

Linköping Studies in Science and Technology
Licentiate Thesis No. 1408

Self Assembled Monolayers for Quartz Crystal Microbalance based Biosensing

Annica Myrskog



Linköping University
INSTITUTE OF TECHNOLOGY

Division of Sensor Science and Molecular Physics
Department of Physics, Chemistry and Biology
Linköping University

Linköping 2009

During the course of the research underlying this thesis, Annica Myrskog was enrolled in Forum Scientium, a multidisciplinary graduate school at Linköping University, Sweden.

© Copyright 2009 Annica Myrskog

LiU-TEK-LIC-2009:16
ISBN: 987-91-7393-587-6
ISSN: 0280-7971

Printed in Sweden by LiU-Tryck, Linköping, 2009

Abstract

The work in this thesis has been focused on developing surfaces for use in biosensor systems, especially for quartz crystal microbalances. The surfaces were prepared by adsorption of organosulfur molecules onto gold substrates, so called self assembled monolayers (SAMs). By chemical synthesis these thiols can be specifically tailored to provide surfaces with desired properties. The investigated surfaces were all based on thiols terminated with carboxylic acid groups to render hydrophilic surfaces onto which desired proteins can be covalently attached.

In order to increase the performance of two dimensional carboxyl surfaces, a method for improving the immobilization of proteins to the surface was investigated. The immobilization levels of antibodies were increased by using N-hydroxysulfo-succinimide (sulfo-NHS), instead of N-hydroxy-succinimide (NHS), as stabilizer of the amine reactive intermediate formed by reaction of the carboxyl groups with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The negatively charged sulfo-NHS intermediate promotes the attraction of overall positively charged proteins and enables immobilization also at low pH. In addition, the orientation of the immobilized antibodies was shown to be dependent on the pI of the antibody and to have a profound effect on the subsequent interaction with the antigen.

The organization of carboxyl terminated SAMs can be poor due to the repulsion between the polar terminal groups. By using acidified ethanol as solvent during the assembly step of monolayer formation, the organization in carboxyl terminated alkyl and oligo(ethylene glycol) SAMs was improved. However, the carboxyl groups were found to be converted to ethyl esters, the rate being related to the acid strength. Furthermore, the long-term stability of carboxyl oligo(ethylene glycol) SAMs was investigated. Here, the effect of alkyl chain length on the storage stability was of interest. A short alkyl chain was shown to have a profound negative effect on the storage stability of the SAM, resulting in decomposition and loss of functionality over time compared to when thiols with longer alkyl chains were studied.

I would like to acknowledge...

My main supervisor Bo Liedberg for opening up the fascinating world of SAMs for me and for guidance and support during this time.

Thanks to Attana and Teodor Aastrup for financial support and giving me the opportunity of working with the QCM. At Attana several people have been of great help. Most of all Henrik Anderson my co-supervisor, thanks for help and discussions through these years, without your help the outcome would not have been as good. My boss and latest co-supervisor Björn Ingemarsson and chemistry group at Attana; Karin, Kribu, Helena, Lena, Gunnar, Zhichao, Andréas and Mia are also acknowledged.

Former and present members of the Sensor Science and Molecular Physics group during my time at IFM; Emma E, Daniel K, Daniel A, Tobias E, Olof A, Mattias Ö, Andréas L, Christian U, Jenny C, Sophia F, Feng-I T, Goran K, Karin E, Thomas E, Fredrik B, Erik M, Hung Hsun L, Lan B, Patrik N, Andreas C, Timmy F, Rodrigo P, Kajsa U, Cecilia V, Maria A, Linnéa S, Robert S, Luminita S, Magnus F, Annika B, Ye Z, Chun-Xia D, Alexander O, Shankara N, my diploma worker Karin Elovsson. Thanks also to Kristina B.

My room-mates at IFM; Sara, Linnéa, Feng-I and especially Olle for the help with this thesis.

Ramūnas Valiokas for all valuable discussions on OEGs and help with paper III. Thanks also Živilė Ruželė for the OEG-thiols.

Pia Blomstedt, Anna Maria U and Susann Å for help with numerous administrative issues and for nice talks. Agneta Askendal and Bo Thunér for help with laboratory issues when ever needed.

Stefan Klintström, for your enthusiastic work within Forum Scientium.

Of course my family; mom, dad, Martina and Henrik, without your endless support and encouragement this would never have been possible. Claes, my love, thanks for always being there for me and believing in me!

TABLE OF CONTENTS

1	Introduction	1
2	Protein binding	3
	2.1 Non-specific protein adsorption	3
	2.2 Protein immobilization	4
	2.3 Antibodies	6
3	Self-Assembled Monolayers	9
	3.1 Preparation methods	10
	3.2 Mixed SAMs	11
	3.3 Oligo(ethylene glycol) SAMs	12
	3.4 Long-term stability	12
4	Techniques	15
	4.1 Null-ellipsometry	15
	4.2 Contact angle goniometry	16
	4.3 Infrared Reflection Absorption Spectroscopy	17
	4.4 Quartz Crystal Microbalance	18
5	Summary	21
6	Papers	23
7	References	27

1 Introduction

There is a fundamental interest in monitoring and understanding interactions occurring around and within ourselves. Biological interactions in the body determine our physical condition and are studied in order to improve medical treatments and for the development of new drugs. Advanced equipment for molecular detection is therefore needed and specifically, biosensors are devices that can be used to study biomolecular interactions. A biosensor is made up of two elements, a bioactive element where the biological interaction occurs and a transducer which converts the signal to a measurable quantity. The bioactive surface often involves immobilized antibodies, receptors, enzymes, DNA or whole cells that will react selectively with an analyte and result in a signal detected by the transducer. The obtained signal can thereafter be translated into different amounts for example changes of mass, refractive index or colour.¹ A biosensor, however, does not necessarily imply complicated equipment, as demonstrated by the commonly used biosensors for glucose sensing and home pregnancy tests.

The study of biomolecules on a sensor surface is highly dependent on the choice of interfacial chemistry attaching the biospecific molecules to the surface. The surface chemistry should allow for sufficient amounts of the biomolecules to be immobilized and, equally important, the molecules should retain their biological activity in the immobilized state. Also, the surfaces should enable studies of the interaction of interest without the background noise of other, non-specific, binding events. A frequently used approach to achieve this is the use of self-assembled monolayers (SAMs) that provides highly defined interfaces with the desired properties depending on the choice of organosulfur tail group. To suppress non-specific binding, oligo(ethylene glycol), OEG, containing SAMs have been successfully applied in laboratory studies.²

For biosensor surfaces to be of commercial use, the long term stability is of great importance. From a surface production perspective, it is vital that the surfaces can be produced in advance and have a shelf lifetime of at least several months. During storage, the surface structure and properties should not change in a way negative for the sensor functionality. Also, if the molecules used to form the surface layers are expensive and difficult to synthesize it is of great benefit to be able to use the same solution for extended periods of time.

The aim of the work presented in this thesis has been to develop sensor surfaces suitable for use in Quartz Crystal Microbalance (QCM) biosensors. The performance of the sensor surfaces should be highly reproducible and they should exhibit low non-specific binding in protein rich media. Specifically, preparation methods for SAM biosensor surfaces have been studied and optimized and the storage stability for OEG containing, low non-specific binding surfaces assessed by means of infrared reflection absorption spectroscopy, ellipsometry and QCM. Methods for immobilization of antibodies on SAM sensor surfaces have been optimized and the relationship between antibody orientation and binding capacity examined.

2 Protein binding

When a substrate is immersed in a protein containing media protein adsorption will rapidly occur on its surfaces. In a biological context, the adsorbed proteins will govern the subsequent cellular response towards the surface.² To understand what the driving forces for protein adsorption are, it is of great importance to study these events on different surfaces. The outcome of such studies will, for example, support the development of new protein resistant coatings which can be used for biomaterials, biosensors, marine biofouling and food processing.

2.1 *Non-specific protein adsorption*

Spontaneous adsorption of proteins is the easiest way to achieve surface bound proteins. Adsorption is affected by hydrophobic, electrostatic and van der Waals interaction forces between the protein and surface, as well as rearrangement of the protein structure. The process of protein adsorption can be divided into several steps. Initially the protein has to be transported to the surface to enable attachment. The transport will be diffusion limited closest to the surface, even for surfaces placed under continuous flow. Upon adsorption the proteins can change their conformation, although this process might also take place some time after the adsorption. This partial denaturation may affect the biological activity of the protein. The last step is desorption and transport away from the surface. However, most proteins adsorbed on the surface will not desorb unless exchanged or displaced by another protein.^{3, 4}

The spontaneous adsorption of proteins to surfaces is often unwanted in biosensor applications since it leads to lower specificity. A common approach used to prevent the non-specific adsorption is the preadsorption of another protein, for example bovine serum albumin, on the surfaces. Possible drawbacks with this strategy are denaturation of the preadsorbed protein and replacement of the protein with other proteins.² Also, surfactants can be used to reduce the non-specific binding. For instance, the non-ionic surfactant Tween 20 has been shown to prevent hydrophobic interactions between surfaces and proteins⁵, and is therefore frequently used in biosensor studies. For studies in protein rich solutions, additional strategies to reduce non-specific binding may be necessary. Such strategies include the

incorporation of ethylene glycol (EG) units on the sensor surface (described in more detail later in this thesis). Whitesides and co-workers⁶⁻⁸ investigated around 60 different terminal groups, including EG, and found that the surfaces best capable of resisting non-specific protein binding were hydrophilic, hydrogen-bond acceptors, not hydrogen-bond donors, and had a neutral overall electrical charge.

2.2 Protein immobilization

Covalent coupling of proteins and molecules to surfaces is a way to assure the firm attachment of a protein on the surface without risk of protein replacement or desorption. Common chemical compounds which can be used for covalent attachment are hydroxyl-, carboxyl- and amine-terminated SAMs.⁹ In this thesis, carboxylic acid terminated SAMs were used in combination with one of the most frequently used strategies for immobilizing proteins, often referred to as amine coupling. Figure 2.1A shows a schematic of protein immobilization onto a carboxylated surface by means of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) mediated amine coupling. With this reaction the carboxylic acid groups can be linked covalently to free primary amines in proteins, such as the side chain of lysine or the N-terminal. This reaction has been utilized for immobilization in **papers I, II and III**. Possible drawbacks with this method include a loss of protein activity due to chemical modification of the protein at or close to its active sites and the risk of multiple attachment sites potentially altering the three dimensional structure of the protein and restricting its ability to undergo the structural changes associated with its biological function. Also, when immobilized on two dimensional surfaces random or unfavourable orientation of the protein on the surface may result in poor exposure of relevant interaction sites on the protein.^{10, 11}

The orientation of immobilized antibodies and their antigen binding capacity on a two dimensional carboxyl surface was investigated in **paper I**. It was shown that orientation of antibodies on the surface varied from random to ordered depending on the antibody properties and the immobilization pH. Also, the use of N-hydroxysulfo-succinimide (sulfo-NHS) instead of NHS as an intermediate on the surface was investigated. As seen in Figure 2.1B sulfo-NHS has a negative charge which will aid in the attraction of overall positively charged proteins to surfaces with few carboxylic acids as well as to surfaces displaying poor attraction when

activated with the neutral NHS. The improvement in immobilization level when using sulfo-NHS instead of NHS on a two dimensional carboxyl surface was found to be significant as illustrated in Figure 2.2. In addition, immobilization at a very low pH was shown to be possible, which in turn would be useful for immobilization of acidic proteins.

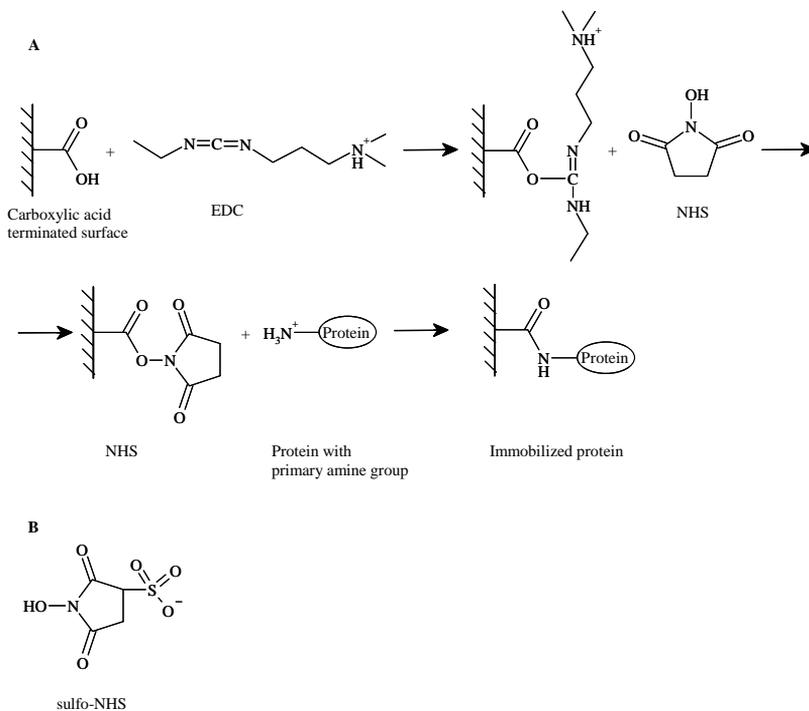


Figure 2.1. A) Schematic picture illustrating the covalent coupling of a protein to a carboxylic acid surface via EDC and NHS. B) Chemical structure of sulfo-NHS.

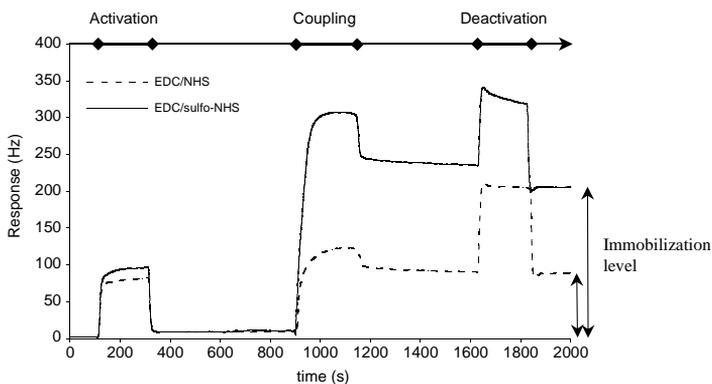


Figure 2.2. QCM data of the immobilization of anti-myoglobin 7005 on an Attana carboxyl surface either activated with EDC/NHS or EDC/sulfo-NHS.

2.3 Antibodies

Antibodies are a group of proteins that has received an immense interest because of their ability to specifically bind other biomolecules. For instance, antibodies are widely used in diagnostics for detection of disease markers and they are being increasingly studied within pharmaceuticals development. In vivo, antibodies are responsible for recognizing and binding foreign molecules, so called antigens, thereby leading to an immune response. The interaction with its antigen is very specific and every antibody recognizes only a single or a few antigens. Collectively, however, antibodies can in practice recognize virtually any molecule. Despite their binding diversity, all antibodies are structurally similar. The common Y-shaped structure of an immunoglobulin antibody, IgG, is depicted in Figure 2.3. The molecule is made up of the F_c part at the base which is linked via the hinge region to the two arms. The arms are referred to as F_{ab} fragments and it is the region in the terminal end of the F_{ab} fragments that is responsible for the binding of the antigen.¹²

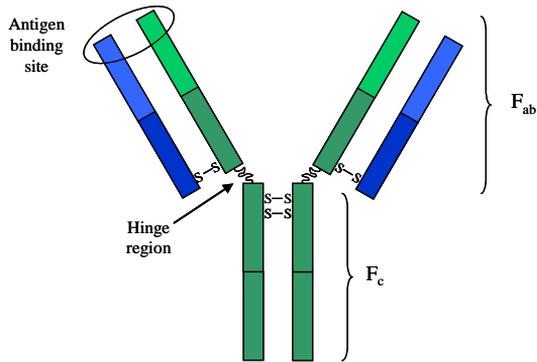


Figure 2.3. The general structure of an IgG antibody. The carbohydrates have been excluded.

Since antibodies have such high specificity and can also be engineered to recognize a desired target molecule they are widely used as recognition elements in biosensor applications. In all three papers within this thesis antibodies were immobilized on the studied surfaces and used as model systems for functionality studies.

3 Self-Assembled Monolayers

In 1983 Nuzzo and Allara were the first to show that organic disulfide molecules spontaneously formed organized monolayers on gold surfaces.¹³ Since then self-assembled monolayers (SAMs) of alkylthiolates have been extensively investigated.^{14, 15} The formation of a SAM from diluted thiol concentrations consists of two phases; a fast initial phase followed by a slower second phase (Figure 3.1). During the fast phase which takes only a few minutes from immersion the SAM reaches 80-90% of its final thickness and close to the limiting value for the contact angle. The subsequent slow phase spans over a period of several hours during which the contact angle and thickness reaches their final values. The alkylthiolates in the SAM proceeds from an unstructured, gauche, conformation during the first phase to forming a dense and well-ordered, all-trans, layer during the second phase. The forces that drive the SAM towards a dense and well-ordered layer are the interchain interactions, for example dipole-dipole and van der Waals forces.¹⁴

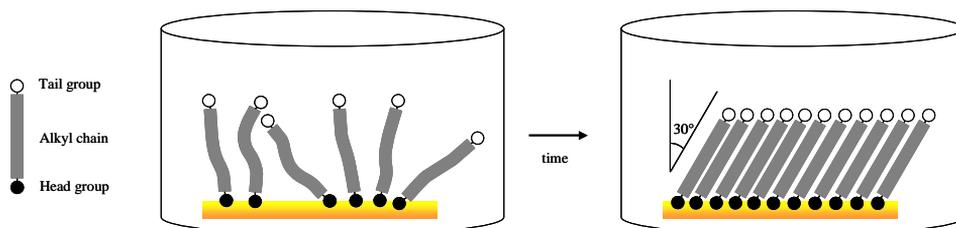


Figure 3.1. Schematic picture describing the formation of a SAM from a diluted alkylthiol solution. The initial unordered SAM transforms to a dense and well-ordered SAM with incubation time.

On an Au (111) surface the thiolate superstrate will adopt the structure of the underlying gold substrate, a $(\sqrt{3}\times\sqrt{3})R30^\circ$ hexagonal structure. The sulfur binding sites are situated approximately 5 \AA from each other and due to the distance between the adjacent alkylthiolates the chains will be tilted to optimize the van der Waals interactions between the neighbouring methylene groups. The tilt will be approximately 30° from the surface normal as illustrated in Figure 3.1 and every molecule will occupy an area of 21.4 \AA^2 .^{14, 15}

The molecular structure of the thiol can relatively easy be varied by synthesis and thereby enable fine-tuning of the surface-liquid interface. It has been shown that the length of the

alkyl chain affects the ordering within the SAM. Thiols with short alkyl chains, $n < 8$, result in less dense SAMs compared to those with longer alkyl chains, $n \geq 9$.¹⁶ By exchanging the terminal group the functionality and wettability can be exactly controlled. If bulky end-groups are inserted the packing density may however be affected.

3.1 Preparation methods

When assembling SAMs the preparation method is of great importance in order to achieve high quality monolayers. Factors that may influence the rate of adsorption as well as the structure of the SAM include choice of solvent, temperature, concentration, molecular structure of the thiol, immersion time and cleanliness of the substrate.¹⁵ For example, if the thiols concentration is reduced, a longer incubation time is required.¹⁷ Decreasing the concentration has also been shown to reduce defects within the monolayer.¹⁸ As already mentioned, the terminal group of the alkyl thiols may have an impact on the assembly of the SAMs. For carboxylic acid terminated SAMs it has been reported that unstructured SAMs will be formed due to the repulsion between the charged carboxylate groups. As a means of getting well-ordered carboxyl SAMs, it has thus been suggested that the thiol solution should be acidified.^{19, 20} The effect of the addition of acid in the thiol solution has been studied in **paper II**, where it is shown that the organization within the SAMs is improved by acidification. Moreover, it was demonstrated that the carboxylic acid groups are rapidly converted to an ethyl ester in the presence of an acid, as reported earlier by others.¹⁹ In this work however, the esterification was also observed in the presence of a weaker acid, which has not been previously reported. Figure 3.2 shows a graph illustrating the formation of the ethyl ester in the presence of hydrochloric acid (HCl) or acetic acid (HAc) as a function of solution age and incubation time. It is obvious that the rate of esterification is strongly dependent on the strength of the acid.

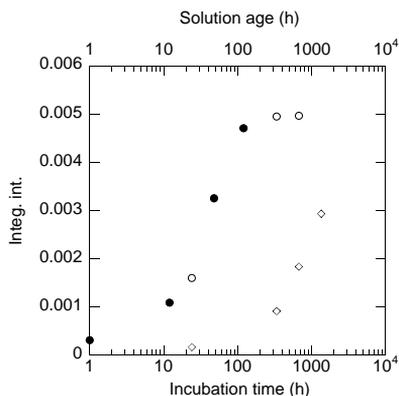


Figure 3.2. Integrated intensity of a CH_3 peak calculated from infrared reflection absorption spectra of SAMs formed from thiol solutions containing HCl or HAc. The filled symbols: ● HCl belong to the lower x-axis and the open symbols: ○ HCl and ◇ HAc to the upper x-axis.

3.2 Mixed SAMs

To enable variation and tailoring of the surface properties two or more thiols can be mixed in solution and subsequently co-adsorbed on the surface. This method could be of use when for example spatial distribution between a functional group is needed to prevent steric hindrance. The ratio on the surface between the different thiolates is however often not the same as the ratio in solution. Depending on the molecular structure, length and terminal group different enrichments on the surface can be seen.²¹⁻²⁴

A risk when co-adsorbing thiols from solution is the formation of islands of the respective thiols on the surface. Even though phase separation into macroscopic islands of the different thiols usually is not observed, it probably occurs to some extent on a smaller scale.^{21, 22} One strategy to achieve truly mixed SAMs on the molecular level, is by co-adsorption at elevated temperatures. In this case, well mixed monolayers can be accomplished with thiols of different lengths and terminal groups.^{25, 26}

3.3 Oligo(ethylene glycol) SAMs

SAMs containing different numbers of ethylene glycol (EG) groups, $(\text{CH}_2\text{CH}_2\text{O})_n$, are highly attractive due to their protein repellent properties.^{15, 27} This property makes these surfaces interesting in applications where low non-specific binding is required, for instance biomaterial and biosensor surfaces in contact with biological fluids.

The EG groups attached to the alkyl chain of a thiol can adopt three different types of conformations when adsorbed on a surface. As for the alkylthiols, the EG-parts can lack any specific order, being amorphous, or they can be closely stacked in a zig-zag formation, all-trans. Additionally, the EG units can adopt a helical conformation at the surface provided that a sufficient amount of EG units are incorporated. The required number depends on the general structure of the thiol.²⁸⁻³⁰ The adopted conformation of the EG groups has been shown to have a major impact on the protein resistance of the surface.^{30, 31} The reasons to why oligo (ethylene glycol), OEG, SAMs are able to hinder protein adsorption are widely debated. It is commonly attributed to the ability of water molecules to penetrate into the EG part of the SAM and to the coordination of water molecules to the EG groups.³²⁻³⁶ An increased number of EG units has been observed to give increased protein resistance.^{8, 35, 37} Furthermore, the specific proteins investigated, as well as the temperature, may have a significant impact on the results and should therefore be chosen with care.³⁸

3.4 Long-term stability

For SAMs to be useful as commercial biosensor surfaces, long term stability is a prerequisite. After storage the surfaces must retain their properties and display satisfactory performance for relevant applications. The stability of monolayers can be investigated in several ways. For example, the structure and potential reorganization of the monolayers due to storage can be characterized with different surface sensitive techniques whereas functionality tests with a biosensor are necessary to determine the impact of SAM degradation on the functional performance. The latter is the critical property, since retained sensor capability despite rearrangement in the SAM means that the structural changes are not of great importance.

Studies performed regarding the temperature stability of SAMs have shown that monolayers of alkylthiolates starts to desorb at 70°, although the rate of desorption is dependent on the temperature, length of the adsorbate as well as the present medium.¹⁷ The stability can however be increased by introducing lateral binding within the SAMs, for example amide bonds.^{39, 40}

The major cause for a lowered shelf-life of SAM surfaces is degradation of the monolayer by the oxidation of the sulfur-gold bond. Upon exposure to air different time frames have been reported before observing oxidative species on the surfaces, from hours⁴¹ to weeks³⁰. Of the different components in air: O₂, N₂, ozone and water, ozone has been shown to be the primary cause of oxidation of the thiolates.⁴¹ However, storage in air filled sealed vessels is still possible due to depletion of the ozone over time leading to a cessation of the oxidation.⁴² Exposure to UV-light will also result in oxidation of the SAMs, as utilized in photooxidation.^{43, 44} However, oxidized species on the surface are not necessarily easily removed.⁴¹ The oxidation rate due to either ozone or UV-light is strongly dependent on the alkyl chain length, a longer alkyl chain will decrease the rate profoundly. This can probably be attributed to increased organization which restricts the active oxygen from penetrating the SAM down to the sulfur-gold bond.^{41, 43, 44} Also, varying the terminal group of the SAM have a big impact on the oxidation rate. For example, a polar end-group decreases the oxidation rate compared to a non-polar one. This is probably due to hydrogen bonding between the terminal groups.⁴⁴ The oxidation of the sulfur headgroup and desorption of thiolates is primarily initiated at defect sites, domain boundaries and vacancy islands, and can from there spread into the well-ordered domains.^{45, 46} Therefore, the substrate should be chosen with care, as SAMs prepared on rougher gold surfaces will oxidize faster.⁴⁷ When working with EG containing SAMs it should also be mentioned that the EG chain can be susceptible to oxidation in air leading to loss of EG units.^{30, 48}

For biosensor applications, the surfaces must often retain their stability for longer periods of time. However, only a few studies have been done where the surface reorganization and functionality are investigated in parallel. A recent study⁴⁹ on the stability of a mixed EG-thiol surface compared different wet and dry storage media. The dry storage media (air or N₂) resulted in retained biosensor properties for at least 30 days, while ethanol resulted in sulfur oxidation, desorption of thiols and decreased sensing capabilities including increased non-specific binding. To investigate this further, the effect of structural changes upon storage in

relation to the biosensor performance of the surfaces was investigated in **paper III** for a set of three types of OEG-thiol surfaces. The thiols were comprised of six ethylene glycols connected to alkyl chains of three different lengths and with an amide linker group as seen in Figure 3.3. The results showed an increased stability of SAMs with longer alkyl chains with respect to thiol decomposition, desorption and functionality. Figure 3.4 illustrates the immobilization capacity before and after storage on the three different SAMs.

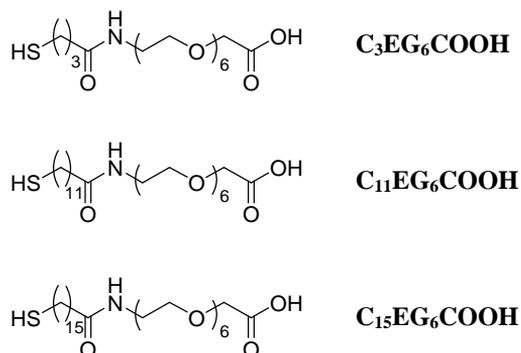


Figure 3.3. The molecular structure of the three thiols investigated in paper III.

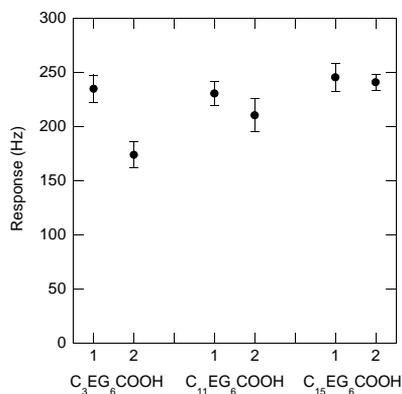


Figure 3.4. QCM data of the immobilization of anti-myoglobin 7005 • on the fresh and stored C₃EG₆COOH, C₁₁EG₆COOH and C₁₅EG₆COOH SAMs. 1 corresponds to the fresh surfaces and 2 to the surfaces after storage.

4 Techniques

In this section, the surface characterization techniques employed within this thesis are described in brief.

4.1 Null-ellipsometry

Null-ellipsometry is an optical technique which enables measuring the complex refractive index and thickness of thin layers, for example SAMs, on reflecting substrates. It is a fast non-destructive technique with the ability to measure layer thicknesses of up to 100 nm at sub-Angstrom resolution. Figure 4.1 shows schematically the experimental configuration of a null ellipsometer. Typically a HeNe laser is used as light source. The light is first linearly polarized via an adjustable polarizer before passing through the compensator and turned into elliptically polarized. The elliptically polarized light is then reflected at the substrate and passed through a second polarizer, called the analyzer, before reaching the detector. The actual experiment consist of iteratively adjusting the angles of the polarizer and analyzer until the light reaching the detector is extinguished and the nulling condition is achieved, hence the term null-ellipsometry.⁵⁰

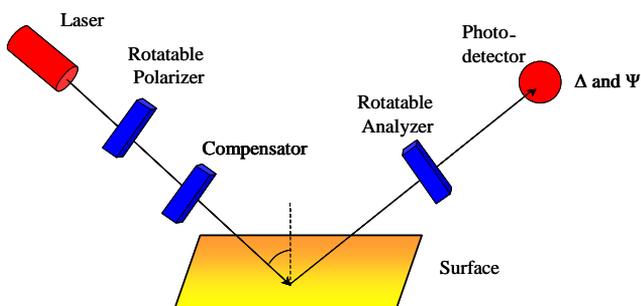


Figure 4.1. Schematic picture illustrating the set up of a null-ellipsometer.

The thickness of the adsorbed films can be determined since the state of polarization of light reflected from a surface, with or without an adsorbed film, will be changed. The change is dependent on the optical properties of the surface. This change in polarization for the parallel (p) and perpendicular (s) polarized light upon reflection at the surface is indirectly measured by the ellipsometer by measuring the angles of the polarizer, compensator and analyzer. The ellipsometer measures the ratio between the complex reflection coefficients for the p- and s-polarized light, R_p and R_s :

$$\rho = \frac{R_p}{R_s} = \tan \Psi \cdot e^{i\Delta} \quad (\text{Eq 4.1})$$

Wherein Δ gives the phase difference for the s- and p-polarized light after reflection while $\tan\Psi$ is the ratio of the amplitude change of the respective reflection coefficients. When the optical properties of the ambient, the adsorbed film and the substrates are known, the measurement of the ellipsometric angles Δ and Ψ enables the calculation of the film thickness using different mathematical methods such as for example the McCrackin algorithm.⁵¹

For the measurements in this thesis the SAMs were assumed to be isotropic uniform films with a refractive index of $N=1.5+0i$.^{16, 17}

4.2 Contact angle goniometry

Contact angle measurements can be performed to yield information on the physicochemical properties of the outermost layer of the surfaces. In contact angle goniometry, a drop of liquid is placed on a substrate and the wetting properties, the hydrophobicity or hydrophilicity of the surface, are examined. The contact angle, θ , is defined as the angle between the surface and the tangent of the droplet (Figure 4.2). The contact angle is related to the surface tension γ by Youngs equation (Eq. 4.2.), where SV denotes the surface tension at the surface-vapour interface, SL surface-liquid and LV liquid-vapour interfaces, respectively.

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\theta \quad (\text{Eq. 4.2.})$$

In addition to measuring the static contact angle it is also possible to measure the advancing and receding contact angles, i.e. the contact angle when the liquid is expanding or retracting. From the hysteresis, the difference between the advancing and receding contact angles, information on the surface homogeneity and roughness can be gained.⁵² With the use of contact angle measurements indications of structuring and organization of SAMs can be obtained. For SAMs the contact angles have been shown to reflect the properties of the outermost part of the monolayer only, functional groups buried below 5 Å from the exposed surface does not affect the contact angles with water.⁵³

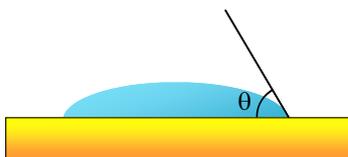


Figure 4.2. Schematic picture of a contact angle measurement.

4.3 Infrared Reflection Absorption Spectroscopy

Infrared reflection absorption spectroscopy (IRAS) can be used for characterizing thin films adsorbed on reflecting surfaces, for example metals.⁵⁴⁻⁵⁶ With IRAS it is possible to gain information on included functional groups, orientation, conformation and interactions like hydrogen bonds. In practice, the infrared light is reflected off the surface and the absorption is measured. The absorption is the result of excitation of specific molecular vibrations within the thin film. This type of excitation can only occur if the dipole moment of the molecular vibration is changed during the vibrational motion. The vibrational modes of a molecule are dependent on the constituents and their surrounding and therefore all molecules have unique vibration spectra.

A schematic picture of the reflection of infrared light at the surface is shown in Figure 4.3 below. The incident light beam can be divided into the two components, the light polarized parallel (p) and perpendicular (s) to the plane of incidence. Upon reflection, off the metal surface, the light will be phase shifted. The s-polarized light is phase shifted $\sim 180^\circ$ for all angles of incidence resulting in cancellation of the incident and reflected s-polarized light at

the surface. The phase shift upon reflection of the p-polarized light, however, is strongly dependent on the angle of incidence, θ . For high angles of incidence the phase shift is close to 90° and the incident and reflected beams interact constructively with each other yielding a strong electric field, E_R , oriented perpendicular to the surface.

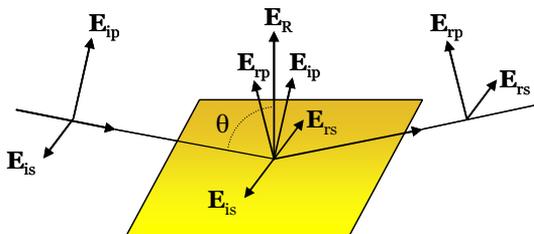


Figure 4.3. Schematic picture of the parallel (p) and perpendicular (s) components of the incident (i) and reflected (r) light at the surface in IRAS.

Due to the cancellation of the s-polarized component at the surface only vibrational modes with a component of the transition dipole moment perpendicular to the surface will be seen in IRAS. This is called the surface selection rule (Eq 4.3):

$$I \propto |E \cdot M|^2 = |E|^2 |M|^2 \cos^2 \psi \quad (\text{Eq. 4.3})$$

As follows by the equation, the absorption intensity for a vibrational mode will depend on the angle Ψ between the electric field, E , and the transition dipole moment M . Hence, information regarding the molecular orientation can be elucidated from IRAS measurements.

4.4 Quartz Crystal Microbalance

A quartz crystal microbalance (QCM) instrument functions as an exceptionally sensitive scale, capable of detecting small mass changes in real time without the need for molecular labelling. The sensor is made up of a quartz crystal sandwiched between two electrodes, usually gold. The technique is based on the converse piezoelectric effect meaning that when an electric field difference is applied over a piezoelectric element, here the quartz crystal, it

will result in a deformation, as seen in Figure 4.4, where the displacement direction is dependent on the applied potential. If instead an alternating voltage is applied over the two electrodes the crystal is made to mechanically oscillate. The crystal will be at resonance when the thickness of the crystal is an odd integer of the wavelength of the induced shear wave with anti-nodes present at the electrode surfaces. The resonance frequency is given by Equation 4.4 below where n is the overtone number, v_p the propagation velocity of acoustic waves in quartz and d_q the thickness of the crystal.⁵⁷ The $n=1$ mode is called the fundamental frequency and this is the parameter measured in all QCM experiments in this thesis. The crystals commonly used are so called AT-cut since they are relatively temperature stable within the interval used for biosensor measurements.

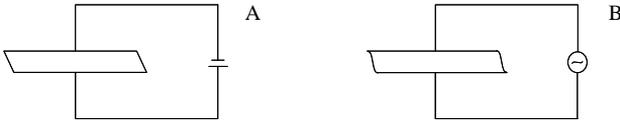


Figure 4.4. Picture of the displacement of the quartz crystal when a potential difference is applied. A) a DC current and B) an AC current.

$$f_n = \frac{v_p n}{2d_q} \quad (\text{Eq. 4.4})$$

As something adsorbs to the crystal the thickness of the crystal will increase and consequently the frequency will change, as given by Equation 4.4. In air and vacuum the observed frequency change was shown by Sauerbrey⁵⁸ in 1959 to be proportional to the mass change as follows (Eq. 4.5):

$$\Delta f = \frac{-2f_0 \Delta m}{A \sqrt{\mu_q \rho_q}} \quad (\text{Eq. 4.5})$$

Δf and Δm corresponds to the frequency shift and mass change respectively, while f_0 denotes the frequency of the crystal prior to adsorption, A the electrode area, μ_q the piezoelectric stiffened shear modulus of quartz and ρ_q the density of quartz. Equation 4.5 is however only valid for thin, rigid and uniform films. For in situ measurements water associated with the

bound molecules will also affect the signal leading to overestimations of adsorbed mass if the Sauerbrey equation is used.^{58,59} Other studies, however, have shown that the linearity between frequency and mass is essentially retained for studies of biomolecules although with a different coefficient for the frequency–mass relationship depending on the biological system.⁵⁹ Following the work of Nomura in 1982⁶⁰ who showed that the crystal can be used when immersed in liquid the application areas for QCM has increased enormously. Except for monitoring mass changes on surfaces the QCM can give information on affinity, kinetics from the binding curves, and it has been used for studying protein interactions, lipids, DNA hybridization, bacteria and cells. In addition to measuring the resonance frequency, the energy loss, or dissipation can be monitored to provide information of the viscoelastic properties of the adlayer. Dissipation monitoring has been found valuable for studies of biointerfaces by assisting analysis of complex events occurring at such surfaces.⁶¹

5 Summary

In this thesis, methods for preparation of carboxylated SAM sensor surfaces have been carefully examined in view of preparation conditions and thiol chemical structure to yield sensor surfaces with reproducible immobilization levels, low non-specific binding and long storage stability. Methods for immobilization of antibodies on SAM sensor surfaces have been optimized and the use of EDC/sulfo-NHS reagents has been proven to increase immobilization levels significantly. Sulfo-NHS mediated amine coupling is clearly a good alternative when the attraction between the surface and the protein is low, such as for two dimensional surfaces with a limited amount of carboxyl groups.

For the preparation of carboxylated SAMs for sensor surfaces, acidification of solutions containing carboxyl terminated thiols will result in improved structural organization of the SAMs both in the alkyl and EG subunits. However, the added acid will result in a conversion of the carboxyl groups to an ethyl ester making it a strategy which should be used with care. The use of a weaker acid will lower the conversion rate and is therefore recommended.

The results obtained for the OEG SAMs indicate that surfaces based on these molecules might have a great commercial potential. The non-specific binding was always low for all the systems investigated, which is encouraging for future testing with more complex fluids, for example serum samples. From a surface production and marketing perspective, the storage stability is of major importance. In this context, it was demonstrated that the investigated OEG thiols, containing an amide linker and a sufficiently long alkyl chain close to the sulfur, are long-term stable and thereby suitable as commercial biosensor surfaces.

6 Papers

Papers and graphs in this thesis are reproduced with permission from copyright holders.

Papers included in this thesis

Paper I

Optimizing immobilization on a two dimensional carboxyl biosensor surface: pH dependence of antibody orientation and antigen binding capacity

Anderson, H.; Myrskog, A.; Ingemarsson, B.; Pei, Z.

Submitted

Short description

A method for immobilization of antibodies on a two dimensional carboxyl surface was optimized. Factors affecting the immobilization were shown to be the surface pK_a , pI of the antibody and pH of the immobilization buffer. To improve immobilization EDC/sulfo-NHS was used as activation reagent. This was also shown to enable immobilization at very low pH, of importance for acidic proteins. The antigen binding capacity was shown to follow the immobilization level in most cases and the orientation of the antibodies on the surface proved to have a profound impact on the antigen binding capacity.

Author's contribution: Laboratory work in relation to development and optimization of immobilization method. Development and preparation of sensor surfaces. Minor part in manuscript preparation.

Paper II

Esterification of self-assembled carboxylic acid-terminated thiol monolayers in acid environment: A time dependent study

Myrskog, A.; Anderson, H.; Ingemarsson, B.; Liedberg, B.

Submitted

Short description

Carboxylic acid terminated SAMs are widely used as anchor layer for immobilizing molecules but the structure of these surfaces may be compromised due to the presence of a polar end group. To ensure optimal surface structure and thereby stability, different acid treatments were investigated with respect to effect on the structuring and functionality on two different carboxyl SAMs, an alkylthiol and an OEG-thiol. Improvements in the crystallinity, packing and orientation in both the alkyl and ethylene glycol subunits were seen due to the lower pH. However, the results showed rapid conversion of the carboxylic acids to ethyl esters. This was also shown to occur in the presence of a weaker acid at a much lower rate.

Author's contribution: All the laboratory work and writing.

Paper III

On the stability of carboxylic acid-terminated self-assembled monolayers: Influence of varying alkyl chain length

Myrskog A.; Ruželė Ž.; Anderson H.; Aastrup T.; Valiokas R.; Liedberg B.

In manuscript

Short description

In this paper the storage stability of three carboxylic acid terminated SAMs was investigated. The molecules had varying alkyl chain length and contained ethylene glycol units to minimize non-specific binding. Structural and functional changes due to storage were investigated. The alkyl chain length was shown to have a great impact on the structural order within these SAMs. The EG units adopt an unordered structure for the short alkyl chain thiol. The structure did not improve when HCl was added during monolayer formation. However, better organization in both the alkyl and ethylene glycol parts were achieved from acidified thiol solutions for the longer alkyl chain thiols. These structural differences were shown to have a profound impact on the surface stability but not on functionality. The short alkyl thiol was less stable over time and the binding capacity of this surface was reduced upon storage. Despite the structural differences the non-specific binding was low on all surfaces.

Author's contribution: All the laboratory work and writing, except for the synthesis of the three EG-thiols.

Papers not included in this thesis

Surface-confined photopolymerization of pH-responsive acrylamide/acrylate brushes on polymer thin films

Dunér, G.; Anderson, H.; Myrskog, A.; Hedlund, M.; Aastrup, T.; Ramström, O.

Langmuir 2008, 24, 14, 7559-7564

7 References

- [1] Thévenot, D. R.; Toth, K.; Durst, R. A.; Wilson, G. S., Electrochemical biosensors: Recommended definitions and classification. *Biosensors and Bioelectronics* **2001**, *16*, (1-2), 121-131.
- [2] Mrksich, M.; Whitesides, G. M., Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. *Annual Review of Biophysics and Biomolecular Structure* **1996**, *25*, 55-78.
- [3] Norde, W., Adsorption of proteins from solution at the solid-liquid interface. *Advances in Colloid and Interface Science* **1986**, *25*, (C), 267-340.
- [4] Wahlgren, M.; Arnebrant, T., Protein adsorption to solid surfaces. *Trends in Biotechnology* **1991**, *9*, (6), 201-208.
- [5] Wang, Z. H.; Viana, A. S.; Jin, G.; Abrantes, L. M., Immunosensor interface based on physical and chemical immunoglobulin G adsorption onto mixed self-assembled monolayers. *Bioelectrochemistry* **2006**, *69*, (2), 180-186.
- [6] Chapman, R. G.; Ostuni, E.; Takayama, S.; Holmlin, R. E.; Yan, L.; Whitesides, G. M., Surveying for Surfaces that Resist the Adsorption of Proteins. *Journal of the American Chemical Society* **2000**, *122*, (34), 8303-8304.
- [7] Holmlin, R. E.; Chen, X.; Chapman, R. G.; Takayama, S.; Whitesides, G. M., Zwitterionic SAMs that resist nonspecific adsorption of protein from aqueous buffer. *Langmuir* **2001**, *17*, (9), 2841-2850.
- [8] Ostuni, E.; Chapman, R. G.; Holmlin, R. E.; Takayama, S.; Whitesides, G. M., A Survey of Structure-Property Relationships of Surfaces that Resist the Adsorption of Protein. *Langmuir* **2001**, *17*, (18), 5605-5620.
- [9] Sullivan, T. P.; Huck, W. T. S., Reactions on monolayers: Organic synthesis in two dimensions. *European Journal of Organic Chemistry* **2003**, (1), 17-29.
- [10] Johnsson, B.; Lofas, S.; Lindquist, G., Immobilization of proteins to a carboxymethyl-dextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Analytical Biochemistry* **1991**, *198*, (2), 268-277.
- [11] Bonroy, K.; Frederix, F.; Reekmans, G.; Dewolf, E.; De Palma, R.; Borghs, G.; Declerck, P.; Goddeeris, B., Comparison of random and oriented immobilisation of antibody fragments on mixed self-assembled monolayers. *Journal of Immunological Methods* **2006**, *312*, (1-2), 167-181.
- [12] Creighton, T. E., *Proteins: structures and molecular properties*. 2:nd ed.; W.H. Freeman and Company: New York, 1993.
- [13] Nuzzo, R. G.; Allara, D. L., Adsorption of bifunctional organic disulfides on gold surfaces. *Journal of the American Chemical Society* **1983**, *105*, (13), 4481-4483.

- [14] Ulman, A., Formation and structure of self-assembled monolayers. *Chemical Reviews* **1996**, 96, (4), 1533-1554.
- [15] Love, J. C.; Estroff, L. A.; Kriebel, J. K.; Nuzzo, R. G.; Whitesides, G. M., Self-assembled monolayers of thiolates on metals as a form of nanotechnology. *Chemical Reviews* **2005**, 105, (4), 1103-1169.
- [16] Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D., Spontaneously organized molecular assemblies. 4. Structural characterization of n-alkyl thiol monolayers on gold by optical ellipsometry, infrared spectroscopy, and electrochemistry. *Journal of the American Chemical Society* **1987**, 109, (12), 3559-3568.
- [17] Bain, C. D.; Troughton, E. B.; Tao, Y. T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G., Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold. *Journal of the American Chemical Society* **1989**, 111, (1), 321-335.
- [18] Valiokas, R. Interfacial Design and Characterization of Oligo(ethylene glycol) Self-Assembled Monolayers. PhD Thesis no. 666, Linköping University, Sweden, 2000.
- [19] Arnold, R.; Azzam, W.; Terfort, A.; Wöll, C., Preparation, modification, and crystallinity of aliphatic and aromatic carboxylic acid terminated self-assembled monolayers. *Langmuir* **2002**, 18, (10), 3980-3992.
- [20] Wang, H.; Chen, S.; Li, L.; Jiang, S., Improved Method for the Preparation of Carboxylic Acid and Amine Terminated Self-Assembled Monolayers of Alkanethiolates. *Langmuir* **2005**, 21, (7), 2633-2636.
- [21] Bain, C. D.; Evall, J.; Whitesides, G. M., Formation of monolayers by the coadsorption of thiols on gold: Variation in the head group, tail group, and solvent. *Journal of the American Chemical Society* **1989**, 111, (18), 7155-7164.
- [22] Bain, C. D.; Whitesides, G. M., Formation of monolayers by the coadsorption of thiols on gold: Variation in the length of the alkyl chain. *Journal of the American Chemical Society* **1989**, 111, (18), 7164-7175.
- [23] Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M., Formation of self-assembled monolayers by chemisorption of derivatives of oligo(ethylene glycol) of structure HS(CH₂)₁₁(OCH₂CH₂)_mOH on gold. *Journal of the American Chemical Society* **1991**, 113, (1), 12-20.
- [24] Nelson, K. E.; Gamble, L.; Jung, L. S.; Boeckl, M. S.; Naeemi, E.; Golledge, S. L.; Sasaki, T.; Castner, D. G.; Campbell, C. T.; Stayton, P. S., Surface Characterization of Mixed Self-Assembled Monolayers Designed for Streptavidin Immobilization. *Langmuir* **2001**, 17, (9), 2807-2816.
- [25] Chen, S.; Li, L.; Boozer, C. L.; Jiang, S., Controlled chemical and structural properties of mixed self-assembled monolayers of alkanethiols on Au(111). *Langmuir* **2000**, 16, (24), 9287-9293.
- [26] Li, L.; Chen, S.; Jiang, S., Molecular-scale mixed alkanethiol monolayers of different terminal groups on Au(111) by low-current scanning tunneling microscopy. *Langmuir* **2003**, 19, (8), 3266-3271.

- [27] Prime, K. L.; Whitesides, G. M., Self-assembled organic monolayers: Model systems for studying adsorption of proteins at surfaces. *Science* **1991**, 252, (5010), 1164-1167.
- [28] Valiokas, R.; Svedhem, S.; Svensson, S. C. T.; Liedberg, B., Self-Assembled Monolayers of Oligo(ethylene glycol)-Terminated and Amide Group Containing Alkanethiolates on Gold. *Langmuir* **1999**, 15, (10), 3390-3394.
- [29] Vanderah, D. J.; Arsenault, J.; La, H.; Gates, R. S.; Silin, V.; Meuse, C. W.; Valincius, G., Structural Variations and Ordering Conditions for the Self-Assembled Monolayers of HS(CH₂CH₂O)₃₋₆CH₃. *Langmuir* **2003**, 19, (9), 3752-3756.
- [30] Harder, P.; Grunze, M.; Dahint, R.; Whitesides, G. M.; Laibinis, P. E., Molecular Conformation in Oligo(ethylene glycol)-Terminated Self-Assembled Monolayers on Gold and Silver Surfaces Determines Their Ability To Resist Protein Adsorption. *Journal of Physical Chemistry B* **1998**, 102, (2), 426-436.
- [31] Vanderah, D. J.; Valincius, G.; Meuse, C. W., Self-Assembled Monolayers of Methyl 1-Thiahexa(ethylene oxide) for the Inhibition of Protein Adsorption. *Langmuir* **2002**, 18, (12), 4674-4680.
- [32] Wang, R. L. C.; Kreuzer, H. J.; Grunze, M., Molecular Conformation and Solvation of Oligo(ethylene glycol)-Terminated Self-Assembled Monolayers and Their Resistance to Protein Adsorption. *Journal of Physical Chemistry B* **1997**, 101, (47), 9767-9773.
- [33] Herrwerth, S.; Eck, W.; Reinhardt, S.; Grunze, M., Factors that Determine the Protein Resistance of Oligoether Self-Assembled Monolayers - Internal Hydrophilicity, Terminal Hydrophilicity, and Lateral Packing Density. *Journal of the American Chemical Society* **2003**, 125, (31), 9359-9366.
- [34] Pertsin, A. J.; Grunze, M., Computer simulation of water near the surface of oligo(ethylene glycol)-terminated alkanethiol self-assembled monolayers. *Langmuir* **2000**, 16, (23), 8829-8841.
- [35] Li, L.; Chen, S.; Zheng, J.; Ratner, B. D.; Jiang, S., Protein Adsorption on Oligo(ethylene glycol)-Terminated Alkanethiolate Self-Assembled Monolayers: The Molecular Basis for Nonfouling Behavior. *Journal of Physical Chemistry B* **2005**, 109, (7), 2934-2941.
- [36] Zheng, J.; Li, L.; Chen, S.; Jiang, S., Molecular simulation study of water interactions with oligo (ethylene glycol)-terminated alkanethiol self-assembled monolayers. *Langmuir* **2004**, 20, (20), 8931-8938.
- [37] Prime, K. L.; Whitesides, G. M., Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): A model system using self-assembled monolayers. *Journal of the American Chemical Society* **1993**, 115, (23), 10714-10721.
- [38] Benesch, J.; Svedhem, S.; Svensson, S. C. T.; Valiokas, R.; Liedberg, B.; Tengvall, P., Protein adsorption to oligo(ethylene glycol) self-assembled monolayers: Experiments with fibrinogen, heparinized plasma, and serum. *Journal of Biomaterials Science, Polymer Edition* **2001**, 12, (6), 581-597.

- [39] Tam-Chang, S. W.; Biebuyck, H. A.; Whitesides, G. M.; Jeon, N.; Nuzzo, R. G., Self-assembled monolayers on gold generated from alkanethiols with the structure RNHCOCH₂SH. *Langmuir* **1995**, 11, (11), 4371-4382.
- [40] Valiokas, R.; Östblom, M.; Svedhem, S.; Svensson, S. C. T.; Liedberg, B., Thermal stability of self-assembled monolayers: Influence of lateral hydrogen bonding. *Journal of Physical Chemistry B* **2002**, 106, (40), 10401-10409.
- [41] Schoenfisch, M. H.; Pemberton, J. E., Air stability of alkanethiol self-assembled monolayers on silver and gold surfaces. *Journal of the American Chemical Society* **1998**, 120, (18), 4502-5413.
- [42] Willey, T. M.; Vance, A. L.; Van Buuren, T.; Bostedt, C.; Terminello, L. J.; Fadley, C. S., Rapid degradation of alkanethiol-based self-assembled monolayers on gold in ambient laboratory conditions. *Surface Science* **2005**, 576, (1-3), 188-196.
- [43] Hutt, D. A.; Leggett, G. J., Influence of adsorbate ordering on rates of UV photooxidation of self-assembled monolayers. *Journal of Physical Chemistry* **1996**, 100, (16), 6657-6662.
- [44] Cooper, E.; Leggett, G. J., Static secondary ion mass spectrometry studies of self-assembled monolayers: Influence of adsorbate chain length and terminal functional group on rates of photooxidation of alkanethiols on gold. *Langmuir* **1998**, 14, (17), 4795-4801.
- [45] Yang, G.; Amro, N. A.; Starkewolfe, Z. B.; Liu, G. Y., Molecular-level approach to inhibit degradations of alkanethiol self-assembled monolayers in aqueous media. *Langmuir* **2004**, 20, (10), 3995-4003.
- [46] Poirier, G. E.; Herne, T. M.; Miller, C. C.; Tarlov, M. J., Molecular-scale characterization of the reaction of ozone with decanethiol monolayers on Au(111). *Journal of the American Chemical Society* **1999**, 121, (41), 9703-9711.
- [47] Lee, M. T.; Hsueh, C. C.; Freund, M. S.; Ferguson, G. S., Air oxidation of self-assembled monolayers on polycrystalline gold: The role of the gold substrate. *Langmuir* **1998**, 14, (22), 6419-6423.
- [48] Han, S.; Kim, C.; Kwon, D., Thermal/oxidative degradation and stabilization of polyethylene glycol. *Polymer* **1997**, 38, (2), 317-323.
- [49] Jans, K.; Bonroy, K.; DePalma, R.; Reekmans, G.; Jans, H.; Laureyn, W.; Smet, M.; Borghs, G.; Maes, G., Stability of Mixed PEO-Thiol SAMs for Biosensing Applications. *Langmuir* **2008**, 24, (8), 3949-3954.
- [50] Tengvall, P.; Lundström, I.; Liedberg, B., Protein adsorption studies on model organic surfaces: An ellipsometric and infrared spectroscopic approach. *Biomaterials* **1998**, 19, (4-5), 407-422.
- [51] McCrackin F.L., *A FORTRAN program for the analysis of ellipsometer measurements*. NBS Technical Note 479: Washington DC, 1969.

- [52] Israelachvili, J., *Intermolecular and surface forces*. 2:nd ed.; Academic Press: London, 1992.
- [53] Bain, C. D.; Whitesides, G. M., Depth sensitivity of wetting: Monolayers of ω -mercapto ethers on gold. *Journal of the American Chemical Society* **1988**, 110, (17), 5897-5898.
- [54] Francis, S. A.; Ellison, A. H., Infrared spectra of monolayers on metal mirrors. *Journal of the Optical Society of America* **1959**, 49, (2), 131-138.
- [55] Greenler, R. G., Infrared study of adsorbed molecules on metal surfaces by reflection techniques. *The Journal of Chemical Physics* **1966**, 44, (1), 310-315.
- [56] Umemura, J., In *Handbook of Vibrational Spectroscopy: Sampling techniques*, Chalmers, J. M.; Griffiths, P. R., Eds. John Wiley & Sons: Chichester, 2002; Vol. 2, pp 982-998.
- [57] Janshoff, A.; Galla, H. J.; Steinem, C., Piezoelectric mass-sensing devices as biosensors - An alternative to optical biosensors? *Angewandte Chemie - International Edition* **2000**, 39, (22), 4004-4032.
- [58] Sauerbrey, G., Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur mikrowägung. *Zeitschrift für Physik* **1959**, 155, 206-222.
- [59] Muratsugu, M.; Ohta, F.; Miya, Y.; Hosokawa, T.; Kurosawa, S.; Kamo, N.; Ikeda, H., Quartz crystal microbalance for the detection of microgram quantities of human serum albumin: relationship between the frequency change and the mass of protein adsorbed. *Analytical Chemistry* **1993**, 65, (20), 2933-2937.
- [60] Nomura, T.; Okuhara, M., Frequency shifts of piezoelectric quartz crystals immersed in organic liquids. *Analytica Chimica Acta* **1982**, 142, (C), 281-284.
- [61] Höök, F.; Kasemo, B., In *Piezoelectric Sensors*, Steinem, C.; Janshoff, A., Eds. Springer: Berlin, 2006.