Synthesis of Substituted Alkanethiols Intended for Protein Immobilization

-Chelate Associated Photochemistry (CAP)

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During the course of the research underlying this thesis, Lan Bui was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.
To those who believed in me............
ABSTRACT

The first and main part of this thesis is focused on the design and synthesis of photo-activable and metal chelating alkanethiols. Chelate associated photochemistry (CAP) is a novel concept of combining two well-known protein (ligand) immobilization strategies to obtain a sensor surface of covalently bound ligand with defined orientation. This includes nitrilotriacetic acid (NTA) which is used to capture and pre-orientate histidine-tagged proteins to the sensor surface, followed by UV activation of a neighboring photo-crosslinking agent, benzophenone (BP), to covalently bind the ligand in this favorable orientation. Our results (paper 1) indicate that up to 55% more activity of the ligand is achieved with the CAP concept compared to the activity of the randomly oriented ligand (immobilized only by BP). This also yields a surface that is more robust compared to if only NTA is used. The photo cross-linking with benzophenone (BP) adduct is limited to a distance range of 3Å, it is therefore favorable to capture the ligand before reacting with surface bound BP-adduct. The surface consists of an excess of ethylene glycols (known for its protein-repellent properties) to prevent non-specific protein binding, thereby increase the specificity of the sensor surface. With this obtained surface chemistry we hope to contribute to the development of large-scale screening systems and microarrays based on His-tagged labeled biomolecules. This will be used in a number of applications such as proteomics-related applications, including drug discovery, the discovery of lead compounds and characterization of protein-protein interactions.

The second part of this thesis describes the effect of the synthetic N-(3-oxododecanoyl)-L-homoserine lactone (30-C12-HSL) on eukaryotic cells. 30-C12-HSL is a natural occurring signal substance in the bacterium Pseudomonas aeruginosa, and this signal molecule is involved in the regulation of bacterial growth. Pseudomonas aeruginosa has been considered as a common cause of infections in hospitals, especially in patients with compromised immune systems. Since the 30-C12-HSL can diffuse freely cross the cell membranes, it is expected to have influence on the host cell behaviour. Herein, we study how the eukaryotic cells respond to the bacterial signal molecule, 30-C12-HSL. Our results (paper 2) indicate that 30-C12-HSL disrupt the adherens junctions in human epithelial cells. The disruption is caused by a hyperphosphorylation of the adherens junction proteins (protein complex between epithelial tissues). This suggests the bacterial signals are sensed by that the host cells.
List of papers

Paper 1

Title: Oriented protein immobilization by chelate associated photochemistry

Authors: Emma Ericsson, Lan Bui, Peter Konradsson, Karin Enander, Bo Liedberg

Journal: In manuscript

Author’s contribution: Design, synthesis, and characterization of CAP structures. Performed self-assembly on gold substrates, surface characterization and some studies of photo cross-linking on ellipsometry.

Paper 2

Title: The junctional integrity of epithelial cells is modulated by Pseudomonas aeruginosa quorum sensing molecule through phosphorylation-dependent mechanisms

Authors: Elena Vikström, Lan Bui, Peter Konradsson and Karl-Eric Magnusson


Author’s contribution: Synthesis of 30-C12-HSL.
Abbreviations

ACN  Acetonitrile
BP   Benzophenone
CAP  Chelate associated photochemistry
DCM  Dichloromethane
DIPCDI Diisopropylcarbodiimide
DIPEA Diisopropyl ethylamine
DMF  N, N-Dimethylformamide
EDC  1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
HOAc Acetic acid
HOBt 1-hydroxybenzotriazole
HPLC High performance liquid chromatography
IRAS Infrared reflection absorption spectroscopy
KSAc Potassium thioacetate
MALDI Matrix assisted laser desorption/ionization
MeOH Methanol
MsCl Methane sulfonylchloride
NaOMe Sodium methoxide
NMR Nuclear magnetic resonance
NTA Nitrilotriacetic acid
OEG Oligo ethylene glycol
EtOAc Ethylacetate
SAM Self-assembled monolayer
SPR Surface plasmon resonance
TEA Triethylamine
TFA Trifluoroacetic acid
TLC Thin layer chromatography
UV Ultraviolet
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General Introduction

Organic chemistry is the study of compounds composed of carbons, which includes almost everything around us including ourselves, vitamins, proteins, medicines and a large number of other everyday essentials. Organic chemical reactions occur continuously in nature, creating molecules and complexes, often referred as biosynthesis (reactions within a living organism). The coupling of amino acids with each other to form peptides and proteins is one of many examples. Research within organic chemistry has led to new discoveries of both naturally occurring and synthetic materials, and it plays a central role in medicine, bioengineering, biotechnology, material science and other disciplines. The first synthesis of an organic molecule was urea, by Friedrich Wöhler in 1828 and since then, synthesis of organic molecules has continued¹. Today, synthetic chemists can synthesize extremely complicated structures, but are still far from being as effective and precise in our synthetic methods as nature. Most of the medicines on the market today are synthesized by chemists. But still, we got most of our inspirations from nature. The reasons why we use synthetic molecules instead of the natural occurring materials are: nature does not make substances in high enough quantities, to possibly replace existing materials with more environmental friendly materials, find compounds with better stability and functionality, and to find new unknown structures with interesting activity. Synthetic organic chemistry involves design, synthesis, and analysis of a target molecule for different applications. Medicinal chemistry, which involves synthesis of compounds, is one of the biggest research areas².

Chemistry today is often involved in projects between the boundaries of chemistry and its connections with other sciences, such as biology, environmental science, mathematics, biotechnology and physics. For understanding the main causes of infectious diseases, microbiologists study the nature of infections, mainly caused by bacteria, viruses, fungi and parasites. Organic synthesis of signal substances (paper 2) from bacteria can be used to verify the mechanisms of disease and illness and to determine how the substance affects our body and hence find ways to cure the disease.

Another example of using synthetic organic molecules is the creation of new materials and to modify solid surfaces to obtain desired properties and controlled surface chemistry such as wettability, protein repellency, and adhesion. The ability to assemble and organize organic molecule on solid substrates has been utilized to develop novel sensing materials.
Alkanethiols form very organized monolayers on gold substrates. The monolayer can then serve as an interface layer between a metal surface and a species present in solution for molecular recognition (paper 1). This is applied in many areas such as drug screening, protein-protein interactions and in clinical diagnostics. The need of high-throughput analysis methods in pharmaceutical and biotechnology industries is substantial.

## 2 Biosensors

A biosensor consists of a recognition element (sensing surface), and a transducer (figure 1). The sensing interface consists of bound recognition molecules, ligands (e.g. enzymes, antibodies, receptor proteins) that recognize the target molecules (e.g. substrates, antigens, signal substances). The recognition event (e.g. changes in temperature, reflective index, color or absorbance) is converted into an electronic signal via suitable transducers.

![Figure 1. Schematic illustration of a biosensor technique. The analyte binds to the surface and the reaction generates a signal that is correlated to the concentration of analyte bound to the surface.](image)

In the development of biosensors, the immobilization of biomolecules at interfaces plays a crucial role. Glucose biosensor is one of the first biosensors commercialized, and it is one of the most commonly used biosensors in our daily life. An enzyme (glucose oxidase) is immobilized on the sensing surface that will break down the blood glucose and the signal is then converted to the amount of glucose in the blood. Biosensors are used in many areas for example, in drug discovery, environmental control of pollutants, food industry (detection of contaminants) and there is an overwhelming need to improve them. The current trend is miniaturization and mass production by applying micro- and nanofabrication techniques. Success in this endeavor depends on advancements in biology, biochemistry, chemistry and
physics. Therefore, interdisciplinary cooperation is essential for successful development of biosensors.

Proper surface chemistry is crucial in order to obtain highly stable, selective and sensitive sensor surfaces for studies on biomolecular interactions. The immobilization of the recognition molecule has to be site specific and controlled so that the corresponding analyte can be bound without steric restrictions. Non-specific adsorption of proteins must be minimized in order to ensure that the interaction of interest is the only one being monitored. Self-assembled monolayers (SAMs) have been frequently used to modify surfaces for various sensor applications for study of biomolecular interactions because they are easy to prepare and form well-defined thin films on gold surfaces.

3 Self assembled monolayers

Self-assembled monolayers (SAMs) are thin films of organic molecules adsorbed on metal surfaces. The most widely investigated SAM compounds are those with a sulfur-based anchoring group (mostly alkanethiols, dialkyl disulfides or thioether) that are assembled onto gold surfaces and was introduced by Nuzzo et al. in 1983. These organosulfur compounds are known to chemically adsorb spontaneously onto solid surfaces, especially gold, to form well-organized monolayers through the formation of gold-sulfur bonds. The sulfur head group is believed to bind as a thiolate to gold. Besides gold substrates, there are a number of other surfaces that can be used (e.g. silver, copper, aluminium and silica surfaces). The gold-sulfur system is one of the most studied SAMs, and gold is an inert metal that does not oxidize at room temperature with atmospheric O2 which make it stable to handle. The advantages of SAMs are the ease of preparation, stability and the possibility to introduce different chemical functionalities.
Alkanethiol self-assembly on gold is easy to perform and can be done both in liquid and gas phase\textsuperscript{15}. The adsorption is generally performed in 10-1000 μM solutions of thiols or disulfides in different solvents depending on the nature of the thiol. The adsorption of alkanethiols on gold is suggested to be divided into two steps (figure 2a). First the sulfur adsorbs onto gold, which takes just a few seconds to complete. The second step (orientation and ordering) takes hours up to days to complete, depending on the concentration of the solution, the solvent being used, the chain length of the alkanethiol and the functional group. The stability of the monolayer is governed by inter- and intramolecular forces within the film. SAMs provide as model surfaces a useful tool for studying the physical-organic chemistry of biomolecular recognition\textsuperscript{3}. This gives them a wide range of applications in many areas of modern material science. Some examples are within bio- and chemical sensing, wetting control, and microelectronics\textsuperscript{16}. There are a number of techniques, e.g. surface plasmon resonance\textsuperscript{17} (SPR), ellipsometry\textsuperscript{18} and infrared reflection absorption spectroscopy (IRAS)\textsuperscript{19,20}, available for analyzing the SAM composition, mass coverage of the surface and thermodynamics of binding events.

Figure 2. (a) Schematic illustration of SAM preparation. The clean gold substrate is incubated in the ethanol solution containing thiols. The adsorption step is fast (seconds), but the organization step can take hours-days to give a well ordered monolayer. (b) Structure of monolayer.
3.1 Protein resistance of ethylene glycols

Proteins adsorb spontaneously onto solid surfaces when coming into contact via intermolecular forces, mainly ionic bonds or hydrophobic polar interactions\textsuperscript{21}. The protein-surface interaction depends on the surface properties and the molecular properties of the protein (e.g. its size, shape, charge and structural stability\textsuperscript{22, 23}). The spontaneous adsorption of proteins is not desirable in design and preparation of surface coatings that requires high reliability and reproducibility. Nonspecific adsorption of proteins must be minimized in order to ensure that the interaction of interest is the one being monitored. It can cause major problems in applications where the analyte concentration is low in presence of nonspecifically bound molecules\textsuperscript{6}. Prime et al\textsuperscript{24} introduced oligo ethylene glycol (OEG) for SAM surface modifications to prevent nonspecific adsorption of proteins and cells and it has been widely investigated during the last two decades\textsuperscript{25}.

\[ \text{Figure 3. General structure of ethylene glycol.} \]

The protein resistant properties of the alkane thiol monolayers on gold SAMs depend mostly on the hydrophobic interactions with proteins. The protein resistance increases with increased hydrophilicity\textsuperscript{26} of the monolayer, and with increasing length of the OEG tail group. Also the functional groups (–OH, -CH\textsubscript{3}, -CH\textsubscript{2}CH\textsubscript{3} etc) of the OEG tail have a large influence on the surface ability to resists protein adsorption. Besides the inner hydrophilicity, the terminal hydrophilicity and wettability are also important factors for protein adsorption. For contact angles higher than 70°, an increase of protein adsorption is observed\textsuperscript{23}. The properties of protein resistant monolayers can be summarized into five characteristics: they should (1) be hydrophilic (2) include hydrogen-bond acceptors (3) not include hydrogen-bond donors (4) their overall electrical charge should be neutral and (5) water must be able to penetrate the monolayer\textsuperscript{27}. 
3.2 Protein immobilization

The most challenging step in biosensor development is the immobilization step of the recognition molecules\(^{28}\). The sensor surface should be of suitable material such as a noble metal and the organic film have protein resistant functionalities. The proteins immobilized for the sensing event should be oriented in such way that the binding sites are exposed to the analytes in the sample solution\(^{21}\) and with maintained activity. There are large varieties of protein immobilization strategies and they can be classified into three categories: physical adsorption, covalent and bioaffinity immobilization\(^{6}\).

Covalent immobilization of biomolecules to the surface is usually prepared by coupling to a NHS-ester activated carboxylic acid functionalized surface\(^{29}\). The coupling occurs with an amine group anywhere on the biomolecule to form a stable amide bond. The covalent immobilization provides long term stability of the biomolecule with high coverage density, but it can also lead to denaturation of the proteins\(^{6}\). The coupling usually occurs with unmodified proteins, which have several functional groups that can react with the NHS-activated surface. The immobilization will be random and the activity reduced\(^{30}\). Drawbacks of covalent immobilization methods are that the proteins are often randomly oriented and partly denaturated\(^ {31}\). The non-covalent immobilization occurs either by electrostatic, hydrophobic or hydrophilic interactions between the surface and the biomolecule. The third immobilization strategy is by affinity interaction, often between antigen–antibody pairs.
3.2.1 Non covalent immobilization using affinity chemistry

Immobilized metal ion affinity chromatography (IMAC) was first introduced by Porath et al\textsuperscript{32} in 1975 and is nowadays a standard technique for protein purification\textsuperscript{21}. Nitrilotriacetic acid (NTA) is one of the most commonly used chelating agents and is conjugated to a solid (chromatographic resins or magnetic beads). The NTA moiety is chelated to transition metals like Ni\textsuperscript{2+}, Co\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+}. NTA occupies four of the six possible co-ordination sites (figure 5), which leaves the remaining two sites for any electron-donating groups such as histidines\textsuperscript{33, 34}. A protein mixture is passed through the column, where proteins of interest, modified with a histidine tag have high affinity for the NTA/Ni\textsuperscript{2+} complex\textsuperscript{35}.

![Figure 5. NTA coordinates metal ions (M\textsuperscript{2+}) through nitrogen and oxygen in tentradentate fashion, giving a complex with high affinity for histidine tags.](image)

The use of IMAC-like approaches for the immobilization of proteins on NTA-tailored surfaces has previously been used to capture proteins on solid surfaces for various sensor applications\textsuperscript{35}. This protein immobilization technique is fully reversible by the addition of competitive compounds (like imidazole or histidine)\textsuperscript{36} and it is based on the specific interactions between the biological target molecule and NTA-tailored surface. The protein is attached in a specific manner, with a favorable orientation for biomolecular interactions with an analyte in solution\textsuperscript{27}. The reversibility of this immobilization strategy is a disadvantage in applications of biosensor interface development. The attached recognition molecules are not sufficiently stable to withstand washing, regeneration and storage of such sensor surfaces.
3.2.2 Covalent immobilization using photo cross-linking chemistry

Upon activation with light at a defined wavelength, photo cross-linkers convert to highly reactive species (radicals) that can covalently link to a neighboring molecule\(^\text{37}\). There are many photo-activable cross-linking agents developed for protein immobilization on the market\(^\text{38}\). Most of them contain aryl diazirines, aryl azides, or benzophenone derivatives\(^\text{39}\). Benzophenone as photo cross-linking agents has several advantages\(^\text{40}\). Firstly, they are chemically more stable than diazo esters, aryl azides, and diazirines. Secondly, they can be manipulated in ambient light and can be activated at 350-360 nm, avoiding protein damaging wavelengths\(^\text{41}\). Thirdly, they react preferentially with unreactive C-H bonds (within a radius of 3.1 Å centered on the keton oxygen\(^\text{42}\)), even in the presence of solvent, water and bulk nucleophiles.

The BP cross-linking reaction is initiated by UV light at 350-360 nm, producing a very reactive diradical species (figure 6). The insertion of biradical BP moiety into C-H bond occurs in two steps. First, a hydrogen radical is abstracted (H-abstraction), which is dependent on the distance between the ketyl radical and C-H bond. Formation of the diradical is reversible, if there is nothing it can form a covalent bond with, it can regenerate to its initial structure\(^\text{43}\). The second step is the recombination of alkyl and ketyl free radicals, generated by H-abstraction, into a covalent bond\(^\text{44}\).

There are different ways to use the BP-photo cross-linking approach. The BP can either be bound to the surface\(^\text{45}\) and photo cross-linked to the biomolecule in solution, or they can be attached on biomolecules in solution\(^\text{46}\) and photo cross-linked to the surface. The main drawback with BP photo cross-linking is the lack of specificity, since the reaction occurs anywhere on the protein. Attachment of the recognition molecule may occur too close to the
binding site or in some cases it may denature the protein. In biosensor applications, this could lead to reduced activity, reduced sensitivity and low responses of the sensor signal.

3.2.3 Chelate associated photochemistry

The biomolecules are usually bulky and the common approach to control the surface density is by the use of a large excess of an inert filler molecule (often ethylene glycols)\(^\text{47}\), illustrate in figure 7. Long chain organic alkanethiol molecules chemically adsorb onto gold surfaces spontaneously and form densely packed monolayers\(^\text{48}\). The alkanethiol head-groups can be modified into any functionalities of interest to get the desired chemical properties. To get a surface with sensing properties, the recognition molecule has to be attached in such a way that their three-dimensional structure, functionality and binding sites are retained\(^\text{49,35}\). Chelate associated photochemistry (CAP) is a combination of the both immobilizations strategies mentioned above (section 3.2.1-3.2.2). With this approach, the recognition molecules can be immobilized in an addressable way with high specificity.

![Figure 7. Schematic structure of CAP surface](attachment:figure7.png)
The goal of this surface chemistry was to develop a robust immobilization strategy for covalent attachment of biomolecules onto a solid surface for development of large-scale screening systems, microarrays and proteomic applications. The basic idea was to combine the two well known strategies of metal chelating chemistry of nitrilotriacetic acid (NTA)\textsuperscript{50} to pre-orient the recognition molecules and finally photo cross-link them to the surface using a nearby photo-labile group, benzophenone (BP)\textsuperscript{51}, to obtain a chelated-associated photochemistry (CAP) surface (figure 8).

Figure 8. Schematic illustration of the chelate associated photochemistry (CAP) approach of site specific immobilization of proteins. (a) Ni\textsuperscript{2+}-loaded CAP surface is exposed to a mixture of proteins. (b) Rinsing to remove nonspecific binding proteins. (c) Covalent linkage to the pre oriented protein was induced by UV activation of the Photoreactive benzophenone group. (d) Control of the surface activity is performed with an analyte with affinity to the bound protein.

The recognition molecule is modified with a histidine-tag (6-10 histidine residues) to the C or N terminal. A mixed monolayer containing filler molecules with OEG terminated alkanethiolates will prevent nonspecific protein binding. NTA functional groups will be loaded with Ni\textsuperscript{2+} containing buffer before the His-tagged ligand is passed through and captured. Running buffer is used to wash away any excess of proteins. Irradiation at ~360 nm initiates the photochemical crosslinking and the protein is covalently bound to the surface.
4 Synthesis of monolayer building blocks

The different lengths of OEG in the filler molecule, NTA, and the photo-labile group in these monolayers have a substantial effect on reactivity and accessibility of the recognition molecule to the surface. Triethylene glycol was used as a linker for the filler molecule and the NTA-derivative. The photo-labile group (BP) is composed of a longer ethylene glycol (EG₃) linker (OEG₈) to be able to “reach out” to the biomolecule. The goal was that the BP moiety will react close to or on the histidine tag to ensure that the binding does not interfere with the binding site of the protein.

4.1 Filler molecule

The synthetic strategies for modification of ethylene glycols (2-7) have been reported earlier by Svedhem et al52 (Scheme 1). The protected thiol (9) was first introduced by a S_N2 reaction with potassium thioacetate (KSAc) in DMF. The reaction was straightforward in room temperature for 1 hour (scheme 1). This alkyl chain was used as building block for all alkanethiols and disulfides.

Synthesis of filler molecule (11) was based on amide coupling between acetyl-protected 12-mercaptododecanoic acid (8) to an amine functionalized OEG (4). Deprotection of 10 using NaOMe in methanol gave the OEG₃-alkanethiol (11) upon neutralizing with Dowex.
Scheme 1. Synthesis strategy for OEG-terminated alkanethiol and disulfide.

4.2 Benzophenone derivative

Benzophenone terminated alkanethiol (18) was synthesized for photo-immobilization of biomolecules on surfaces (scheme 2). 4-hydroxybenzophenone 13 was first coupled to tetraethylene glycol via a Mitsunobu-like coupling followed by an elongation with a mesylated tetraethylene glycol (5) at the hydroxy end. Reduction of the azide 15 using PPh3 in methanol gave BPEG8NH2 (16). Amide coupling to acetyl-protected 12-mercaptopdodecanoic acid (8) followed by deprotection with NaOMe in methanol resulted in the BP-thiol (18).
4.3 Nitrilotriacetic acid derivative

The synthesis of the NTA-derivative (scheme 3) was performed in a mixture of solvents (EtOH, acetone and DCM), because of the difference of polarity of NTA moiety and the alkane chain moiety (21). Deprotection of the Tert-butyl group was performed in TFA. The carboxylic acid (21) was converted to NHS-ester by DCC and NHS in DCM. The urea-derivative precipitate was removed by filtration and reaction with \(N_{\alpha},N_{\alpha}-\text{Bis(carboxymethyl)-L-lysine hydrate} \) without further purification. Deprotection as earlier using NaOMe in methanol gave the NTA-thiol (23) as product.
4.4 Asymmetric disulfide

Scheme 4. Synthesis of BP/NTA, an asymmetric disulfide.

The asymmetric disulfide synthesized in two steps (scheme 4). First, the thiol group 18 was activated into a more reactive functional group using 2-aldrithiol. The thiol-ester was purified using preparative LCMS before reaction with 23. The disulfide 26 was purified using preparative LCMS.
5 Surface characterization techniques

In this section a brief description of the various surface characterization techniques that have been used in this thesis is given.

5.1 Water Contact angle

A contact angle is a quantitative measure of the wetting of a solid by adding a liquid drop (usually water) on a solid surface. The angle between the drop and the surface is called Young’s contact angle ($\theta$) and it depends on three interfacial tensions $\gamma$; liquid-vapor ($\gamma_{lv}$), solid-liquid ($\gamma_{sl}$) and solid-vapor ($\gamma_{sv}$) (figure 9). The dynamic sessile drop method was used, which measures both advancing ($\theta_a$) and receding ($\theta_r$) contact angles. The difference between these angles, (the hysteresis (H)), shown in eq.2, can be used to characterize surface heterogeneity, roughness, and mobility. On extremely hydrophilic surfaces the water droplet will spread out completely and the contact angle will be $0^\circ$.

\[
\cos \theta = \frac{\gamma_{sv} - \gamma_{sl}}{\gamma_{lv}} \quad \text{(eq. 1)}
\]

\[
H = \theta_a - \theta_r \quad \text{(eq. 2)}
\]

Figure 9. The angle between a liquid drop and a solid surface ($\theta$) is usually measured with water as liquid.

5.2 Null ellipsometry

Null ellipsometry is an optical method for determining film thickness on a flat surface. By measuring the change in the polarization state that occurs upon reflection of a polarized light beam, the refractive index and thickness of an adsorbed layer can be
measured. A typical setup of an ellipsometry experiment is sketched in figure 10. The sensitivity of an ellipsometer is such that a change in film thickness of a few Angstroms (Å) is usually easy to detect.

Figure 10. Illustration of ellipsometric setup.

5.3 Surface plasmon resonance

Surface plasmon resonance (SPR) is commonly used for analyses of biomolecular interactions in real time without the use of labeled molecules. SPR is an optical technique which monitors the changes of the refractive index of the monolayer coverage of the metal surface. The method is based on the reflection of a polarized laser beam at the interface of the metal layer and the monolayer. The sensorgram provides information on the interaction of analyte binding to surfaces.
6 Results

6.1 Surface characterization

The synthesized molecules were immobilized on gold substrates forming monolayers. The monolayers were characterized with ellipsometry, contact angle and infrared reflection absorption spectroscopy (IRAS). Mixed monolayers were used for the study of photochemistry and metal chelating chemistry. The surface plasmon resonance (SPR) technique was used to study protein immobilization and analyte interactions on mixed monolayers (BP/NTA/OEG). The evaluation of the photochemistry and the chelating chemistry each by them self to optimize their reaction parameters before examination of the combination, the CAP surface.

6.2 Protein photo-crosslinking to benzophenone containing monolayer

The film thickness of photo cross-linked His-tagged ligand to a benzophenone containing monolayer was measured by ellipsometry. A clean gold substrate was incubated in an ethanol solution containing disulfides of BP and OEG (20 µM respectively 80 µM) for at least 24 hours. Film thickness was measured before incubation with ligand in a buffer solution for 10 minutes. After irradiation or incubation in darkness, the surface was rinsed with buffer and milli-Q water and dried with nitrogen. An increase in film thickness (4.5-7 Å) was obtained when the monolayer was incubated in protein solution followed by an irradiation (360 nm) time of 10 seconds to 30 minutes (figure 11). As a control, the same experiments were performed in the darkness, no increase in film thickness was observed, showing that the protein binding was not physically adsorbed, but covalently immobilized via photo cross-linking to the BP moiety on the surface. The increase in film thickness occurred after only 10 seconds of irradiation (360nm) which is good for the biosensor applications, since long irradiation times gives a higher temperature and can cause denaturation and damage of biomolecules.
Figure 11. Data from ellipsometry measurements of benzophenone-functionalized gold surfaces. The results show no increase in film thickness without UV irradiation.

6.3 Nitrilotriacetic acid chelating chemistry

Figure 12. Surface plasmon resonance sensorgram (SPR) showing injections of ligand, analyte and an elution buffer containing imidazole. The binding of ligand and analyte are reversible.

Binding of His-tagged ligand to NTA/Ni$^{2+}$ containing surface (2% NTA-thiol and 98% EG$_3$-thiol) was measured using a SPR technique. The step like responses (figure 12) correspond to changes in the refractive index for injections of ligand, analyte and an elution buffer containing imidazole. First, His-tagged ligand was captured on the surface which gives rise to the increase in response. The next step was an injection of the analyte and finally, both...
ligand and analyte was eluted from the surface upon injection of imidazole, which competes with the histidines in binding to the NTA/Ni\(^{2+}\)-complexes.

6.4 Chelate associated photochemistry

Immobilization of His-tagged ligand was investigation of CAP surface (containing 10% BP/NTA-disulfides and 80% EG\(_3\)-disulfides) with SPR technique. Results are showed in figure 13.

First, His-tagged ligand was captured by NTA/Ni\(^{2+}\), then irradiation with UV light at 360 nm, to photo cross-link the ligand to BP-moieties on the surface. Treatment with imidazole was performed to remove any noncovalent bound ligands. Analyte interaction studies were monitored on SPR. Two control experiments were performed, one where the ligand was immobilized via photo cross-linking and the other where only immobilized via coordination to NTA was used. CAP surface shows an increased analyte response, up to 55% compared to an analyte immobilized with photochemistry alone.

![Figure 13. SPR data, for binding of analyte to His-tagged ligand on a CAP surface. Analyte binding is higher with CAP (when both BP and NTA are used, solid line) than with either chemistry used by itself (BP dashed lines; NTA dotted lines).](image)
7 Synthetic molecule for bacterial signaling

*Pseudomonas aeruginosa* is a bacterium often found in soil, plants and animals, and they are the most common cause of infectious diseases in individuals with compromised immune systems, such as patients with cancer, severe burns or AIDS. *P. aeruginosa* can grow within a host without harm until they reach a certain concentration where they then cause infections. The infections are usually respiratory infections, urinary tract infections, osteomyelitis (infection in bone), and infections of burns and soft tissues. *P. aeruginosa* control their cell growth through a cell-density mechanism called quorum sensing, which is the density dependant regulation of gene expression. The infection caused by the bacteria is mostly dependant upon the cell-to-cell communication based on the quorum sensing mechanism. The quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) was synthesized (scheme 5) to study its effect upon eukaryotic cells. Evaluating the mechanisms of how the bacterial autoinducers manipulate gene expression in mammalian cells may yield new therapeutic methods for the prevention and treatment of bacterial infections.

![Scheme 5](image)

Scheme 5. Synthesis of the quorum sensing molecules 30-C12-HSL and 30-C10-HSL.

The synthetic strategy for 30-C10-HSL and 30-C10-HSL has been described elsewhere. The synthetic 3O-C12-HSL was used to study the cell communication human cells (paper 2). Especially the effect of 30-C12-HSL’s effect on the adhesion junction (AJ) associated proteins, E-cadherin and β-catenin. Degradation of both proteins was observed after 2 and 4 hours incubation in C12-HSL, but the effect was reversible. The level of E-cadherin and β-catenin could return to normal after 5 hours treatment. The degradation could be prevented by introducing protein phosphatase and kinase inhibitors.

Epithelial cells are joined to each other by forming complexes of proteins (E-cadherin and β-catenin). This junction is dependent on the level of phosphorylated tyrosine, serine and...
threonine. Treatment of 30-C12-HSL to eukaryotic cells causes delocalization, degradation and loss of expression of the junction proteins through phosphorylation of tyrosine, serine and threonine.
8 Conclusions and future perspectives

Photo-activable and metal chelating thiols and disulfides have been synthesized and characterized. In ethanol solutions, these thiols/disulfides spontaneously form monolayers on gold, with well-defined structures. With chelate associated photochemistry, the ligand was pre-orientated before cross-linking to the surface. Our results (paper 1) showed that with chelate associated photochemistry, we achieved up to 55% higher activity of the surface bound ligand than for ligand immobilized with benzophenone or NTA/Ni\(^{2+}\) alone. We have developed a technique for immobilization of biomolecules on solid surfaces. The immobilization is performed in two steps. First, pre-orientation of the biomolecule ensures that the binding site is fully exposed to the solution for analyte binding. Second, the ligand is photo cross-linked for covalent attachment of the biomolecule to the surface.

With the CAP surface chemistry, we contribute to the development of large-scale screening systems, microarrays, based on His-tagged labeled biomolecules. Additional improvement of the experimental setup is under development to enable real-time detection of the protein immobilization and the subsequent analyte interaction.

The surface chemistry needs to be evaluated further to obtain the optimal composition of the various components (BP/NTA/OEG) to increase the interaction with the analyte. The nonspecific binding needs to be reduced, which is necessary for the resolution and sensitivity of the biosensor interface.

The bacterial signaling molecule 30-C12-HSL was synthesized for the study of cell communication. This small molecule can freely diffuse across cell membrane in the bacterium and can be sensed by the host cells. Results (shown in paper 2) indicate that the 30-C12-HSL disrupt the adherens junctions in human epithelial cells. The mechanism is through hyperphosphorylation of the adherens junction (AJ) proteins, E-cadherin and β-catenin, which lead to dissociation of the AJ complex. This disruption is reversible. The levels of the both E-cadherin and β-catenin returned after 5 hours 30-C12-HSL-treatment and reached control level after 24 hours.

The use of organic synthesis can be applied in many areas of basic research, medicine and technology. Synthesis of both natural and synthetic compounds with different functionalities that can provide scientists tools to solve problems, modification of surfaces with a recognition molecules or small molecules for study of infectious deseases as shown here.
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10 References


