 5 kDa in molecular weight. Values are from one measurement only and
are presented for the seventh overtone.

<table>
<thead>
<tr>
<th></th>
<th>SA, $\Delta f_{n=7}$ (Hz)</th>
<th>SA, $\Delta D_{n=7}$ (10^{-6})</th>
<th>bBSA, $\Delta f_{n=7}$ (Hz)</th>
<th>bBSA, $\Delta D_{n=7}$ (10^{-6})</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH-PEG, 5kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 % biotin</td>
<td>6.7</td>
<td>0.12</td>
<td>0.5</td>
<td>0.15</td>
</tr>
<tr>
<td>10% biotin</td>
<td>19.5</td>
<td>-0.09</td>
<td>-0.07</td>
<td>0.1</td>
</tr>
<tr>
<td>SH-PEG, 3 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 % biotin</td>
<td>7.5</td>
<td>2.8</td>
<td>0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>10% biotin</td>
<td>20.5</td>
<td>-0.19</td>
<td>0.04</td>
<td>0.03</td>
</tr>
</tbody>
</table>

To test the specificity of the interaction between SA and bBSA, SS-OEG layers with 1 %
biotin content were subjected to non-biotinylated BSA after SA immobilization, as described
in section 4.2.2. Mean values from these measurements, obtained from 5 separate
measurements, for frequency and dissipation shifts are $\Delta f_{n=7} = 0.445 \pm 0.39$ Hz and
$\Delta D_{n=7} = 0.090 \pm 0.085 \times 10^{-6}$ respectively. This is to be compared with the specific values for
bBSA immobilization showed for the same biotin concentration and polymer in figure 5-2
where the frequency shift is highly reproducible in the magnitude of $\Delta f_{n=7} = 17$ Hz.

In connection with the conformation studies of plasminogen, presented in sections 4.2.3 and
5.1.3, SS-OEG with 1 % biotin content and immobilized SA/bBSA acted as a reference
surface in surface plasmon resonance measurements. This resulted in dry mass values for the
immobilized SA of 196 ± 20 ng/cm^2. Satisfactory bBSA adsorption was only obtained at one
case and resulted in a signal corresponding to 60 ng/cm^2. That is to be compared with the
mass values, calculated with the Sauerbrey relation, for the SA and bBSA immobilization in
QCM-D that is 407 and 301 ng/cm^2 respectively corresponding to the negative frequency
shifts of 23 and 17 Hz observed in figure 5-2.
5.1.3 Immobilization and subsequent ligand interaction of plasminogen

There was a need for a more applied, and challenging test system for evaluating the robustness in performance of the developed protocol for biotinylated QCM-D crystals. This led to the development of a case study with the goal to detect conformational changes in the protein plasminogen. All plasminogen experiments were performed with SS-OEG/SS-OEG-biotin treated gold crystals with 1 mol % content of biotinylated SS-OEG in the incubation solution. To be able to distinguish conformation changes from bulk effects during ligand exposure there was a need for good reference systems that were comparable to plasminogen. Streptavidin and bBSA were used as references since they are both proteins and in the same size scale as plasminogen, 70-90 kDa. Results are presented below from the QCM-D part of the plasminogen experiments with the two different ligands ligand-X (fig. 5-4a-b) and EACA (fig. 5-4c-d). The SA and bBSA immobilization resulted in frequency and dissipation shifts in accordance with the SA/bBSA results presented in figure 5-2 for SS-OEG. As can be seen from the blue curves in fig. 5-4a and 5-4c corresponding to the biotinylated plasminogen, this protein gave larger shifts in both frequency ($\Delta f = -36.3 \pm 1.10$ Hz) and dissipation ($\Delta D = (1.71 \pm 0.10) \times 10^{-6}$) upon immobilization than SA and bBSA. At ~ 2000 s, when SA is subjected to bPLG and bBSA, the binding of plasminogen can be seen to show a slower binding kinetics visualized as the slope of the blue plasminogen curves.

For both ligand-X and EACA, the reference subtracted plots (fig. 5-4b and 5-4d) show a positive dissipation shift that increases with increasing ligand concentration without reaching saturation. In both the reference subtracted plots the two references SA and bBSA can be seen to give similar results. An interesting observation is the difference in off-kinetics between ligand-X and EACA, which can be seen in the right side of the ligand square pulses in fig. 5-4b and 5-4d. Here ligand-X is shown to release from plasminogen with slower kinetics than EACA.
Figure 5-4 In a) and c) QCM-D normalized frequency (circles) and dissipation (squares) values are shown for the bPLG (blue), SA reference (green) and bBSA (red) for the seventh overtone. Results from the rising concentration series of ligand-X (a) and EACA (c) are presented at the times indicated by the arrows. Sensograms b) and d) show the resulting dissipation signal at ligand exposure when the references have been subtracted. The reference subtracted data has been offseted to show the absolute changes in dissipation.
SPR measurements on the bPLG model system proved to be challenging and it was difficult to obtain acceptable results. Due to this fact, results are only presented in fig. 5-5 for the reference subtracted data for bPLG, with bBSA as reference, at 10 µM of ligand-X and 1mM for EACA. These SPR measurements were conducted as a comparison to QCM-D results and the results for ligand-X at 10 µM and EACA at 1 mM have large similarities with the data presented for QCM-D in fig 5-4. Of course they show different parameters, dissipation and response units, but both figures indicate slow dissociation kinetics of the ligand-X (fig. 5-5a) in the rightmost part of both square pulses and a faster kinetics for the EACA (fig. 5-5b). Biotinylated PLG could be immobilized with a RU response of approx. 1700, which corresponds to a mass value of approx. 170 ng/cm$^2$. As can be seen in fig. 5-5a the 10 µM ligand-X interaction induced a 25 RU response and when the solution was changed back to buffer the signal instantly retreated 15 RU instantly and another 10 RU over a time of 400 s. The 1mM EACA produced a RU shift of 20 RU units and instantly returned to the same baseline when the ligand solution was exchanged to buffer.

![Figure 5-5](image)

**Figure 5-5** Reference subtracted data for the interaction between bPLG and 10 µM ligand-X (a) and 1mM EACA (b) Biotinylated BSA functioned as a reference and was exposed to the same ligand solutions.
5.1.4 Storage tests

Storage tests were made to test the long-time stability of the biotinylated crystals. This was made for both SS-OEG with 1 % of SS-OEG-biotin (fig. 5-6a) and for SH-C11-OEG with 10 % of SH-C11-OEG-biotin (fig. 5-6b). All results presented here in fig. 5-6 were obtained from biotinylated crystals stored in normal air environment in a petri dish.

Storage for 5 h resulted in SA/bBSA immobilization that highly correlates with the results for direct use presented in sections 5.1.1-5.1.2. Although, an important difference is that the protein repellant properties of SH-C11-OEG have been altered and resulted in adsorption of FBS by $\Delta f_{n=7} = -21.1$ compared to $\Delta f_{n=7} = -4.7$ at direct use (fig. 5-1).

After 7 days of air storage both the SS-OEG and the SH-C11-OEG system showed retained SA/bBSA immobilization, but with a slight decrease from $\Delta f_{n=7} = -23$ to $\Delta f_{n=7} = -21$ Hz for SS-OEG. The FBS adsorption on the one-week stored crystals suggests that SS-OEG still have protein repellent properties, if not as pronounced as after 5h of storage as indicated by the negative frequency shift of -2.4 Hz.

After 14 days of storage the SS-OEG surfaces still seem to have largely retained function with a frequency decrease of -17.3 Hz upon SA immobilization and -14.6 Hz when bBSA is subsequently adsorbed. Specific SA binding to the 10 % biotinylated SH-C11-OEG has been reduced to half after 14 days of storage and also the adsorption of FBS on this crystal is close to that of clean Au (fig. 5-1).

Figure 5-6 Normalized frequency shifts for the seventh overtone obtained from QCM-D measurements of 1 % biotinylated SS-OEG (a) and 10 % biotinylated SH-C11-OEG (b) after storage in air from 5h (0.25 days), 7 and 14 days. Results from adsorption tests of SA, bBSA and FBS are presented in red, blue and green respectively.
5.1.5 Regeneration

To allow for several sequential measurements on one and the same biotinylated crystal tests were made to remove SA and bBSA from 1 % biotinylated crystals as described in 4.2.5. The results from tests with high temperature treatment (50 °C) under flow of water in the E4 instrument are shown in fig. 5-7. During the temperature step both dissipation and frequency responses experienced large shifts. When the system had cooled down to room temperature again a persisting small frequency shift of approximately 6 Hz could be observed for all the crystals while the dissipation returned to the same value as before.

![Graph showing dissipation and frequency responses](image)

Figure 5-7 Dissipation (squares) and normalized frequency (circles) values for the seventh overtone obtained when crystals with layers of SS-OEG (red), SA (green), bBSA (yellow) and a clean Au crystal (blue) were subjected to 50 °C for 1h.

A test was also made with flowing 8 M Guanidine*HCl at pH 1.5, according to 4.2.5 over 1 % biotinylated SS-OEG and 10 % biotinylated SH-C11-OEG with SA/bBSA (fig. 5-8). On both systems SA/bBSA were immobilized at the same levels as reported in fig 5-2 and 5-3. After treatment with Guanidine*HCl the SS-OEG system retreated to a level 5 Hz under the original baseline while SH-C11-OEG mostly were subjected to an increase in dissipation of $1.1 \times 10^{-6}$ and also drift were observed in the frequency of SH-C11-OEG after Guanidine*HCl exposure.

SS-OEG was inert to re-immobilization of SA and bBSA while 18 Hz of SA and 7 Hz of bBSA could be immobilized on the regenerated SH-C11-OEG surface.
**5.2 Structural characterization**

**5.2.1 Electrochemical impedance**

As described in section 4.3.1 electrochemical impedance allows for the calculation of surface coverage of alkane thiol SAMs on a metal surface. Measurements were performed with the EQCM-D setup described in 2.3.5. The resulting impedance spectra are shown below in fig. 5-9. By modeling the QCM-D surface with the adsorbed thiol layer as a capacitor and the buffer as a resistor in series, shown in fig. 5-9 (and fig 2-6) capacitance values could be obtained for clean Au, SH-C11-OEG and SS-OEG.

**Figure 5-9** Impedance spectra obtained against the frequency from electrochemical impedance measurements for clean Au, SH-C11-OEG and SS-OEG. Capacitance values modeled for the different systems are also shown. All values were obtained with a constant voltage of 10 mV.
To obtain surface coverage values, $\Theta$, for the adsorbed thiol layers calculations were made according to Eq. 12 and resulted in $\Theta_{\text{SH-C11-OEG}} = 0.98$ and $\Theta_{\text{SS-OEG}} = 0.85$. This can be compared with values presented by Menz et al from impedance spectroscopy measurements on SH-C11-PEG systems with PEG chains of 350 and 2000 Da in molecular weight. They calculated $\Theta_{\text{SH-C11-350PEG}} = 0.97$ and $\Theta_{\text{SH-C11-2000PEG}} = 0.94$. [28]

5.2.2 Ellipsometry and contact angle goniometry

Ellipsometry measurements were made according to 4.3.2 in order to evaluate that the thickness of the OEG/PEG thiol layers, by modeling with the Cauchy model, were in the expected range and to evaluate if multilayer formation could be observed. The thickness values, $d$, presented in table 5-5 can be compared with values presented by Vanderah et al at 10-15 Å for similar OEG thiols with five to six ethylene glycol units. [44, 45] Palestrosdemange et al have reported thickness values of 20-25 Å for SH-C11-OEG with four ethylene glycol units. [46]

The hydrophobic/hydrophilic behavior of SS-OEG, SH-C11-OEG and SH-PEG were measured with contact angle goniometry in static mode in accordance to 4.3.3. These $\Theta_c$ can be compared with earlier reports that states angles of 31.5° for SH-C11-(EG)$_6$-OH and 78° for SH-C11-EG6-OCH$_3$.[46]

Table 5-5 Thickness values, $d$, obtained from ellipsometry measurements on dried samples of SS-OEG and SS-OEG are presented as mean values of 6 values measured on 3 different crystals for each system. Ellipsometry measurements were only performed once on the SH-PEG system. Contact angle measurements were made in static mode and the values presented as $\Theta_c$ are mean values from 3 different crystals for each SAM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$d$(Å)</th>
<th>$\Theta_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-OEG</td>
<td>14.6 ± 0.94</td>
<td>33.75 ± 1.10°</td>
</tr>
<tr>
<td>HS-C11-OEG</td>
<td>20.4 ± 0.42</td>
<td>53.0 ± 2.26°</td>
</tr>
<tr>
<td>HS-PEG</td>
<td>42</td>
<td>28.1 ± 0.2°</td>
</tr>
</tbody>
</table>
6 Discussion

6.1 Stability considerations

With few exceptions, all QCM-D measurements with SS-OEG and SH-C11-OEG systems were performed without any significant drift present in the output signal that would impair the interpretation of the results. The SH-PEG systems experienced substantial initial negative frequency drift of ~ -10 Hz/1h in flow of HBS-N on a few occasions. This drift is to be expected and can be related to the long PEG chains present in the SH-PEG SAM. Before mounting, the surface is dried in nitrogen gas which dehydrates the SAM. Then, when HBS-N buffer is flown over the SH-PEG SAM, the PEG chains couple larger amounts of water than SS-OEG and SH-C11-OEG through hydrogen bonding and this induces a negative frequency shift as the oscillating mass is increased. Drift can be an issue for the stability of the system if it is uncontrolled and arises randomly. The drift in SH-PEG measurements is expected but cannot be controlled or quantified in a good way and could potentially compromise the stability of a commercial product.

Results from the regeneration procedure with increased temperature to 50 °C during 1 h of water flow (5.1.5) are discussed more in detail in section 6.5, but the SS-OEG SAM and the subsequent binding of SA and bBSA can from these results be suggested to be stable at higher temperatures. This is based upon the low frequency shifts obtained after the high temperature step, which show no desorption of SS-OEG, SA or bBSA.

During the Guanidine*HCl treatment that was suggested as a regenerative procedure both biotinylated SAMs of SH-C11-OEG and SS-OEG, with subsequent SA and bBSA bound, were treated at low pH = 1.5, and high ion concentration, [Guanidine*HCl] = 8 M. From the low frequency shifts after Guanidine*HCl exposure and rinsing with HBS-N (5.1.5) it can be suggested that SH-C11-OEG with bound SA and bBSA is more resilient to low pH and high ion concentrations than SS-OEG. These results are discussed more in detail in 6.5 but it is an interesting observation that, from a stability point of view, SH-C11-OEG is more suitable if the application environment is suspected to be challenging.
6.2 Molecular characterization

Characterization of the molecular structure of the SAM layers has not been the focus of this master’s thesis work; emphasis has more been on the functional studies of the adsorbed mixed monolayers. Still, the molecular structure is responsible for the function of the monolayers, which is of course important. In future studies of these SAMs, there are several techniques that should be considered for more in detail characterization of the adsorbed monolayers:

- X-ray photoelectron spectroscopy (XPS) gives information about the atomic composition and orientation in a monolayer. [47] This technique could be used to establish the relation between solution and surface concentration of biotinylated SS-OEG that has been reported to be non-linear for SH-C11-OEG. [9]

- Atomic force microscopy (AFM) is a surface sensitive technique for topographical imaging on the nanometer scale. [47] AFM would enable studies of protein coverage and give information of possible domains being created in the SAM that would separate biotinylated and not biotinylated EG thiols. An aspect that could induce domains is the presence of an amide bond in all biotinylated derivatives (fig. 1-1) that could give rise to hydrogen bonding.

- Infrared spectroscopy (IR) can provide information about the chemical composition of the compounds present at a surface. [39] This could be used to confirm that the thiols present in the SAMs have the structure suggested in fig. 1-1 and that no undesired oxidation or other structure-altering reactions have taken place. IR can also be used to characterize the conformation and orientation of the molecules in the SAM. For example, OEG/PEG in a helical and in all-trans configuration will have distinctly different IR spectra.

One structural parameter investigated during this thesis was the SAM thickness, measured by ellipsometry. This was performed to verify that the thicknesses of the SAMs were in the correct magnitude and to exclude multilayer formation. Dry sample thicknesses of the three different SAMs and the comparison with earlier publications presented in 5.2.2 showed that the formed SAMs have thicknesses expected for monolayer surface coatings. This proves that the incubation procedure performed satisfactory, although the presence of small fractions of other molecules than the expected ones cannot be out ruled as neither the purity of the thiols, nor the molecular composition of the surface structures were tested.
6.3 Protein repellence of OEG and PEG-modified surfaces

From the results presented in 5.1.1 it is obvious that the surface modifications based on SS-OEG and SH-PEG have excellent protein repellant properties. No mass uptake is observed in either of the studies (the frequency shifts are zero or slightly positive) upon serum exposure. In contrast, the SH-C11-OEG system showed a serum protein adsorption that was 9% of that on a clean Au substrate.

These results can be compared with results from the contact angle measurements, see table 6-1, which presents an interesting explanation for the difference in non-specific protein adsorption, namely the difference in wettability of the SS-OEG, SH-PEG and SH-C11-OEG. All systems have contact angles < 90°, which has been said to indicate hydrophilic behavior, see section 2.6, but the angle of 53° for the SH-C11-OEG is nearly twice the angles for SS-OEG and SH-PEG which indicates more hydrophobic properties. Prime et al, reported that the protein adsorption to SH-C11-OEG SAMs were largely dependant on the presence of hydrophobic regions, in their case from domains of CH₃ end groups in the SAM, [6] and the higher contact angle of SH-C11-OEG could indicate that such regions exist in these SAMs. One hypothesis could be that the highly hydrophobic alkane chains are exposed at the gold grain boundaries or between domains in the SAM because of the 20-30° angle of the molecules in the SH-C11-OEG monolayer.

Table 6-1 Comparison between non-specific serum adsorption, see 5.1.1 and contact angle values, see 5.2.2 for SS-OEG, SH-C11-OEG and SH-PEG.

<table>
<thead>
<tr>
<th>System</th>
<th>FBS adsorption (Hz)</th>
<th>Contact angle (Deg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-OEG</td>
<td>0.71 ± 0.86</td>
<td>33.75 ± 1.10</td>
</tr>
<tr>
<td>SH-C11-OEG</td>
<td>-4.7 ± 1.09</td>
<td>53.0 ± 2.26</td>
</tr>
<tr>
<td>SH-PEG</td>
<td>1.91 ± 1.05</td>
<td>28.1 ± 0.2</td>
</tr>
</tbody>
</table>
The surface hydrophilicity can, to a first approximation, be used to predict protein resistance. Generally, the more hydrophilic the surface, the less protein will adsorb as described above. There is one outstanding exception to this ‘rule’, represented by EG-based surface modifications such as those in this study. These are generally more protein resistant than more hydrophilic surfaces. [48] To integrate this theory into this project one could look at the differences in protein repellence between the different SAMs from the perspective of the molecular structure. The most striking difference is the presence of a hydrophobic alkane chain on the SH-C11-OEG. Alkane chains enable the SAM to be more highly ordered on the Au surface, see fig. 2-1, with a 20-30° tilt angle and this leads to a more densely packed OEG layer. As has been explained earlier in section 2-1 the nature of protein repellence of EG derivatives has been thought to strongly depend on the coupling of H₂O which acts as a steric barrier for proteins to adsorb to the surface. The more densely packed SH-C11-OEG cannot couple as much water as the more loosely packed SH-PEG and SS-OEG and this could be a plausible explanation for the higher protein repellent properties for the systems lacking the C11 alkane chain. Furthermore, the exact conformation of the OEG chains has been suggested to be important for the protein resistant properties. [13]

6.4 Specificity of biotinylated surfaces

The most important property to establish for the product development part of this master’s thesis was the specific immobilization of streptavidin to biotin-modified sensor surfaces. The main reason for this was that if this project was to end up in a commercialized product, specific biotinylated protein binding would be the reason for customers to purchase it. Results from the SA/bBSA assay in 5.1.2 show that surfaces modified with SS-OEG, SH-C11-OEG and SH-PEG were all able to specifically bind streptavidin resulting in frequency shifts between -20 and -25 Hz. By using the Sauerbrey relation (Eq. 2) and the dimensions for SA and the sensor surface, stated in sections 2.2 and 2.3.2, this can be converted to coverage ratios of 1.00-1.25, which indicates that a monolayer of SA has been immobilized. This can be used as a strong argument for the specificity of the adsorbed SAMs, since non-specific SA binding would induce further SA to be adsorbed by non-specific interacting with already bound SA. This is of course dependent on the protein repellent properties discussed in section 6.1 that ensures that no SA will bind if biotin is absent in the SAM. Thus, a monolayer of SA can be assumed to specifically bind to all SAMs of the different biotinylated thiol EG derivatives at appropriate biotin concentration. The low dissipation shifts in table 5-1 support
the assumption that SA is functional on the surface since it is a relatively rigid protein that should not induce a large dissipation shift upon adsorption.

One aspect that has to be taken into consideration is that even though immobilized SA is considered to be a sterically stable and compact protein, a lot of water will be coupled by hydrogen bonding. The degree of hydration can be assessed by comparing the calculated mass of adsorbed SA from QCM-D and SPR, since SPR measures the dry mass while QCM-D measures the mass of both protein and coupled water. From QCM-D the adsorbed SA was calculated to 407 ng/cm$^2$ and to 196 ng/cm$^2$ from the SPR results. This suggests a hydration of approximately 50% in agreement with what has been reported earlier by Edvarsson et al. [22] This can be suggested to undermine the statement that a monolayer of SA is adsorbed to the surface because 50% hydration suggests that 50% less SA is adsorbed. That is of course a relevant question, but the 50% hydration should more be considered as information about how SA is distributed on the surface. Probably the coupled water and the availability for free biotin groups limit the maximum adsorption of SA to 50% of complete surface coverage and should thus be considered as a monolayer.

When biotin concentration is discussed throughout this study it is discussed in a way that suggests that the biotin concentration in solution is the same as on the surface. However, this does not have to be true and a slight deviation from linearity has been reported by Nelson et al for a SH-C11-EG4-OH system. [9] There it is suggested that the equilibrium of adsorption is somewhat shifted towards the surface. Also, in the model made for SA immobilization as a function of biotin concentration, Eq. 19, the maximum SA immobilization was calculated to occur at 3%, instead of 1%, of biotin coverage at the surface. This is a simplified model, but indicates that the equilibrium for the biotinylated SS-OEG is shifted towards the surface and that the real biotin concentration is approx. 3%. This is not a major problem for the discussion, but it should be pointed out that the exact values for the biotin surface concentration can deviate from the one stated. If it would be of interest for further studies to know the exact surface biotin concentration it could be measured with for example x-ray photoelectron spectroscopy (XPS) that measures the elemental composition and orientation on the surface. [47]

The biotin fraction in the incubation solutions required to immobilize a monolayer of SA differed between the different SAMs (fig. 5-2, 5-3, table 5-2) with SS-OEG showing the lowest amount of biotin, 1%, needed in solution. It was expected that SS-PEG would require
higher biotin concentration than SH-OEG because of the higher flexibility of the longer PEG chains. This flexibility makes it possible for some of the biotin groups to “hide” in the PEG layer making them unavailable for the SA. Therefore SH-PEG demands a higher total biotin content to be able to present a sufficient amount of biotin to the SA (fig. 6-1).

Figure 6-1 Schematic images of SAMs of biotinylated SS-OEG (left) and SH-PEG (right) showing that the biotin groups (red boxes) are more exposed in the SS-OEG SAM than in the SH-PEG due to the high flexibility of the PEG chains. Images not in scale.

The observation that SH-C11-OEG needed 10% of biotin content to adsorb a monolayer of SA is unfortunately not 100% reliable. This is due to two reasons; firstly these SAMs were made by mixing molecules from two different manufacturers with different molecular structure, see section 3.2, with the Prochimia molecules being newly bought while the metoxy capped tetra(ethylene glycol) undecathiol system from Assemblon had been stored in a refrigerator for one year. The ethylene glycol (EG) chain of the two thiol systems were different in length with four EG units for the non-biotinylated and six EG units for the biotinylated. Mixing of two such diverse molecules can lead to domains being created in the SAM since more favorable interactions are experienced between molecules of the same type. Secondly, when the dry material of biotinylated SH-C11-OEG from Prochimia was diluted in ethanol upon delivery, a white unspecified substance could be seen in the solution. In the belief that this was precipitates of the thiol compound the solution was subjected to further dilution, heating, ultra sonication and vortexing to dissolve the solid compound without any success. This left one option, namely that some contamination was left in the thiol container from the manufacturer. This has not been confirmed by an analytical method but it cannot be excluded as an important source of error for the quality of the biotinylated solutions of the SH-C11-OEG system.
One interesting aspect from the biotin concentration study of the SS-OEG/SS-OEG-biotin system is that SA adsorbed on surfaces with 0.4-1% biotin attracted 20% more bBSA than those with 10% biotin. This is likely due to the fact that a higher biotin concentration on the surface increases the chance of SA being blocked by an excess of free biotinylated SS-OEG chains close to SA that can occupy all the biotin binding domains of the protein and disable the subsequent binding of bBSA. [7]

Perhaps the most striking difference between the SS-OEG/SS-OEG-biotin, SH-C11-OEG/SH-C11-OEG-biotin and SH-PEG/SH-PEG-biotin SAMs that allow for the immobilization of one monolayer of SA, is the subsequent bBSA binding. On both SS-OEG and SH-C11–OEG the bBSA adsorption produced a frequency shift of -17 Hz while it only resulted in frequency shifts close to zero for SH-PEG. This phenomenon has been observed and discussed earlier by Huang et al [7] where they describe how the high flexibility of the PEG chains allow several biotin moieties from the SAM to bind to the SA (see previous paragraph) which, as in the case with 10% biotinylated SS-PEG, can lead to blocking of all binding sites on the SA. Another possibility is that the bound SA proteins can sterically block the biotin binding domains on other neighboring bound SA proteins as another effect of the flexibility in the PEG chains. Additional optimization could perhaps have revealed the biotin concentration in solution that enables maximum adsorption of SA and bBSA, since 1% biotinylated PEG at least seems to enable some bBSA adsorption, see table 5-3, and an optimal concentration would probably be found between 1-10% of biotin. But this was not considered meaningful since SS-OEG showed such promising results and also the SH-PEG system would probably not produce as easily interpretable results by QCM-D due to the viscous nature of the longer polymer chains.

Based on the results and discussion of both serum protein binding and SA/bBSA binding, 1% biotinylated SS-OEG was chosen as the system of choice for further studies and product development, since it show low non-specific serum binding and reliable SA/bBSA immobilization.

The SA/bBSA experiments were performed with stable baselines for both SA and bBSA, which raised the need for a more challenging model system that could test the robustness of the biotinylated surface. This was performed with the case study of immobilization and ligand interaction of plasminogen discussed in 6.6.
6.5 Regeneration

Regeneration, in the context of this project, means the possibility to remove SA from the biotinylated SAM or alternatively biotinylated molecules from the SA, which would enable repeated use of the same crystal. An attractive regeneration strategy to break the biotin-SA bond has been presented by Holmberg et al [42], achieved by raising of the temperature under a flow of water, mostly due to the simplicity of the procedure. This approach was tested for 1 % biotinylated SS-OEG (5.1.5 and fig- 5-7). The results from these experiments show that no SA or bBSA was removed from the SS-OEG. In fig. 5-7, a small positive frequency shift can be observed for all crystals, except for the clean gold. This shift can originate from instrumental effects of the high temperature step and it is therefore not chemically interpreted. This does not correlate with the results presented by Holmberg et al where 40 % of biotinylated DNA was released from SA coated beads after 300 s at 40 °C and ~ 60 % if the temperature was further raised to 60 °C. Reasons for this difference is a matter of speculation, but one explanation could be the difference in how the SA is bound to the surfaces in the two different systems. For Holmberg et al the SA was covalently immobilized to magnetic beads with a polystyrene coating which differs from the biotin-mediated immobilization done in this study, see fig. 2-2. A common way to obtain covalent attachment of proteins [49], or cross-linking in for example collagen matrices [50], is to activate carboxylic acid groups by exposure to N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) which converts the carboxylic acid groups into reactive esters that easily reacts with amino groups (or other nucleophiles) on an arbitrary protein, e.g. SA or collagen. This immobilization technique does not result in an oriented immobilization of the protein, and can lead to conformational changes in the protein structure. It is probably a technique similar to the EDC/NHS immobilization method that has been used to immobilize the SA to the magnetic nanoparticles. This could have altered the conformation of SA, and thus also the affinity for biotin. If the biotin-binding domains have been altered in such a way that the biotin affinity has been decreased that could explain why Holmberg et al succeeds in removing the biotinylated DNA strands at such high yield at 40-60 °C. Another possibility is that they have used a mutated form of SA that has a lower affinity for biotin that enables removal by raised temperature. By immobilizing SA to the biotinylated SS-OEG SAMs as done in this master’s thesis project, a more native structure of the protein is assumed, and that would also lead to a higher affinity interaction between the SA and additional biotinylated molecules such as bBSA.
Another regeneration procedure that was tested was to expose the biotinylated SS-OEG and SH-C11-OEG to Guanidine*HCl. Guanidine*HCl is a well known reagent for denaturing proteins that has been used since the 1970ies [51] until today [52]. Kim et al investigated if 8 M Guanidine*HCl at pH = 1.6 would allow for reuse of avidin coated columns for immunoaffinity with biotinylated antibodies incorporated [43]. Kim et al succeeded to regenerate the avidin coated columns up to 20 times for repeated immobilization of biotinylated antibodies. These results made us wonder if this denaturing agent would be possible to use to regenerate the biotinylated SAMs with immobilized SA and bBSA more successfully than the temperature treatment above. Results from the measurements presented in fig. 5-8 indicate that both SA and bBSA could be removed from 1 % biotinylated SS-OEG SAM but not to the same extent for SH-C11-OEG SAM, after being subjected to a flow of 8 M Guanidine*HCl for approx. 1 h at pH = 1.6. This suggests that the biotinylated SS-OEG SAM binds to the SA/bBSA complex more loosely than to SH-C11-OEG. Also, one possibility is that the SS-OEG binds more loosely to the gold surface because of the lack of hydrophobic interactions between alkane chains in SH-C11-OEG.

In fig. 5-8 it can also be seen that it is not possible to repeat the immobilization of SA or bBSA after the denaturation of proteins adsorbed to the SS-OEG SAM. This is likely due to that the relatively rough Guanidine*HCl treatment at pH = 1.6 has removed or destroyed the biotin moieties at the surface, or at least altered the affinity of the biotin head groups which disables their SA binding properties. On the SH-C11-OEG SAM additional SA and bBSA can be re-immobilized but this is most likely due to hydrophobic interactions between partly denatured bBSA or SA on the surface and the newly introduced solutions of the same proteins.

The only system that seems to allow for regeneration to some extent is the 1 % biotinylated SS-OEG if the Guanidine*HCl treatment could be altered to milder conditions so the biotin head groups could stay intact. The proper way to achieve this is probably to decrease the concentration of Guanidine*HCl and also raise the pH, since this most likely affects the biotin groups. A possible way to obtain regenerative properties of SA immobilized to 1 % biotinylated SS-OEG SAM could be to use SA that has been mutated to have a lower affinity for biotin. This could enable easier removal of SA and subsequently immobilized biotinylated compounds by temperature increase that is suggested to affect the SAM more moderately than the Guanidine*HCl treatment.
6.6 Case study: Immobilization and subsequent ligand interaction of plasminogen

The purpose of the biotinylated plasminogen (bPLG) model system was to investigate the response when bPLG, immobilized to SA on a 1 % biotinylated SS-OEG SAM, was exposed to different low molecular weight compounds and how this could be interpreted regarding protein structure of bPLG. It has earlier been reported that the ligand EACA induces conformational changes in plasminogen from a condensed, globular state to a more loose extended conformation [53]. This conformational change from a globular to an extended state was hypothesized to induce a positive dissipation shift in QCM-D. For these studies the two ligands EACA and ligand-X were used, where ligand-X is supposed to be more potent to induce conformational changes of plasminogen, and also to have a slower kinetic behavior upon dissociation from PLG compared to EACA.

From the results presented in 5.1.3 it can be concluded that bPLG is readily immobilized onto SA on 1 % biotinylated SS-OEG SAMs (a frequency shift of -36.3 ± 1.1 Hz in QCM-D) which is a good indication for the robustness of the system. The immobilization is approx. 13 Hz lower than for SA which is to be expected since the molecular weight of PLG is ~85 kDa compared to ~60 kDa for SA. In this context, it should be mentioned that the SPR response of approx. 170 ng/cm$^2$ upon bPLG binding indicates that the bPLG is highly hydrated since the frequency shift obtained in QCM-D corresponds to 643 ng/cm$^2$ following the Sauerbrey relation. RU shifts for EACA are 20 RU for 1 mM, which corresponds to approx. 2 ng/cm$^2$ that in turn is approx. 1 % of the mass shift for the bPLG immobilization. EACA has a molecular weight of 131 Da which is 0.1 % of the molecular weight for plasminogen. This indicates that 10 EACA units bind to one plasminogen protein. This is in the same range as theoretical values that states that up to 6 ligands can bind to one plasminogen unit and induce the conformational change. [53] One possible explanation for the small difference could be that the EACA ligands also non-specifically binds to PLG by electrostatic interactions with charged amino acid side chains, since EACA has a zwitter ionic structure. The small EACA shift of 2 ng/cm$^2$ would correspond to a frequency shift of 0.11 Hz and this explains why only dissipation shifts are observed in fig. 5-4 at ligand binding. [54] This observation also suggests that the hydration of bPLG is not dramatically
changed upon ligand binding since no large negative frequency shifts were observed. The molecular weight of ligand-X is unknown and therefore no similar comparison can be done for this ligand, but it can be concluded from fig. 5-4c that the level of hydration of bPLG is not highly affected by ligand binding. Dissipation results in 5.1.3 show that bPLG is affected upon ligand binding and especially in fig. 5-4 for the QCM-D measurements a positive dissipation shift can be observed for both ligand-X and EACA when the two reference systems with bBSA and SA are subtracted. It should be mentioned that the ideal reference surface would be one with immobilized biotinylated plasminogen that is mutated to not interact with EACA and ligand-X. Such a protein was not available and therefore surfaces with immobilized bBSA and SA were considered as valid references. Relative dissipation shifts increase with increasing ligand concentration and the shifts are reversible when changing to buffer between the ligand exposures both for EACA and ligand-X. From these observations it can be speculated that the positive dissipation shifts in fact arise, as was hypothesized, from bPLG undergoing a conformational change upon ligand binding. To strengthen this theory it is important to certify that the dissipation shifts have arisen from ligand binding effects and not bulk effects or other non-specific effects. This can at least be indicated when inspecting the dissociation kinetics of EACA and ligand-X both for the QCM-D and SPR measurements. Ligand-X is known to dissociate from plasminogen with a slower dissociation kinetic than EACA. This can be confirmed by the measurements performed in this work, which also indicate that the observed positive dissipation shifts are results from conformational changes of plasminogen upon ligand binding. Finally one can conclude that the bPLG model system performed very well with the gold crystals coated with SAMs of 1 % biotinylated SS-OEG which strengthens the possibility of a future commercialization
6.7 Next step towards a product

6.7.1 Gold quality

A problem that has been identified throughout this master’s thesis work is the quality of the gold coated QCM-D crystals. For the batch of gold coated sensor used, the gold dissolved, at several occasions, from the QCM-D crystals after ultra sonicaton. Prolonged ultra sonicaton was tested on clean and thiol incubated gold crystals, without observing dissolution. This suggests that it is not the ultra sonicaton itself but rather the incubation in the ethanolic thiol solution or the hydrogen peroxide/ammonia washing procedure that loosens the gold from the titanium adhesive layer on the quartz crystal. As the focus of this master’s thesis was the functionalization and characterization of the thiol SAMs and as this was not investigated further. This however, questions the reliability of the gold crystals as a substrate for thiol chemistry and should be thoroughly investigated before gold crystals modified with thiol procedures are to be commercialized.

6.7.2 Storage

For the possibility to commercialize the biotinylated QCM-D crystals developed during this master’s thesis project, storage is an important parameter. If the biotinylated crystals withstand long shelf time, then production, distribution, and marketing of the product would be easier to perform. As is presented in section 5.1.4 and in fig. 5-6, SS-OEG is indicated as the system that best withstand storage both regarding surface contamination and chemical stability of the SAM for at least 7 days with approximately 90 % retained specific activity and with acceptable protein repellent properties adsorbing only 5 % compared to the clean Au surface shown in fig. 5-1. The SH-C11-OEG system has approximately the same, or even higher, specific activity as SS-OEG after 7 days, but the non-specific binding of serum proteins is substantial, being 27 % of that for clean Au. This leads to the question whether the SA adsorbed onto SH-C11-OEG after 7 days binds specifically to biotin moieties in the SAM or is non-specifically immobilized. After 14 days the SS-OEG has been further affected by the storage and now has approximately 70 % retained specific activity and unspecifically adsorb serum protein to a level of 12 % compared to clean Au. This suggests a more or less linear reduction of the polymer layer function over time, but this has to be investigated further to be confirmed since these are results from only one measurement. SH-C11-OEG seems to have been heavily affected by 14 days of storage and enabled only 40 % of SA to be immobilized.
compared to a fresh crystal. Furthermore, this SA is assumed to be non-specifically immobilized since the non-specific serum adsorption of SH-C11-OEG after 14 days was as high as 90% to that of clean gold which indicates that the SAM has been more or less removed from the surface, probably during the ultrasound sonication or during rinsing in ethanol before the QCM-D measurement. Another possibility is that the more hydrophobic SH-C11-OEG SAM has been the subject for hydrophobic contaminations which impair the protein repellence. A way to investigate this would have been to perform ellipsometry and contact angle measurements before mounting the stored crystals into the QCM-D to measure possible increased hydrophobicity and thickness from contaminations. These procedures could not be performed promptly after one another as suggested above mostly because the instruments were situated at different locations at Chalmers and transportation between the different instruments could have altered the properties of the SAMs.

All storage measurements suggest that SS-OEG is the SAM that is the most resilient to storage in air $\geq 7$ days both with respect to protein repellence and the ability to specifically bind to SA. If there is a desire to store crystals with biotinylated SS-OEG for time periods $> 14$ days then alternative solutions have to be taken into consideration. One possibility that was brought up during our work was to coat the SAMs with a protective layer which is dissolved in water upon use of the sensor. Trehalose is a disaccharide that has the function to stabilize different macromolecular structures in organisms by hampering the degradation of proteins and lipid membranes. [55] The polymer SAMs used in this master’s thesis have a clear macromolecular structure and a hypothesis is that a dried layer of trehalose on top of the SAMs would protect them from degradation and contamination during long time storage. Due to the fact that trehalose is highly water soluble at room temperature ($68.9$ g/100g H$_2$O [55]) it would be possible to put the stored biotinylated crystals into a QCM-D instrument and dissolve the trehalose in situ which would make the biotinylated crystals more user friendly. This is still a hypothesis and was formed at a late stage in this project and could therefore not be tested and optimized. Another way to get around the storage issue and that is to offer the costumers clean Au surfaces, pre-made thiol solutions containing 1% biotinylated SS-OEG and a protocol, making them able to perform the SAM preparation themselves. Of course this narrows down the available customer range since it requires some experience and equipment for surface preparation, but could be a way to ensure the quality of the product. Another tempting solution to the storage problem would be to store the gold coated crystals in a sealed container filled with the SS-OEG incubation solution. This would ensure the SAM quality as long as the surface can be secured in the container in a
satisfying way so that it will not be dislocated. Still, the customer has to be able to ultrasonicate the surface and rinse it in ethanol before mounting it into the QCM-D instrument, but these are easy procedures not requiring any chemicals such as ammonia or hydrogen peroxide that is used in the pre-incubation wash. However, one should be aware that ethanolic alkane thiol solutions have been reported to corrode and dissolve gold by 6% of a gold monolayer after 24 h. [56] This corrosion process was shown to be dependant on thiol concentration and solvent polarity and thus further optimization of the incubation conditions would be needed if this approach was to be tested for a commercial product.
7 Conclusions

Self assembled monolayers of SS-OEG obtained after over night incubation of gold coated quartz surfaces in 0.5 mM ethanolic solution with 1 % SS-OEG-biotin content were proven to be highly reproducible with respect to SA/bBSA binding and protein repellence. SA was adsorbed at a surface concentration of 407 ng/cm$^2$ in a 50 % hydrated monolayer with retained native structure, assumed from the low dissipation shifts at SA adsorption and high subsequent bBSA immobilization.

The precise combination of thiols used in this protocol represents a novel approach to obtain biotinylated SAMs, although similar SAMs have been described in the literature. The presented protocol was proven to perform better than the more extensively studied SH-C11-OEG with respect to non-specific binding of serum proteins, which is probably related to the more hydrophilic properties of the SS-OEG SAMs. Surfaces modified with SS-OEG also bind SA more reproducibly than both SH-C11-OEG and SH-PEG and at lower solution percentage of biotinylated thiol which could be of economical interest for a commercial product, since the biotinylated derivatives are considerably more expensive. SH-PEG proved unsuitable for subsequent immobilization of bBSA, likely due to the high flexibility of the long PEG chains.

Storage > 7 days had a negative influence on protein repellence and specific activity of the SS-OEG-biotin and SH-C11–OEG-biotin coated QCM-D gold crystals. This questions the stability of the SAMs and is, together with the substrate gold quality, the main obstacles before commercialization of biotinylated sensors based on the presented protocol based on SS-OEG. Furthermore, a regeneration protocol of SS-OEG-biotin with respect to SA and/or bBSA binding could not be established.

In a challenging case study, the interaction of biotinylated plasminogen with low-molecular weight ligands (ligand-X and EACA) was studied when immobilized via SA on a SAM of 1 % biotinylated SS-OEG, both by QCM-D and by SPR. Biotinylated plasminogen was readily bound to the sensor surface, although with slower kinetics than bBSA. Experiments were performed using bBSA as reference. Reference subtracted responses upon interaction with ligand-X and ligand EACA were detected in SPR as positive RU shifts in the range of the expected mass uptake (~20 RU). No frequency shifts were detected by QCM-D, but
positive dissipation shifts ($\sim 0.2 \times 10^6$) were taken as indicatives for conformational changes in the protein as the ligands are known to induce large conformational changes in the protein structure. This result, by QCM-D, is unique for structurally defined proteins. Differences in dissociation kinetics between ligand-X and EACA in the QCM-D measurements strengthen the assumption that the positive dissipation shift is due to conformational change from a globular to a more extended state of plasminogen.
8 Future

The spectrum of application fields for surface chemistry that enables specific protein immobilization onto different substrates, while minimizing non-specific protein binding is extensive. In particular, the presented protocol for biotinylation of gold surfaces is very useful in sensor applications. To improve the characterization of the SAMs, it is close at hand to suggest further studies with additional surface sensitive techniques such as infrared spectroscopy (IR), x-ray photoelectron spectroscopy (XPS), cyclic voltammetry, and atomic force microscopy (AFM) if there is a desire for specific protein studies.

An obvious application field for the kind of biotinylated sensor surfaces presented here is various QCM-D assays. Conformational studies of proteins have not been a major application field for the QCM-D technique, but could perhaps get more attention based on the results presented here for the plasminogen experiments.

Of particular interest are combined QCM-D and electrochemical measurements, since the absence of an insulating alkane chain layer is likely interesting for the study of electroactive biotinylated molecules.

The surface modification described here could also be used within different cell culturing applications, where the surface chemistry of the cell culture container, or surface, could be modified with specific proteins, or other functional groups such as carbohydrates. This could enable specific studies of the interaction between cells and a surface with known chemistry and possibly lead to greater understanding of cell adhesion and cell mobility on different substrates, possibly combined with patterning. The SS-OEG-biotin approach could be applicable here as a tool for patterning of quartz surfaces with gold sputtered in areas separated by SiO$_2$ to enable different proteins to be immobilized in different spots on the surface. This kind of surfaces could also work as screening tools for proteomic research and as a base for development of medical devices where the presence of different antibodies in a patient’s blood could be analyzed.

As a final comment, it is very likely that the engineering of molecularly defined surfaces will go hand in hand with miniaturization and more advanced sensor assay development. The present project can be seen as a contribution to this process. The more advanced systems that will be asked for, the better control at the molecular level will be required.
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References


