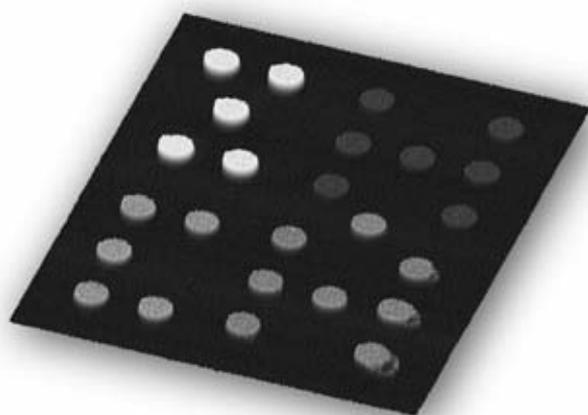


PROTEIN MICROARRAY CHIPS

Goran Klenkar



Division of Molecular Physics
Department of Physics, Chemistry and Biology
Linköping University, Sweden

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The cover page shows an image of the antibody loading of a microarray for the detection of four narcotics in parallel: ecstasy (X), heroin (H), cocaine (C), and amphetamine (A).

During the course of the research underlying this thesis, Goran Klenkar was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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Dedicated to my close ones

ABSTRACT

Life is a thing taken for granted by most. However, it is the life-long quest of many to unravel the mysteries of it. Understanding and characterizing the incomprehensively complex molecular interaction networks within a biological organism, which defines that organism, is a vital prerequisite to understand life itself. Already, there has been a lot of research conducted and a large knowledge has been obtained about these pathways over, especially, the last century. We have seen the fruits of these labors in *e.g.* the development of medicines which have been able to cure or at least arrest many diseases and conditions. However, many diseases are still incurable (*e.g.* cancer) and a lot more work is still needed for understanding them fully and designing successful treatments.

This work describes a generic analytical tool platform for aiding in more efficient (bio)molecular interaction mapping analyses; protein microarray chips. Microarray chips are surfaces with micrometer sized features with the possibility of studying the interactions of many (thousands to tens of thousands) (bio)molecules in parallel. This allows for a higher throughput of analyses to be performed at a reduced time and cost. Protein microarrays have been around for approximately a decade, following in the footsteps of the, so far, more successfully used DNA microarrays (developed in the 1990s). Microarrays of proteins are more difficult to produce because of the more complex nature of proteins as compared to DNA. In our work we have constructed model surfaces which allow for the stable, highly oriented, and functional immobilization of proteins in an array format. Our capture molecules are based on multivalent units of the chelator nitrilotriacetic acid (NTA), which is able to bind histidine-tagged proteins. Furthermore, we have explored an approach for studying lipid membrane bound systems, *e.g.* receptor-ligand interactions, in a parallelized, microarray format. The approach relies on the addressable, DNA-mediated adsorption of tagged lipid vesicles.

In an analogous work we have used the protein microarray concept for the detection of four common narcotics (heroin, amphetamine, ecstasy, and cocaine). The detection is based on the displacement of loosely bound antibodies from surface array positions upon injection of a specific target analyte, *i.e.* a narcotic substance. The proof-of-concept chip can easily be expanded to monitor many more narcotic substances. In addition, we have also been able to simultaneously detect the explosive trinitrotoluene (TNT) along with the narcotics, showing that the chip is a versatile platform for the detection of virtually any type of harmful or illegal compound. This type of biosensor system is potentially envisaged to be used in the fight against crime, terrorism, drug abuse etc.

Infrared reflection absorption spectroscopy together with ellipsometry has been used to characterize molecular layers used in the fabrication processes of the microarray features. Imaging surface plasmon resonance operating in the ellipsometric mode is subsequently used for functional evaluation of the microarrays using a well-defined receptor-ligand model system. This approach allows simultaneous and continuous monitoring of binding events taking place in multiple regions of interest on the microarray chip. A common characteristic of all the instrumentation used is that there is no requirement for labeling of the biomolecules to be detected, e.g. with fluorescent or radioactive probes. This feature allows for a flexible assay design and the use of more native proteins, without any time-consuming pretreatments.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Livet tas för givet av de flesta. Det finns däremot många som ägnar stora delar av sitt liv för att försöka lösa dess mysterier. En del av lösningen ligger i att förstå hur alla molekyler är sammanlänkade i det gigantiska nätverk som definierar den levande organismen. Under det senaste seklet har en hel del forskning utförts för att kartlägga dessa nätverk. Resultatet av dessa mödor kan vi se i de läkemedel som vi har idag och som har utvecklats för att bota eller åtminstone lindra olika sjukdomar och tillstånd. Dessvärre finns det fortfarande många sjukdomar som är obotliga (t.ex. cancer) och mycket arbete krävs för att förstå dem till fullo och kunna designa framgångsrika behandlingar.

Arbetet i denna avhandling beskriver en analytisk plattform som kan användas för att effektivisera kartlägningsprocessen; protein-mikroarrayer. Mikroarrayer är ytor som har mikrometerstora (tusendels millimeter) strukturer i ett regelbundet mönster med möjligheten att studera många interaktioner mellan biologiska molekyler samtidigt. Detta medför snabbare och fler analyser - till en lägre kostnad. Protein-mikroarrayer har funnits i ungefär ett decennium och har följt i fotspåren av de framgångsrika DNA-mikroarrayerna. Man bedömer att protein-mikroarrayerna har en minst lika stor potential som DNA mikroarrayerna då det egentligen är mer relevant att studera proteiner, som är de funktionsreglerande molekylerna i en organism. Vi har i detta arbete tillverkat modellytor för stabil inbindning av proteiner, som lämnar dem intakta, funktionella och korrekt orienterade i ett mikroarray format. Därmed har vi adresserat ett stort problem med protein mikroarrays, nämligen att proteiner är känsliga molekyler och har i många fall svårt att överleva tillverkningsprocessen av mikroarrayerna. Vi har även studerat en metod att tillverka mikroarrayer av proteiner bundna till strukturer, som modellerats att efterlikna cellytor. Detta är särskilt viktigt eftersom många (hälften) av dagens (och säkerligen framtidens) läkemedel är riktade mot att påverka denna typ av proteiner och att studera dessa i sin naturliga miljö är därför väldigt relevant.

I ett annat projekt har vi använt protein mikroarrayer för att detektera fyra vanliga droger (heroin, amfetamin, ecstasy och kokain). Detektionen baseras på användandet av antikroppar som lossnar från platser på ytan när de kommer i kontakt med ett narkotikum. Detta koncept kan enkelt utvecklas till att detektera mer än bara fyra droger. Vi har även lyckats att parallellt mäta förekomsten av en annan typ av förening på mikroarray ytan, nämligen det explosiva ämnet trinitrotoluen (TNT). Detta visar på en mångsidig plattform för detektionen av i princip vilken typ av farlig eller olaglig substans som helst - och på *en* yta! Vi föreställer oss därför att möjliga tillämpningsområden finns inom brottsbekämpning, i kampen mot terrorism och mot narkotikamissbruk etc.

Mikroarrayerna har i denna avhandling utforskats med optiska metoder som tillåter studie av omärkta proteiner, vilket resulterar i så naturliga molekyler som möjligt.

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During my five years at IFM there have been so many people who have helped me in ever so many ways. Now at the end I find it amusing reminiscing about this. First of all I would like to thank **Bo Liedberg**, my supervisor, for taking me under your wings and for helping me in becoming a researcher. It was you who introduced me to the world of surface physics and sparked my interest in the field. Thank you also for believing in me and for motivating me to continue when things seemed to stand still. Another inspiring person it was my honor to work with is **Ingemar Lundström**. Other persons aiding my development into becoming a researcher are: **Knut Johansen**, helping me in the beginning. **Ramūnas Valiokas** for coming to me with brilliant ideas of collaborations and for working together on many (most) of the included projects. **Jacob Piehler** and **Annett Reichel**, it was my pleasure to be able to work together with you - I have learned so much from you. **Per Månsson** and **Ann-Charlotte Hellgren** at Biosensor Applications AB, thank you for your assistance and giving me a chance to work with and develop your systems. **Thomas Ederth** has also been a big help. **Stefan Klintström**, director of Forum Scientium, has helped me a lot during my time at IFM at countless occasions. **Hans Arwin** for giving the Applied Optics course. **Bo Thunér** for helping me with technical issues (especially Tekla). **Pia Blomstedt** for helping me with administrative issues. **Agneta Askendal** deserves big thanks for helping me with practical lab-issues. **Choy-Hsien Li** and **Björn Brian** are diploma workers who have helped me with included projects. **Olle Andersson**, **Ye Zhou**, and **Andréas Larsson** are colleagues who have directly aided me in my work. There are of course countless numbers of other people at IFM and in Forum Scientium who have helped me with discussions concerning work and other things. **Christian Ulrich** is especially acknowledged for playing table tennis with me, providing needed breaks from work. Thank you all, it would have been a bore without you! Last, but not least, I want to thank **my family** for helping me on a more personal level through all these years!

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1 GENERAL INTRODUCTION

During the last century incredible progress was made in understanding how biological organisms work on the most fundamental level, the molecular. This has resulted in important insights into the molecular mechanisms of, for instance, many diseases. Massive projects have sequenced the entire genome of several organisms, *e.g.* the human genome project (HUGO) sequenced the human recently. The ultimate goal with projects like these is developing cures for many diseases and conditions humans suffer from and also increasing life standards in general. Now in the "post-genomic" era there are many issues that still remain to be solved. The biomolecules of a living organism are connected to each other in an extremely complicated network of interactions. These relations and the functional expression of them is what defines the living organism. Understanding these networks is a key to understanding life itself.

Proteins play a central part in these networks as they are fundamentally responsible for regulation of cellular functions.^[1] Interactions between biomolecules give maps of complexities hard to imagine. For example, figure 1.1a shows a protein-protein interaction map cluster, containing ~78% of the yeast (*Saccharomyces cerevisiae*) proteome.^[2] Most of the data behind the produced map has been obtained by systematic two-hybrid assays via high-throughput screens of protein arrays,^[3] figure 1.1b. The human proteome consists of many times more proteins and a similar mapping would require much more work.

Besides being able to determine the connectivity between the biomolecules in an organism, it can be of equal interest to know the characteristics of the connections, *i.e.* the affinities and kinetics (given by the thermodynamics) of the interactions. Detailed knowledge of the connections could lead to *e.g.* better medical diagnosis of patients with diseases and their treatments with better pharmaceuticals.

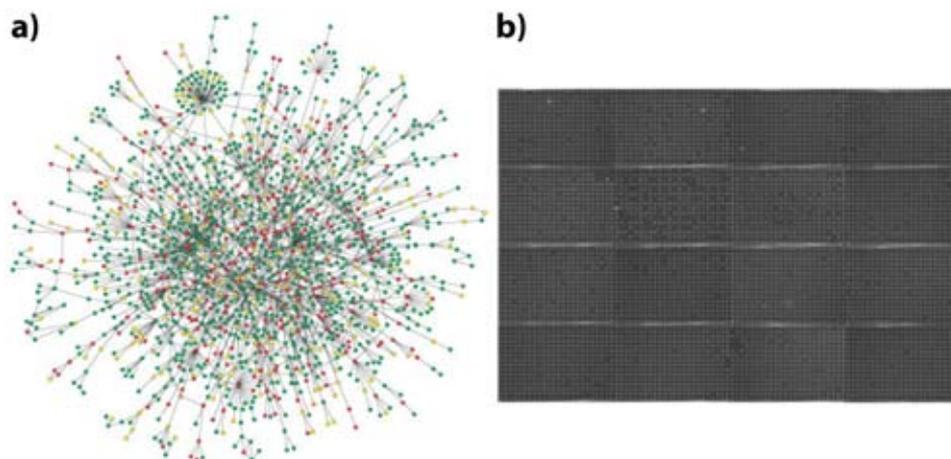


Figure 1.1. a) Map of protein-protein interactions in the largest interaction cluster of the yeast proteome, containing ~78% of its proteome. Each node represents a protein and its color the importance to the organism if removed: red for lethal, green for nonlethal, orange for slow growth, and yellow for unknown. A trend observed is that the higher the connectivity of a protein the more likely it is vital for the survival of yeast. Reprinted by permission from Macmillan Publishers Ltd: Nature, vol 411, p41-42, copyright 2001.^[2] b) Image of the array for the two-hybrid assay, which is the main source of the data on which the map in a) is based. Reprinted by permission from Macmillan Publishers Ltd: Nature, vol 403, p623-631, copyright 2000.^[3]

Biosensors, consisting of a sensor with an immobilized biomolecular recognition layer, have shown to be important tools for obtaining valuable information of the biomolecular interaction networks (including kinetics and affinities), albeit small portions of it. They have, until recently, been limited to a capacity of monitoring only a few interactions simultaneously on one sensor chip. Current approaches, involving miniaturization via microfabrication, have, however, increased the capacity tremendously. The result is microarrays, consisting of many (thousands of) individual spots of immobilized biomolecules. By simultaneously monitoring the interaction of all the spots with analyte solutions, many interactions can be screened rapidly, in parallel, and thus there is a large increase in the throughput of the analyses. Along with the miniaturization there are considerable improvements in sample and time consumptions, *i.e.* potentially large economical benefits.

The existing and potential applications of microarrays are many, *e.g.* in general functional proteomics research, in drug discovery, and in clinical diagnostics. The last example holds especially great potential with the

visualized, ultimate microarray chip, which would reveal any diseases or conditions a patient suffers from with a mere drop of blood sample.

In our work we have developed platforms for the stable, homogenous, and functional immobilization of proteins on microarrays, which is often an underestimated problem. Proteins are complicated three-dimensional structures which can be extremely sensitive to their environment. Many microfabrication procedures impose harsh conditions to proteins and there is a substantial risk for loss of function. Our surfaces have been structurally characterized and functionally evaluated with model protein systems.

We have also presented an approach for addressable adsorption of lipid vesicles, loaded with receptors, and monitored their interaction with ligands. The study of receptors in their native environment has great importance to the pharmaceutical industry, as many drugs are directed towards receptors integrated in cell membranes. However, our approach is also applicable to the addressable immobilization of any type of protein (provided it is equipped with a capture tag) to a surface and tethered to a lipid membrane under very mild conditions, *i.e.* supporting their functional immobilization.

Another application for (protein) microarrays is in trace detection for dangerous or illegal compounds, *e.g.* explosives, narcotics, toxins, and biological and chemical warfare agents. There is a great demand for reliable and efficient sensor systems in today's world, especially with the threat from terrorism. Currently, a very common technique used in transportation security is ion mobility spectrometry, figure 1.2.^[4] Sampling is performed by for instance swabbing with a collection filter on suspected objects or by collecting particles or vapors from investigated persons with airflows. The sample is transferred to the gas-phase and analyzed by first ionizing it and then measuring the time of flight for the sample in an electric field at an opposing drift flow. The time will depend on the charge, size, and geometry of the sample and a compound will give a characteristic spectrogram. The greatest benefits with the method is that it is very rapid (seconds) and its high sensitivity (ng). However, the major drawback is a relatively low selectivity as it can produce false hits with species similar in chemical composition to the real trigger compounds, *e.g.* pharmaceuticals and perfumes. With a biosensor, using *e.g.* antibodies, the performance can be improved due to the high selectivity biomolecular recognition offers. We have in our included work

designed a protein microarray chip for the simultaneous and parallel detection of four narcotics on one surface. The chip is in no way limited to the four narcotics as it has capacity to be expanded virtually endlessly, with the addition of more protein systems. We have also managed to measure explosives on the same chip with excellent results. The chip is therefore envisaged for use in e.g. customs, in airport and public transportation security, within the police force and at correctional facilities, drug interdiction, forensics, warning alerts on military equipment and personnel, and monitoring drinking water reservoirs.

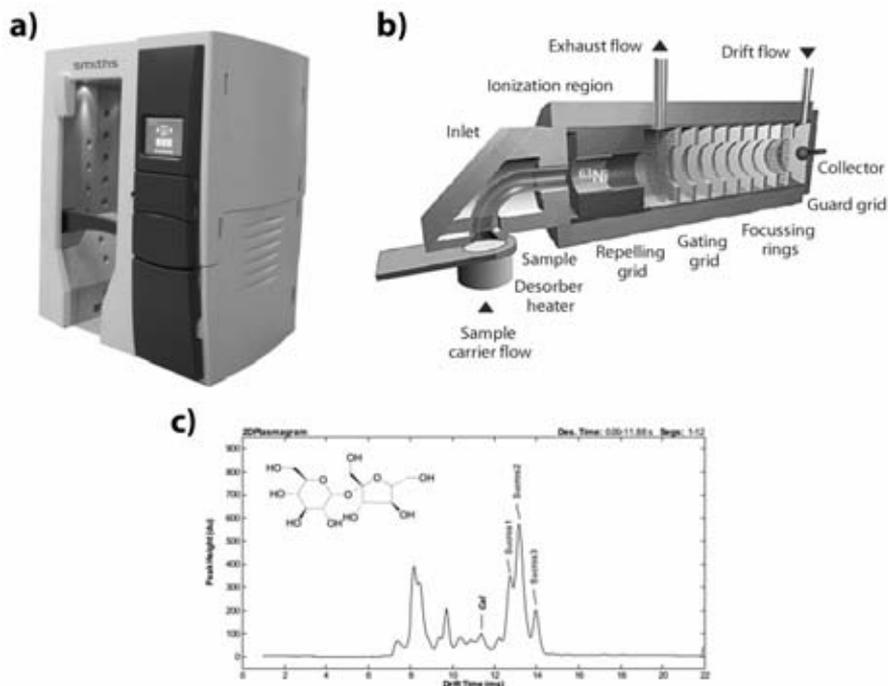


Figure 1.2. Ion mobility spectrometry (IMS), a commonly used technique for trace detection of harmful and illegal substances. a) Image of a Sentinel unit from Smiths, which houses an IMS instrument and air flow system. The airflow is used to dislodge particles from an investigated person in the portal. Particle and vapor traces are then analyzed within seconds. b) Schematic illustration of IMS. The sample is soft ionized by a ^{63}Ni source (β radiation) and electrostatically attracted to the collector. On the way the sample experiences a resistance from the opposing drift flow of a retention gas. The time of flight for the sample will therefore depend on its size, charge, and geometry. c) Example of a recorded IMS run showing the fingerprint spectrum obtained for a compound, in this case sucrose. Reprinted by permission of Smiths Detection.^[5]

The remainder of these chapters will be an introduction to the included papers. First, biomolecules and the basics of biomolecular recognition will be discussed, followed by a chapter devoted to biosensors and monitoring biomolecular interactions with them. The main part of that chapter will deal with the exciting features of (mainly protein) microarrays. The last two chapters are of a more technical nature and describe the surface production procedures and instrumentations used.

2 BIOMOLECULES AND BIOMOLECULAR RECOGNITION

Nature has provided biological organisms with a huge complexity of molecules. These biomolecules interact with each other in an intricate system, which defines the living organism. Understanding the interactions and the pathways between them all is a part of understanding life itself. Science has for a long time tried to untangle the pathways and much progress has been made in, for instance, understanding how different diseases affect an organism on a molecular level and how they can be cured. However, due to the high degree of complexity and interconnectivity the full mapping of the pathways of *e.g.* humans will take many more years.

There are different classes of biomolecules, figure 2.1. The high degree of complexity comes from the many ways in which the molecular subunits, *e.g.* amino acids for proteins, can be combined to form the respective biomolecules. A biomolecule normally has the ability to recognize and bind to other molecules. The simplest form of a biomolecular interaction is the 1:1 interaction of a biomolecule (A) and its binding partner (B) in which they form a complex (A·B)



where k_a and k_d are the association and dissociation rates, respectively. The association constant, K_A , is defined as

$$K_A = \frac{[A \cdot B]}{[A][B]} = \frac{k_a}{k_d} \quad 2.2$$

where brackets indicate the respective species' concentration at equilibrium. A large association constant indicates high relative formation of the complex and thus a high affinity between the two interacting species.

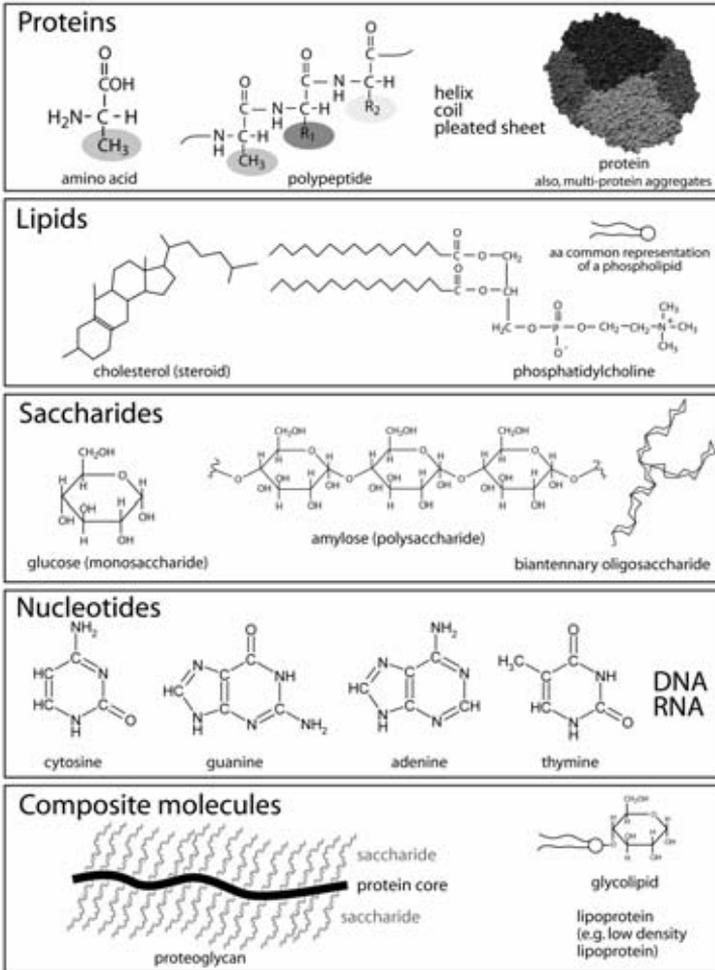


Figure 2.1. Classification of biomolecules, the building-blocks of a biological organism. The enormous degree of complexity stems from the many combinatorial ways the molecules can be connected into macromolecules. Figure redrawn from Castner and Ratner.^[6]

Chemical groups of the biomolecule have complementary, matching groups on the respective countermolecule and bringing them in close proximity is energetically favorable. The enthalpic contributions to the binding come from van der Waal, hydrogen bonding, and electrostatic interactions. On the other hand, bringing two macromolecules in close contact to form one complex, requires overcoming large entropic barriers. There is loss of entropy of free rotation and translation of the separate molecules upon complex formation. In addition, there can be loss of conformational entropy of mobile domains or

sidechains of the molecules. However, these losses are balanced by increased entropy from displaced water at the contact interface between two molecules. Crystallographic X-ray studies of formed complexes in proteins reveal that the water in the interface is almost completely removed by the close shape complementarity of the two joined surfaces.^[7, 8] The remainder of this chapter will cover some important aspects of the biomolecules that have been mainly used in the included papers: DNA, proteins, and lipids.

2.1 DNA

DNA contains the coded information from which the proteins are built up. It consists of two complementary, antiparallel strands which under physiological conditions form the double-helix as described by Watson and Crick in 1953.^[9] Each strand consists of linearly connected subunits of nucleotides, which in turn consists of three parts: a phosphoryl group, a deoxyribose, and a heterocyclic nitrogenous base.^[10] There are four bases, cytosine, guanine, adenine, and thymine (figure 2.1), giving four different nucleotides. Thus, the data encoded in the DNA of an organism lies in the sequence of its nucleotide bases.

The two chains of the DNA double-helix bind to each other with very high specificity due to specific base pairing between the strands. Adenine forms a bond to thymine and guanine bonds with cytosine. The bond consists of hydrogen bonds between two complementary bases as they come in close proximity to each other. There are 2 hydrogen bonds between adenine and thymine and 3 between guanine and cytosine, figure 2.2.^[9]

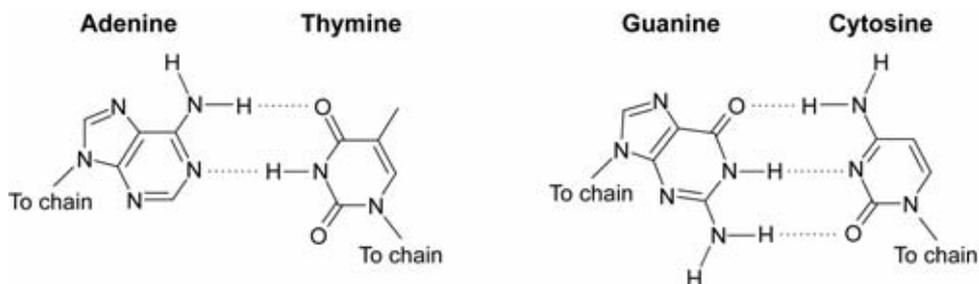


Figure 2.2. Hydrogen bonding (dashed lines) between the base-pairs of DNA.

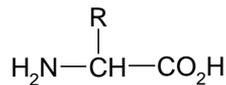
It is quite remarkable that two complementary DNA-strands bind to each other, considering the repulsive negative charges from the phosphate backbone.

The fact that they do bind is due to the hydrogen bonding between base-pairs, as well as stabilizing stacking forces between the baseline-planes involving van der Waal and dipole-dipole interactions. In addition, the negative phosphate-groups are normally shielded by counterions, basic polyamines, and positively charged groups of chromosomal proteins.^[10]

Individually, the binding forces of the base pairs are weak, but when they act together^[7] in a long strand they give a DNA duplex that is highly stable.^[10] To break the duplex (DNA denaturation), one can heat the DNA solution above its melting temperature, T_m . The melting temperature is dependent on the base composition of the strands, high content of G-C base-pairs give a higher T_m . One can also break the duplex by extracting the ions from the DNA solution. This will remove the shielding of the negatively charged backbone and will increase the repulsive forces. For example, in deionized water DNA denatures at room temperature.^[10] A denatured DNA molecule has the capacity to reassemble into a duplex (renature) if the denaturing conditions are removed.

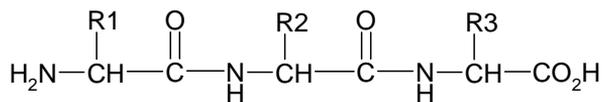
2.2 Proteins

Proteins regulate the biological organism at the molecular level and are produced from the coded sequences of DNA.^[7] There are many different types of proteins, ranging in size and function. They are usually characterized by their function, *e.g.* enzymes, receptors, antibodies, signal transducers, and transporters. Proteins are, just like DNA, a natural polymer, consisting of a sequence of monomers, in this case amino acids. There are 20 different natural amino acids, 19 of them have the general chemical structure



2.3

where R is the side-group which differentiates the amino acids. The 20th amino acid is similar but its amine group is connected to the side-group as well. Although the amino acids differ in chemical structure, they can crudely be categorized as hydrophobic, polar, acidic, and basic. In a protein, the amino acids are connected to each other with peptide bonds in a linear sequence as illustrated by the following three-valued peptide:



2.4

Although a protein consists of a linear sequence of amino acids, it is in no way one-dimensional. Instead, interactions (van der Waal, electrostatic, and hydrogen bonding) between the side-groups and the peptide backbone (and surroundings, *e.g.* water), make the protein fold into a three-dimensional structure which minimizes its total free energy. The folding is very complicated and normally the energy of the folded (native) state does not differ significantly (~5-15 kcal/mol) from unfolded (denatured) states, which makes many proteins sensitive to their environment.^[11] Figure 2.3 shows the intricate three-dimensional structure of the holoenzyme glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus* in three different ways.

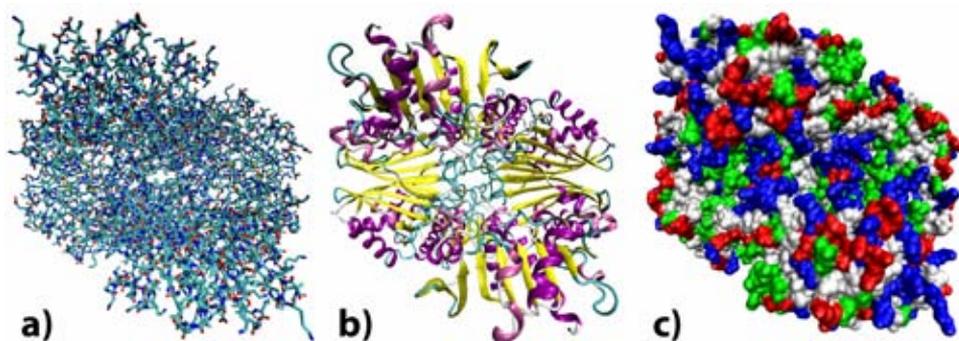


Figure 2.3. Three-dimensional structure of the holoenzyme glyceraldehyde 3-phosphate dehydrogenase from *Bacillus stearothermophilus*. a) All-atom view colored by atom type. b) Cartoon view of the secondary structure. c) Surface-view of the protein showing the solvent-accessible areas, colored by acidic (red), basic (blue), polar (green), and nonpolar (white) residues. The protein structure was obtained in the protein data bank (PDB)^[12] with the PDB-ID 1GD1 and graphically rendered with the virtual molecular dynamics software.^[13] All non-amino acid components have been omitted.

A folded protein is a very dense material, the packing density is about 0.75,^[11] which is comparable to values of up to 0.78 for crystals. As a consequence, the inside of most proteins is more or less out of reach to their surroundings, figure 2.3c. Therefore, most interactions of a protein and its counterpart, the ligand, take place on the surface of a protein, or in grooves or pockets of the surface. The interaction shows both steric and physical

2.2.1 Antibodies

Antibodies are part of the immune system of highly developed biological organisms and act by binding to foreign compounds, termed antigens (i.e. the antibody's ligand). One antibody is usually capable of binding a few antigens very specifically, but the collective antibody arsenal in an organism is capable of recognizing virtually any molecule.^[7]

There are different classes of antibodies, but they are fundamentally similar in their structure. The basic structure is a Y-shaped molecule composed of four chains; two heavy (H) and two light (L).^[7] The chains are held together by disulfide bonds at the hinge-region, which separates the protein into two parts: the F_C stem and the two F_{ab} arms. In figure 2.5 the structure of immunoglobulin G (IgG) is shown. The major part of an IgG consists of domains that are constant (C) in their sequence between different antibodies. However, at the end of the F_{ab} arms there are domains with a highly variable (V) sequence between antibodies. It is at this variable region that the antibody binds its antigen and since there are two F_{ab} arms one antibody can normally bind up to two antigens. Antibodies are remarkable in the wide range of different types of antigens they can recognize and bind. They can bind virtually everything from small molecules (haptens) up to large macromolecules such as other proteins. The recognition mechanisms have been discussed above. For a more extensive review of the antibody-antigen complex, the reader is encouraged to read *e.g.* the review by Davies et al.^[8]

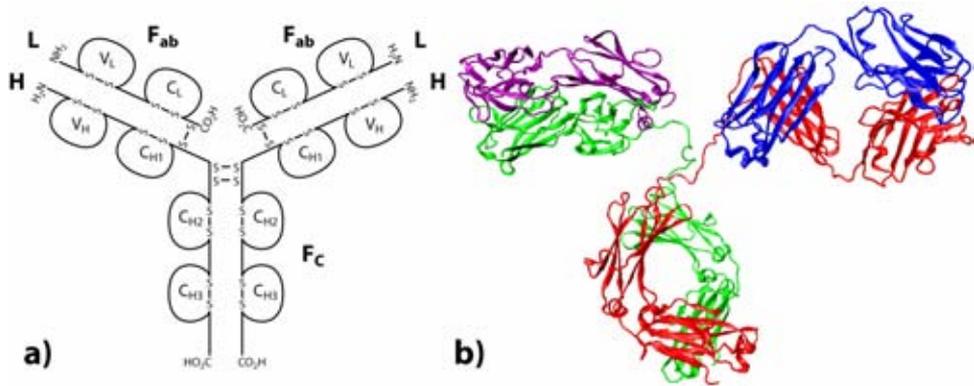


Figure 2.5. The IgG antibody. a) Schematic illustration, adapted from^[7]. b) Perspective three-dimensional view showing the secondary structure colored by chain of a mouse IgG. The structure was obtained in the protein data bank (PDB)^[12] with the PDB-ID 1IGT and graphically rendered with the virtual molecular dynamics software.^[13] The sugars of the C_{H2} domains have been omitted.

The connective segments between the domains of the IgG molecule are quite flexible, and the domains are, consequently, able to some considerable movements relative to each other. This flexibility and its importance in the role of the antibody can be found in a review by Burton.^[15] The features of antibodies discussed above, *i.e.* the high specificity and the ability to recognize virtually any molecule, makes them ideal for many applications in the life science field. One example being in diagnostics where antibody-based immunoassays are the most commonly used diagnostic tests, due to its nearly unlimited applicability and specificity.^[16]

With the advances made in cloning and expressing whole antibodies or antibody fragments in host organisms, *e.g.* bacteria, new opportunities have arisen for engineering antibodies to improve their performance. The improvements for an antibody can involve increased assay sensitivity, decreased antigen cross reactivity, standardized manufacturing and possibility to introduce novel labeling agents. In addition, it is possible to manufacture antibodies against antigens that are normally not possible by conventional means.^[16]

Figure 2.6 shows an interaction study (unpublished results) of an antibody (anti- β 2-microglobulin) with its antigen, β 2-microglobulin. The antibody has been immobilized on a sensor chip and the antigen introduced at different

concentrations, indicated in the figure. The interaction is quite well described with a 1:1 model (equation 2.1, see section 3.3 also) and the calculated association constant is $3.1 \cdot 10^8 \text{ M}^{-1}$.

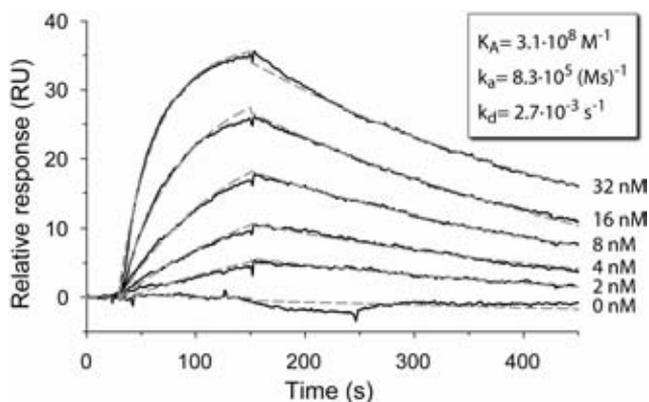


Figure 2.6. Real-time biomolecular analysis of the interaction between anti- β 2-microglobulin (immobilized to the sensor surface) and β 2-microglobulin (analyte). Sensor signals obtained with surface plasmon resonance at different analyte concentrations (whole lines) together with fitted data (dashed). Only during the interval 30-150 s the analyte is introduced to the immobilized protein and during the remaining time buffer is flowed over the surface. The interaction was modelled with an ideal 1:1 interaction and the calculated parameters are shown.

2.2.2 Receptors

Receptors^[1] are an important class of proteins; they are responsible for interacting with signaling molecules and initiating a cellular response to the particular signal. Because of this function, they are major targets for pharmaceutical research and many of today's pharmaceuticals are aimed towards receptors.^[17]

There are different kinds of receptors, of which most are located on the cell surface, integrated into the cell membrane via hydrophobic domains in their structure. The signaling molecule binds to the extracellular part of the receptor which affects the intracellular part and initiates a signaling cascade inside the cell via further interactions with intracellular biomolecules. Since extracellular signal molecules are present at low concentrations, the affinity of the receptors are usually very high towards them ($K_A > 10^8 \text{ M}^{-1}$).

Traditionally, cell-based assays have been used extensively to explore the role of many receptors. However, their usefulness is rather limited by the inherited complexity of a system consisting of cells.^[18] It is therefore advantageous to design molecular assays where it is possible to decrease the number of interacting parameters down to a minimum. In figure 2.7, the real-time interaction study of the ectodomains of the type I interferon receptor with its ligand IFN α 2 on an imaging surface plasmon resonance (SPR) sensor chip is shown.^[19] On this type of novel assay it was possible to show the ternary complex formation of the two receptor subunits and their ligand as the distance between the subunits decreases.

Since most receptors are integrated into cell membranes, it is of great interest to study them in their native environment. Bieri *et al.*^[18] have in a very interesting study, also using SPR, been able to immobilize a G protein-coupled receptor integrated in a lipid bilayer on a surface. They were then successful in following ligand binding, G protein activation, and receptor deactivation.

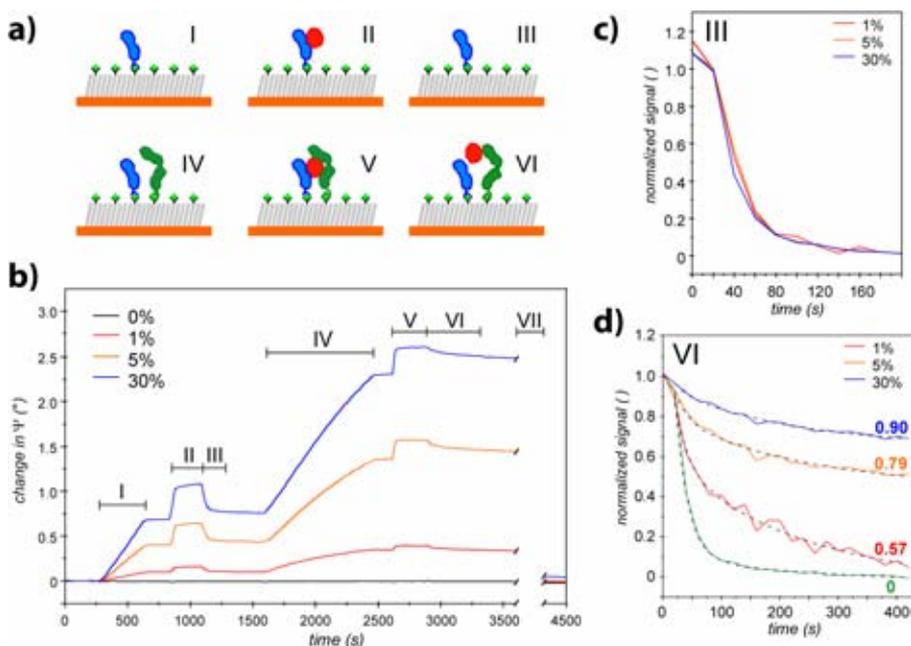


Figure 2.7. The formation of a ternary complex studied with imaging SPR. a) Schematic illustration of the interactions between receptor subunits *ifnar2*-H10 (blue) and *ifnar1*-H10 (green) with the ligand *IFN* α 2-M148A (red). b) Recorded real-time sensorgram on a microarray containing spots of different surface densities of capture molecule (*bis*-NTA, see section 3.2.4). c) Normalized dissociation of ligand from *ifnar2*, no dependence is seen on surface density. d) Normalized dissociation of ligand from *ifnar2* co-immobilized with *ifnar1*, clear dependence on surface density is seen now. This is ascribed to ternary complex formation, increasing with higher receptor subunit surface densities (i.e. shorter distance between the two receptor subunits). The numbers correspond to calculated fraction of ternary complex formation. The figure has been redrawn from Klenkar *et al.*,^[19] paper A, where more details can be found.

2.3 Lipids

Lipids, figure 2.1, are a class of molecules which form the membrane that encloses cells.^[1] A common lipid, the phosphatidylcholine, consists of two fatty chains and a polar head group, i.e. it is amphiphilic - both hydrophilic and hydrophobic. The membrane consists of a bilayer of the phospholipids, where the hydrophilic head groups form the inner and outer surface and the hydrophobic chains form the inside. This results in a barrier, which keeps unwanted molecules from migrating into the cell and intracellular components to escape from the cell. The membrane also consists of other biomolecules of which the receptors are an important class. Roughly 50% of the pharmaceutical

drug targets are membrane-bound receptors^[17] and it is therefore highly interesting to study them in their native environment. Therefore, solid-supported lipid membranes and vesicles have been developed as model systems, mimicking the cell membranes, for the study of interesting membrane-associated biomolecules.^[20] In our work in paper D, we and collaborators from Lund University and Goethe University, Frankfurt, have constructed a microarray platform for the parallel analysis of several lipid-receptor systems, figure 2.8. In our approach we use the concept of self sorting of lipids to array positions on the surface via DNA barcoding. Single stranded DNA (ssDNA) has been pre-immobilized to areas of the array and when vesicles, tagged with complementary DNA (cDNA), are introduced they adsorb to the correct surface position due to the highly specific hybridization between the DNA strands. The vesicles also contain molecules for the capture of histidine-tagged proteins and we have successfully shown that two co-immobilized receptor subunits are able to form a ternary complex with their ligand due to lateral diffusion on the lipid membrane surface.

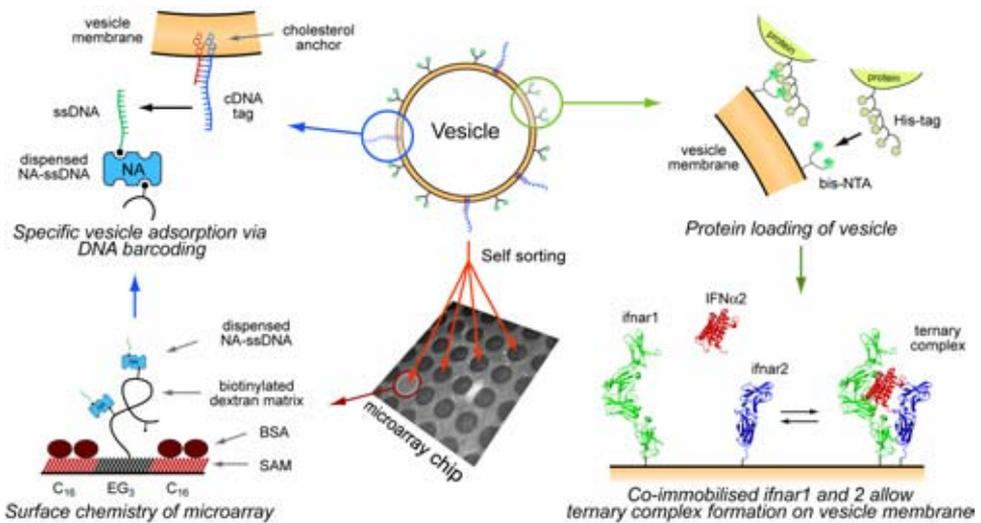


Figure 2.8. Using lipid systems for study of membrane-bound receptors. This illustrates the work in paper D where lipid vesicles self sort on a microarray to predefined areas via DNA barcoding. The vesicles also contain a capture molecule to tether histidine-tagged receptors to the membrane, allowing the study of complex receptor-ligand interactions.

3 BIOSENSORS - MONITORING BIOMOLECULAR INTERACTIONS

The high specificity of biomolecules can be used to improve the performance of existing sensor systems. A biosensor^[21-23] is such a device, consisting of a physical transducer modified with an active surface of a biomolecule, *e.g.* an antibody, making the biosensor selective towards one or a few analytes only. Examples of transducers which can readily be used for biosensing applications are based on electronic, optical, acoustical/mechanical, and calorimetric principles. The first biosensor was described in 1962 and suggested to detect glucose in 1962 with the enzyme glucose oxidase on an electrode transducer.^[24] Since then, a lot of research has been and is still being conducted to create novel biosensors and improve their performance. Biosensors are ideal for molecular assays in which biomolecular interactions are monitored. They are therefore valuable tools in exploring the molecular pathways of biological organisms in a more controlled manner compared to *e.g.* cellular assays. In the most simple case they are designed to detect an analyte and in more advanced designs they monitor more complex (bio)molecular interactions.

Typical applicational areas of biosensors are in clinical diagnostics,^[21, 25] in pharmaceutical research,^[26] and in proteomics.^[27] The driving force for developing better biosensors has mainly been the commercial aspects of successful biosensors, *e.g.* glucose sensors in diagnostics.^[28] In more recent years, biosensors have also been used in trace detection of *e.g.* explosives^[29, 30] and narcotics,^[31, 32] which has a huge potential market. Today, a lot of focus is directed towards developing miniaturized and parallelized biosensors, *e.g.* with protein microarrays,^[33, 34] able to monitor many (thousands) biomolecular interactions simultaneously on a small area ($\sim 1 \text{ cm}^2$).

Most biosensors are based on biomolecules bound to a surface, which is often part of the transducer element. Therefore, an important prerequisite to a successful biosensor lies in manufacturing well-defined and well-behaving

surfaces that are compatible with the biomolecule *i.e.* preserving its functional activity after immobilization. In many cases, an intermediate layer is necessary between the surface and the biomolecule to achieve the desired properties.

3.1 Spontaneous Protein Adsorption to Solid Surfaces

The spontaneous adsorption of proteins to solid surfaces has been the focus of a lot of research and, since it is still not fully understood, still is.^[35-39] Understanding the complicated adsorption mechanisms is, in addition to biosensors, of great interest in *e.g.* biomedical implant research.^[6, 40] There are many enthalpic and entropic parameters governing the adsorption process in a complex system consisting of a surface in a liquid solution of proteins. The complexity is a consequence of the virtually infinite amount of physical and chemical variations proteins (see *e.g.* figure 2.3c) and solid surfaces can have and, furthermore, their interaction with solvent molecules (mainly water and ions) is critical, which complicates matters further.^[39, 40]

The consensus in the field is that the surface energies of the two interacting species, the solid support and the protein exterior, play a key role in protein adsorption.^[39] Also, it is more or less agreed that the process is entropically driven by released water from the surface and the protein as well as the increased mobility of protein side chains upon (limited) conformational changes.^[39, 40] Studies of protein adsorption on surfaces of varying surface energies generally show that protein adsorption is much higher on hydrophobic surfaces.^[37, 38]

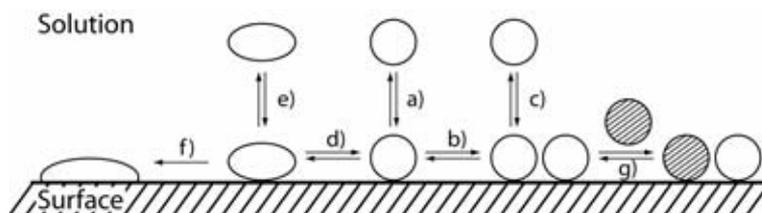


Figure 3.1. Schematic illustration of protein adsorption to a solid surface, showing the involved complexities. Several possible equilibria have to be considered. a) protein adsorption. b) lateral mobility. c) dissociation of a protein in close proximity to another. d) reversible minor conformational changes and e) dissociation in these states. f) further structural changes resulting in irreversible adsorption. g) exchange of the protein with another protein from solution. The figure is redrawn from Mrksich and Whitesides,^[35] who have tried to illustrate protein adsorption in its simplest possible form. However, they stress that it is not complete in any way, rather complicated further by the many different conformations and environments available to a protein adsorbing to a surface.

Figure 3.1 outlines an attempt of Mrksich and Whitesides^[35] to schematically describe protein adsorption in the simplest case: one well-defined protein interacting with a uniform, well-defined surface. Even in this simple case a substantial range of processes is usually involved as can be seen. It is complicated even further by the many varying conformations and environments available to an adsorbed protein. Generally, when a surface is submerged in a protein solution the proteins will adsorb to it to a degree determined by the respective surface energies and environmental parameters. Upon adsorption the protein can undergo some limited conformational changes to its structure,^[37] *i.e.* partial denaturation. But surprisingly often, the biological function of the protein is more or less retained.^[40] On the other hand, it is not uncommon that the function is completely destroyed.^[41]

The adsorption is in most cases irreversible as adsorbed proteins remain on the surface when the surface is moved to a protein-free solution.^[38, 39] Adsorbed proteins can, however, be exchanged for other proteins or molecules (*e.g.* detergents) in the solution.^[39] Another important, general feature of protein adsorption is that since proteins repel each other in solution and do not form aggregates, they will normally only form a monolayer (at most) on the surface.^[39]

A lot of effort has been made to produce surfaces which resist non-specific protein adsorption. In biomedical implant research this is important to *e.g.*

prevent blood coagulation cascades or immunological responses.^[40] It is also of tremendous importance to the food industry and maritime transport to produce surfaces that resist adsorption of proteins (antifouling). In biosensors, it can be used to decrease non-specific biomolecular adsorption and consequently increase the specificity (*i.e.* signal to noise ratio). Some common surface strategies to render surfaces more protein resistant are briefly discussed in *e.g.* a review by Castner and Ratner^[6] and Mrksich and Whitesides^[35]. One of the strategies has already been discussed; a preadsorbed protein layer will further resist the adsorption of other proteins, albeit of low durability. The other strategies mainly employ the use of polymers of either ethylene glycole groups $-(\text{OCH}_2\text{CH}_2)_n\text{OH}$, known as PEGs, or saccharides (*e.g.* dextran). PEGs have proven to be extremely successful in passivating a surface.^[35] Even surfaces modified with molecules containing short oligo ethylene glycols ($n=2-7$) are highly efficient at repelling proteins.^[35, 38]

3.2 Controlled Protein Immobilization

In most biosensor applications it is important to immobilize the biomolecules to a surface in a more controlled way than the physical adsorption discussed in the previous section. The goal is to immobilize proteins steadily over a long time in a functional configuration, as closely resembling its native state and environment as possible. The following sections will discuss strategies aimed towards these goals: the first will deal with covalent immobilization of proteins and the remaining two with non-covalent immobilization.

3.2.1 Covalent Immobilization

Proteins can be coupled to surfaces by the formation of covalent bonds by a variety of chemistries. Reactive groups on the surface (*e.g.* $-\text{OH}$, $-\text{NH}_2$, $-\text{COOH}$) can be coupled to reactive chemical groups of the protein's amino acids with the aid of one or more intermediate coupling chemicals. Many of the most common immobilization schemes can be found in the book by Carr and Bowers.^[42] A very common coupling scheme is shown in figure 3.2, where surface carboxyl groups are coupled to solvent-accessible primary amine groups of a protein.

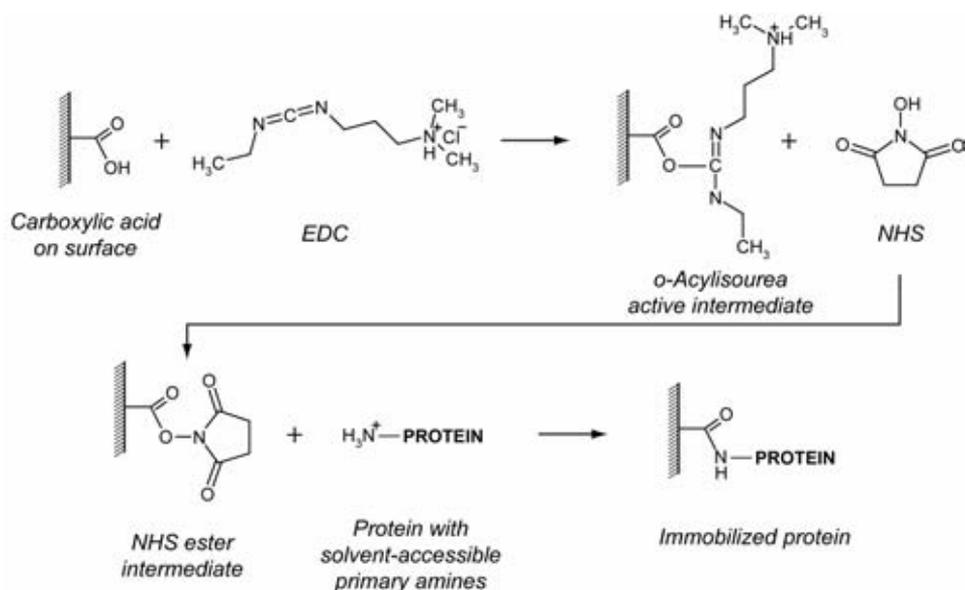


Figure 3.2. Covalent immobilization of amine-containing proteins to carboxyl groups on a surface via the coupling agents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS). Figure adapted from ^[43, 44].

The benefit of a covalent immobilization is that it is stable over a very long time. The major drawback is that since the protein usually has multiple reactive groups on its surface and the chemical reaction is not specific it will result in a random orientation distribution of immobilized proteins, *i.e.* a heterogeneous protein layer. This can result in an impaired protein function.

3.2.2 Immobilization of Biotinylated Proteins

A widely used immobilization strategy of proteins is the avidin-biotin system, figure 3.3. The avidin-biotin coupling has been used in numerous applications; Wilchek and Bayer review this and the principles in more detail.^[45, 46] In brief, the affinity between the vitamin biotin and the protein avidin is extremely high, $K_A=10^{15} \text{ M}^{-1}$, which is the main reason for the popularity. The binding can, for all practical purposes, be considered irreversible, *i.e.* stable over very long time. Avidin, or its bacterial relative streptavidin, consists of four subunits, of which each can bind to one biotin, *i.e.* the interaction is 1:4. The normal protein immobilization strategy is to immobilize avidin on a surface (covalently, via pre-immobilized biotin, or some other immobilization scheme)

and then to introduce a biotin-tagged protein, figure 3.3b. The protein can be biotinylated covalently via a range of protocols, aimed at different chemical groups of it.^[47] Depending on the protein and the specificity of the biotinylation protocol, the immobilized protein layer can be either homogenous or heterogenous. There has been a lot of research in developing new variants of avidin and streptavidin with improved performance for various applications. Some variants even allow for reversible binding of biotin, *i.e.* the biotinylated protein can be released under some conditions.^[47] There are countless examples of where the avidin-biotin system is used to immobilize proteins or other biomolecules. *E.g.* Bieri *et al.*^[18] used it to immobilize their G protein-coupled receptor as discussed in the previous chapter and in one of our studies we have used it to immobilize single stranded DNA (paper D).

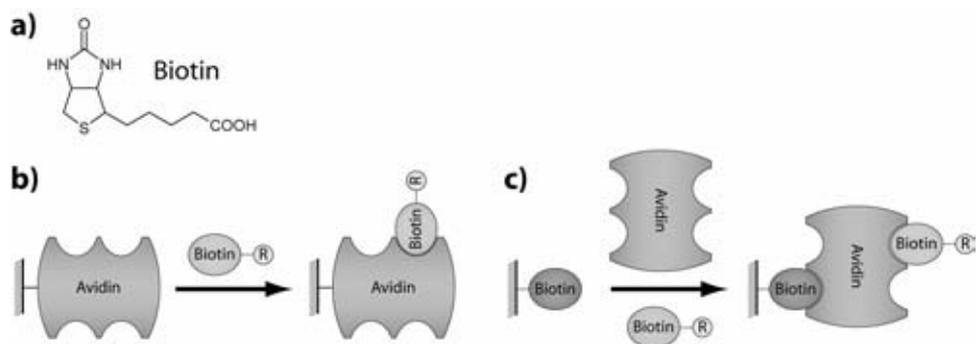


Figure 3.3. Schematic illustration of the avidin-biotin system. a) Chemical structure of biotin. The carboxylic acid end can easily be covalently coupled to another molecule in solution or to a solid support. b-c) Immobilization of a biotinylated molecule, R, either via avidin directly immobilized to a surface (b) or via a surfacebound biotin (c). Figure adapted from Cooper^[26].

3.2.3 Protein Immobilization via Antibodies

The high specificity and affinity of antibodies can be used to immobilize antigen proteins. The antibody can be immobilized with one of the above discussed strategies and its antigen will then be recognized and bound to it as discussed in the previous chapter. The preparation of and some properties of antibody-based protein immobilization can be found in the book edited by Cass and Ligler.^[47] Most importantly, the use of antibodies gives the ability to produce immobilization of highly oriented protein layers, *i.e.* homogenous. However, although the affinity between antibody-antigen can be very high, it is

not of the same strength as *e.g.* avidin-biotin and the immobilized protein suffers the likely risk of dissociating.

3.2.4 Immobilization of Histidine-tagged Proteins

Metal ion complexation was introduced as a method for separating proteins by Porath et al. in 1975.^[48] The interaction with a biomolecule is based on their complex formations with metal ions, which can be immobilized by chelators such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA),^[49] figure 3.4a.

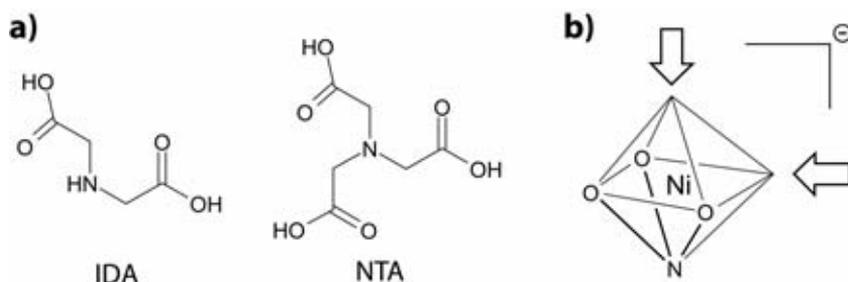


Figure 3.4. a) Chemical structures of the metal chelators iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). b) The octahedral complex schematically illustrated, arrows indicate free binding sites which can be occupied by two histidines. Figure adapted from ^[49, 50].

The chelator is immobilized on a surface and loaded with bivalent metal cations, *e.g.* Ni^{2+} , Zn^{2+} , or Cu^{2+} .^[48, 49] The following discussions will only be based on the NTA-chelator, which is tetradental and forms an octahedral complex with a central metal ion, occupying four of its six coordination sites, figure 3.4b.

Two coordination sites are then available for binding with *e.g.* electron donating groups of the amino acids (histidines or cysteines) of a protein.^[48, 49] Interestingly, the association of one histidine of a protein with NTA is not stable. Instead, for a relatively stable binding, both available sites would have to coordinate to two immediately adjacent histidines or cysteines (on the protein surface). The risk of that existence is, however, very small. Thus, non-specific binding of proteins to NTA is low.^[49]

On the other hand, the requirement of two adjacent histidines can be turned into an advantage as was realized by molecular biologists. By introducing an oligohistidine sequence at the C or N terminus of a recombinant

protein to be expressed in a host organism, one obtains a histidine-tagged protein. This protein can thereafter easily be purified by immobilized metal ion affinity chromatography, *i.e.* captured by NTA in a column.^[51] The binding between NTA and the oligohistidine is reversible and the captured protein can be released by:^[49] (1) introduction of a competitive ligand (imidazole or histidine), (2) addition of ethylenediaminetetraacetic acid (EDTA), which removes the metal ion from NTA, or (3) by protonation of the histidines (pH change). Due to the simplicity, low cost, and high efficiency of the method it rapidly grew in popularity and numerous proteins have been expressed with oligohistidine tags.^[52, 53] Many proteins are therefore available or can be produced with histidine tags when needed. Normally, the tag consists of (at least) six histidines, which gives satisfactory capture by the chelator.^[51] The oligohistidine is a small and flexible tag, which means its effect on the protein's native conformation and function is negligible.^[49, 54] Beside the discussed application of NTA in protein purification, it has successfully been used in detection,^[55, 56] immobilization on surfaces,^[57, 58] and tethering to lipid bilayers^[50, 59] of oligohistidine-tagged proteins.

To summarize, the major benefits of using NTA and oligohistidine tags to immobilize proteins are: (1) the binding is specific, (2) immobilized proteins are uniformly oriented (homogenous layer), (3) the binding is switchable under mild conditions (*e.g.* imidazole), and (4) since the binding agent on the surface (the chelator) is non-biologic it is stable over long time and resistant to harsh treatments. The major drawback is that the binding between an individual chelator and histidine tag is relatively weak and of low stability, $K_a \sim 2 \cdot 10^5$ 1/M.^[50, 60] However, it has been realized that stable immobilization is possible by multipoint attachment on surfaces with high density of NTA chelators.^[57, 58, 61] This is due to *e.g.* hexahistidine tags offer binding sites for up to three NTA units (*i.e.* each NTA coordinates with two histidines via the metal ion as described above) and larger oligohistidine tags to even more.

On a related issue, Rao *et al.* published an article in 1998^[62] on how to increase the affinity between a model receptor and its ligand using multivalent interactions. By designing a trivalent receptor and a trivalent ligand, they were able to increase the binding affinity by ~ 11 orders of magnitude compared to the corresponding monovalent interaction. The achieved binding even surpassed the avidin-biotin interaction by a factor 25. However, contrary to the

avidin-biotin interaction, Rao *et al.* also showed that the multivalent interaction could easily be disturbed (broken) under mild conditions; the addition of a monovalent ligand.

This concept has been adapted recently to NTA-chelators. Molecules based on multivalent chelators, consisting of two to four NTA units, have been synthesized by Lata *et al.* and their binding to oligohistidine-tagged proteins was improved ~3-5 orders of magnitude compared to the monovalent NTA chelator.^[63] Furthermore, the binding is rapidly and fully reversible by the addition of imidazole or EDTA. Multivalent chelators have been used, with excellent results, to tether histidine tagged proteins to lipid membranes,^[64, 65] for their immobilization to surfaces,^[19, 41, 54, 66] and even for labeling them with fluorescent dyes.^[67] In our work we have worked with multivalent NTA molecules immobilized to surfaces (paper A-C) and tethered to lipid bilayers (paper D). Figure 3.5a shows *mono-*, *bis-*, and *tris-*NTA molecules which were used to modify gold sensor surfaces using thiol chemistry, see section 4.1. For discussions regarding the multivalent chelators on surfaces and oligohistidine tags it is important to distinguish between molecular multivalency and surface multivalency as illustrated in figure 3.5b.

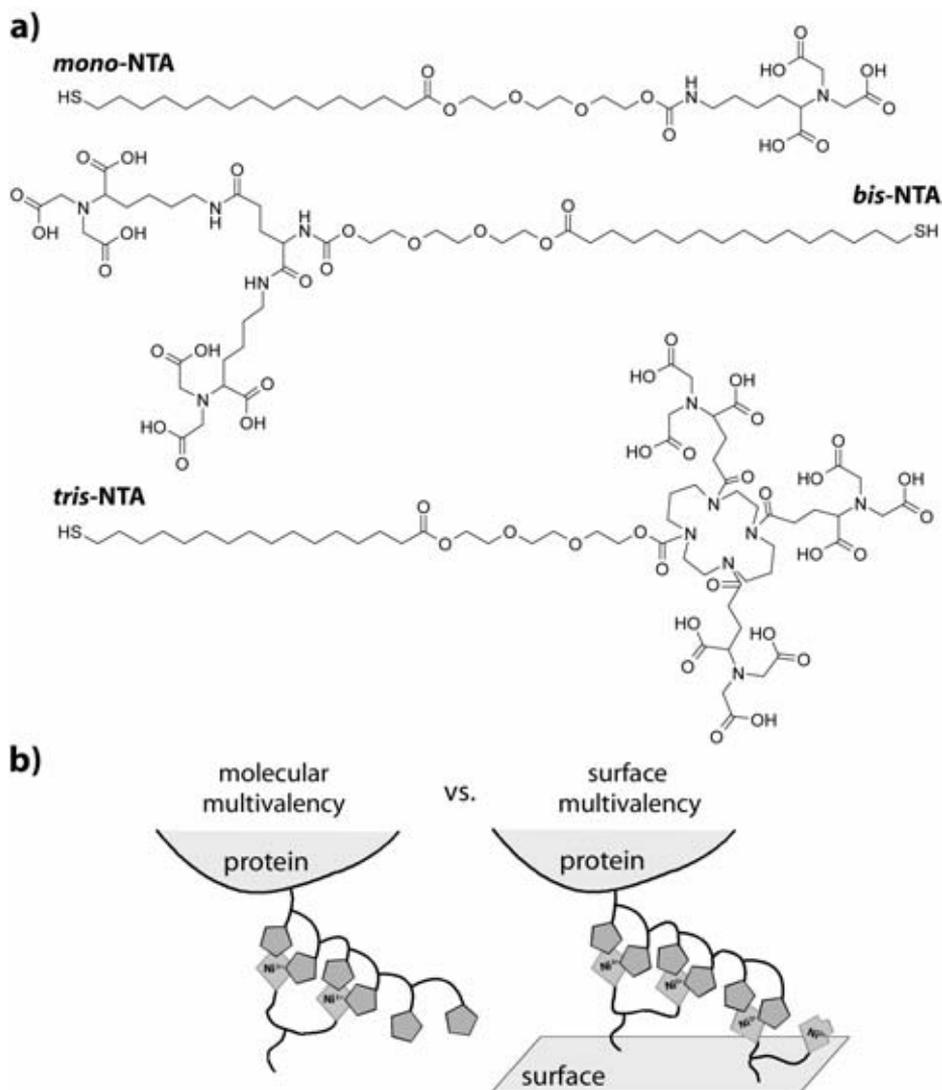


Figure 3.5. a) Structure of *mono*-, *bis*-, and *tris*-NTA alkanethiols for modification of gold surfaces. b) Schematic illustration of recognition of a His₆-tagged protein by a *bis*-NTA (left) and two *bis*-NTAs coupled to a surface (right), representing molecular and surface multivalency, respectively. Redrawn from [66].

3.3 Monitoring Biomolecular Interactions in Real Time

The interaction between a biomolecule and its counterpart can be characterized in terms of the affinity (K_A) and the kinetic rate constants (k_a and k_d) (equations 2.1 and 2.2). The affinity describes the strength of the

interaction and is relatively easy to measure^[68] in equilibrium studies. Kinetics of the interaction, however, provide more information about the interaction, namely the rate of complex formation and dissociation. A cell is a highly dynamic system - it rarely exists in a state of equilibrium. It is therefore important to know the kinetics of its interacting molecules. In figure 3.6, four simulated interaction curves illustrate this importance; each curve has the same affinity constant (K_A) but the k_a and k_d values differ by three orders of magnitude ($K_A = k_a/k_d$). As can clearly be seen, the curves show a huge difference. It is therefore of great benefit to be able to measure the full binding interaction continuously in order to extract the most information possible from the interaction. In addition, kinetic analysis of binding assays allows for fewer experiments to be performed^[47] compared to affinity analysis. This is also illustrated in figure 3.6, where data obtained from a traditional solid phase assay, *e.g.* an enzyme-linked immunosorbent assay (ELISA)^[69] is indicated by the marker points in the simulated binding curves. When a single measurement is undertaken with one concentration of the analyte the traditional assay will give one data point, which on its own is meaningless (unless the assay is a detectional one where only the presence of the analyte is of importance). However, a continuous, real-time measurement can provide direct information on how the analyte binds to and dissociates from the biomolecule. Interaction partners with high dissociation rates can also be studied, see purple curve in figure 3.6. Such interactions are not detected with ELIS because of the rapid dissociation during the rinsing stages. Theoretically, one binding curve is enough to calculate k_a and k_d , which can be used to obtain the K_A (equation 2.2). In practice, however, several measurements with careful design have to be done due to factors that will be discussed below.

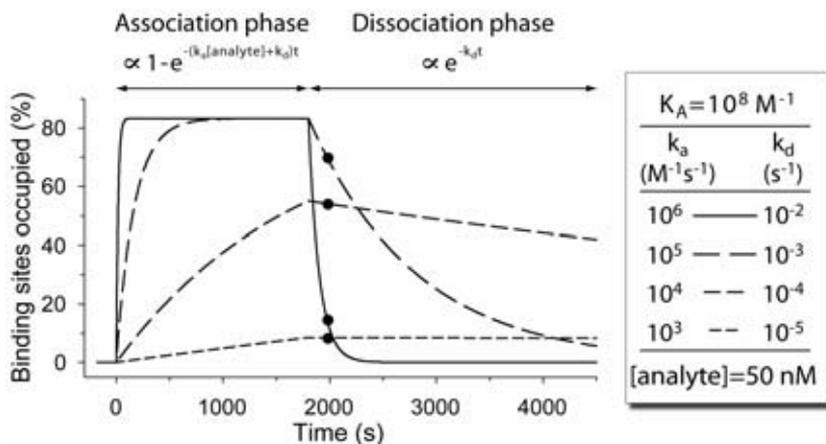


Figure 3.6. Simulated interaction curves between 4 biomolecules and their binding partners (the analyte). All four pairs have the same affinity ($K_A = 10^8 \text{ 1/M}$) but with different kinetics (k_a and k_d). Being able to measure in real time allows resolving their respective behaviors, thereby providing more information than a simple equilibrium (affinity) analysis. Furthermore, a traditional solid phase assay like ELISA would only be able to capture the binding interactions at one point of time, indicated in each curve by a circle marker.

There are numerous (bio-)sensor devices available for measurements in real time (*i.e.* continuous over time) based on *e.g.* electrochemical, quartz crystal microbalance, magnetic, and optical principles.^[21, 22] They can generally be divided into two groups: one that requires some sort of labelling of the molecules in order to detect them and the other that does not. Kinetic information has proven vital in *e.g.* functional studies of the binding sites of proteins^[70] and functional studies of mutated proteins to improve their performance.^[71] For more information on applications of real-time measurements, the reader is encouraged to turn to the recent review by Rich and Myszk.^[27]

The immobilization of a biomolecule (A) on a sensor transducer surface will in the ideal case render all the molecules equally active and all binding sites independent of each other. In this case the biomolecule's complex formation rate with its counterpart (B) can be described by

$$\frac{d[A \cdot B]}{dt} = k_a[A][B] - k_d[A \cdot B] \quad 3.1$$

following the definitions of equations 2.1 and 2.2. The concentration of free biomolecules on the surface can at any time be described by the difference between the total concentration of biomolecules and the complex concentration, *i.e.* $[A] = [A]_{\text{tot}} - [A \cdot B]$. To be able to determine k_a and k_d one has to be able to measure the complex formation continuously and a constant concentration of the counterpart has to be assumed. Solving the differential equation 3.1 yields the expression

$$[A \cdot B] = [A \cdot B]_{\text{eq}} \left(1 - e^{-(k_a[B] + k_d)t} \right) \quad 3.2$$

which describes the association phase (starting at $t=0$) and where $[A \cdot B]_{\text{eq}}$ represents the complex concentration at equilibrium (obtained by setting the complex formation rate to zero in equation 3.1):

$$[A \cdot B]_{\text{eq}} = \frac{k_a[B]}{k_a[B] + k_d} [A]_{\text{tot}} \quad 3.3$$

Biosensors are usually equipped with flow cells attached to a delivery system (*i.e.* a pump). Measurements are normally undertaken in the liquid state since that is the native environment of biomolecules. With the flow system one is able to inject samples over the sensor surface in a controlled way. After ending the injection of a binding partner its concentration over the surface will drop to zero and the first term of the right side of equation 3.1 will vanish. Solving the differential equation yields the expression

$$[A \cdot B] = [A \cdot B]_{t_0} e^{-k_d(t-t_0)} \quad 3.4$$

which describes the dissociation phase and where $[A \cdot B]_{t_0}$ is the complex concentration at the arbitrarily fixed time t_0 during dissociation. The signal from a biosensor is proportional to the A·B complex and hence the association and dissociation phase can be fitted to equations 3.2 and 3.4, respectively, to obtain the k_a and k_d values (see figure 2.6).

Being able to measure the affinity constant (K_A) of an interaction allows for thermodynamic information of the interaction and it can be analyzed in terms of enthalpy and entropy.^[72] However, with the additional possibility to measure kinetics, *i.e.* k_a and k_d , one can also gain thermodynamic information regarding the transition state of the biomolecule while forming the complex with its

counterpart.^[72, 73] This feature makes real-time biosensors invaluable tools for *e.g.* pharmaceutical research where new drugs need to be characterized or discovered.^[26]

In practical work ideal binding curves are not always obtained, which results in poor fits of equations 3.2 and 3.4. The reasons for this can be complicated, but in many cases logical explanations are found, *e.g.*:^[47]

- (1) Non-specific binding to the biomolecule or the sensor surface of the analyte or another compound in the sample solution.
- (2) A heterogenous analyte, existing in multiple forms, with different binding properties.
- (3) An analyte with multiple binding sites, enabling multivalent binding.
- (4) A non-constant analyte concentration.
- (5) The immobilized biomolecule is heterogenous, either by nature or as a consequence of the immobilization procedure (discussed in sections 3.1 and 3.2).
- (6) The biomolecular interaction goes through multiple steps until a more stable complex is formed.

These effects/problems can be pinpointed relatively easy and in many cases be circumvented^[47] or modeled. The fourth point is worth discussing further as it is a common issue with biosensors. Since the biomolecules are immobilized on a surface, they will interact with analytes that are in close proximity to the surface. As they bind their partner, there will consequently be a decrease in the concentration of the free partner at the surface. Analyte molecules from the bulk will diffuse to the surface (aided by the flow system) to replenish the captured analytes. This flux of analyte molecules to the surface (per unit area) is given by

$$j_i = 0.98 \sqrt[3]{\frac{D^2 f}{h^2 w l}} [B] \quad 3.5$$

as described by Sjölander and Urbaniczky.^[74] D is the diffusion coefficient of the analyte, f the volumetric flow rate, h the height of the flow cell, w the width, and l the length. Thus, j_i describes the supply of analytes to the surface and equation 3.1 describes the consumption. The interaction is said to be mass transfer limited when the consumption at the surface is greater than the supply to it. Mass transport effects are most profound in the beginning of an

adsorption phase, since the consumption is at its maximum there. The problem can be avoided by increasing the supply, e.g. by increasing the flow rate, and/or by decreasing the analyte consumption by reducing the concentration of immobilized biomolecules, i.e. decreasing the available binding sites on the surface.

There are, however, more fundamental complications involved in measuring kinetics on surfaces. Since one of the interacting partners is immobilized this will be a completely different situation as compared to the native state (with the exception of membrane-bound biomolecules), where both the interacting partners are free in solution. This problem, however, can be significantly reduced by immobilizing biomolecules on a surface modified with a highly mobile and flexible support, e.g. a dextran matrix,^[75] which more closely resembles the native environment of biomolecules. Due to the discussed complications involved in measuring kinetics on surfaces there has been criticism to it as measurements in many cases produced diverging results.^[11] The criticism is justified; there are many problems that can occur and it is therefore important that one knows about them and how to identify them, so that appropriate measures can be taken to obtain the true kinetic parameters. Judging from a lot of (recently) published work, many researchers clearly have not understood these issues as has been reviewed by Rich and Myszka.^[27] However, the review also contains some very good examples of high-quality publications, and it is therefore stressed that with careful experiment design it is possible to obtain reliable data. As an example, Day *et al.* show in a study of a challenging enzyme-substrate system that surface kinetic measurements give highly matching data compared to traditional solution-based methods.^[72]

3.4 Towards Higher Throughput with Microarray Chips

In the previous chapters and sections, biomolecules and their interactions with corresponding binding partner(s) on solid supports have been discussed. Until (fairly) recently, biosensors have only been designed to monitor the interaction of one or a few immobilized biomolecules per sensor surface (chip). However, untangling and functionally characterizing the enormous biomolecular interaction networks of biological organisms call for more efficient techniques. Automated, highly multiplexed binding assays^[76-80] are tools that are suitable and needed for these purposes. Microfabrication procedures^[81] can be used to

produce high-density two-dimensional arrays of immobilized biomolecules. Normally, the samples are delivered to the surface with automated (robotic) subnanoliter liquid dispensing units.^[78, 82-84] The dimensions of the structures are in the micrometer range, which allows for hundreds to (many) thousands of individual biomolecule spots per sensor chip. Thus, high throughput (HT) analysis is provided since (potentially) many thousand (or more) interactions can be followed in parallel by introduction of samples containing complex mixtures of interaction partners. Flow cells and liquid delivery systems can be made with small volumes which, in addition, provide the feature of low sample consumption. A further benefit with microarrays is that it allows "local referencing", *i.e.* reference regions are in close proximity to the specifically interacting regions, which can increase the quality of the assay. Also, negative and/or positive control spots can be included in the array.

Following the success of DNA microarray chips^[77] protein microarrays have been developed.^[22, 33, 34, 76, 80, 85] The DNA chips provide considerable data on gene expression in organisms but it is a general belief that protein arrays are more relevant for analyzing gene function, regulation, and a variety of other applications.^[33, 80, 82, 85] This, since it is ultimately the activity of the encoded proteins that directly manifest gene function, *i.e.* cellular function, in an organism.^[1, 80] Furthermore, the function of a protein is not simply a question of its existence or not, it is also a result of post-translational modifications like phosphorylation, dephosphorylation, glycosilation, of compartmentalization, and/or of interactions with other proteins/molecules.^[1, 33]

Approaches combining label-free detection methods with protein microarrays have been found to be promising.^[86, 87] Several label-free optical principles have been developed for parallel screening of protein interactions.^[88-90] Approaches that require *e.g.* fluorescent labelling are more sensitive and have proven very successful.^[78, 84] They are, however, limited in their flexibility compared to label-free techniques. For instance, the labelling can disrupt the biomolecules' native functions, it is an additional (timeconsuming) step, and fluorescent probes are usually bleached over time rendering real-time measurements difficult (see previous section).

A key problem is immobilizing proteins on arrays while maintaining their function. Unlike DNA, proteins are extremely sensitive to their environment, owing to their intricate three-dimensional native structure with low stabilization

energy (section 2.2). Many immobilization schemes of proteins are incompatible with microfabrication procedures. Therefore, even more care normally needs to be taken when immobilizing proteins on microarrays. An additional problem, as highlighted by Barry and Soloviev,^[91] concerns the actual signals generated by the biointeractions on a protein microarray. By having a variety of different proteins on a surface, there will be a large heterogeneity of binding affinities (and kinetics) to respective analytes. As a result, some immobilized proteins will bind large quantities of their analyte, while others will bind low, which could potentially result in saturated sensor signals or undetectable signals, respectively. It is therefore important when performing quantitative protein profiling to design the sensor and experiments to fall within the dynamic range of the sensor.

Microarrays have a wide range of possible applications, it is not only meant as a tool for gaining insight into the molecular pathways of the cell. Below, the author has summarized four areas, which in his opinion are important and good examples of where (protein) microarrays have been or can be used successfully. DNA microarrays have, despite their high success, been somewhat left out of the discussions below mainly due to the author's focus on proteins.

3.4.1 Functional Proteomics

Currently, methods normally used for proteome analysis include 1D and 2D gel electrophoresis (GE), mass spectrometry (MS), affinity chromatography and more.^[76] Of these, protein separation with 2D-GE and subsequent identification with MS are the core technologies in large-scale proteomics. With the potential of much higher throughput with time and cost savings, protein microarrays have therefore had a great impact in functional proteomic research.^[78, 84]

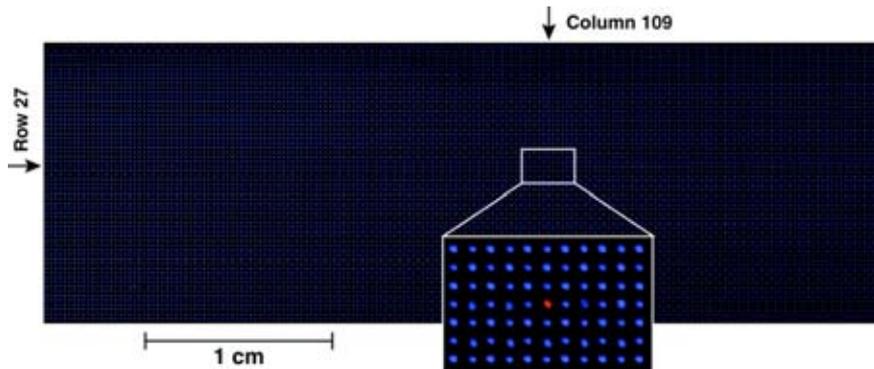


Figure 3.7. A protein microarray consisting of 10,800 spots. Protein G has been printed 10,799 times and a single spot of FRB (FKBP12-rapamycin binding protein) has been printed in row 27, column 109. From ^[78]. Reprinted with permission from AAAS.

Chips have been produced with relatively high densities (e.g. 1600 per cm^2)^[78] of individual protein spots on surfaces, figure 3.7. This is still, on one hand, many times less than high-density DNA microarrays.^[81, 92] The DNA spots are, however, chemically synthesized directly on the chip using photomasks, which allows much higher feature densities. Proteins are much more complicated than DNA and cannot be synthesized chemically with satisfactory results. Therefore, native and functional protein samples are normally delivered to the surface in subnanoliter volumes from a microdispensing unit, which can be computer controlled for rapid and automatic arraying. The size of the dispensed spots will depend on the difference in surface free energy between the dispensed sample and the surface. The spots also need to be separated from each other far enough to avoid contact and resulting cross-contamination. Hydrophobic surfaces allow a higher array density, since dispensed hydrophilic protein solutions will not spread much on them. In cases where hydrophobic surfaces cannot be used, using physical or chemical barriers can increase the potential array density. For instance, in our work we have repeatedly been using chemical barriers of hydrophobic molecules to contain microdispensed samples with excellent results.

A problem when arraying proteins is the difficulty of obtaining functional proteins after immobilization. Since many different proteins are used, it is hard to optimize the conditions for them all simultaneously. The microfabrication procedures can be harsh for the proteins and care has to be taken. Another

common problem when producing protein microarrays is the difficulty in obtaining a large selection of relevant proteins to dispense for the application of interest. Usually a few are available and to demonstrate the concepts of parallel analyses, they are dispensed in multiple spots. For instance, MacBeath and Schreiber have produced arrays of 10.800 spots, but only of a very small set of unique proteins (two),^[78] figure 3.7. Despite this, their work convincingly showed the vast potential of the technique.

Shortly thereafter, Zhu *et al.*^[84] produced a microarray consisting of 5800 unique proteins from the yeast proteome, which corresponds to roughly 80% of all the yeast proteins. The set of proteins was produced with recombinant cloning technology and immobilized either covalently or via a histidine-tag, see chapter 3.2. Signals from proteins immobilized via the histidine tag were found to be superior. They then carried on by screening their protein microarray for interactions with proteins and phospholipids with results in good agreement to previous knowledge on the yeast proteome. The advantages of the microarray chip, they conclude, is that sets of individual proteins can be directly screened in vitro for many activities, such as protein-drug and protein-lipid interactions, enzymatic assays and so on. Also important is that, once the protein microarray is produced, proteome screening is significantly faster and cheaper than existing techniques (see below). Using their concept, they believe it possible to make arrays of up to 100.000 proteins for global proteome analysis in other eukaryotic organisms, including humans.

In a somewhat different approach, Fukui *et al.*^[93] have constructed microarrays of 30-42 oligosaccharides for high-throughput analysis of carbohydrate-protein interactions. The initial results of their experiments was able to give some novel insights into the field. They believe their technology can potentially be used to produce more spots of oligosaccharides. This work is an important achievement as carbohydrate binding proteins are crucial mediators of processes such as protein folding and trafficking. They are also important parts of immunity mechanisms and microbe-host interactions. However, identification and elucidation of the parts on the oligosaccharide chains that are recognized by proteins is very difficult. The diverse and heterogeneous nature of the oligosaccharides of glycoproteins is a key ingredient to this difficulty. Their approach gives a new way for discovering

carbohydrate-recognizing proteins in the proteome and analyzing the many carbohydrate recognition structures of the glycome.

To overcome the problem of shortage of proteins to spot on the array, Ramachandran *et al.*^[79] have dispensed DNA sequences on a surface. Proteins were then translated from the DNA *in situ* on the chip using biochemical reagents and immobilized directly. They were able to produce 29 proteins from the replication system on an array and to map their interactions with query proteins, known to be involved in the replication system of cells.

There is some concern that by using recombinant proteins, or other "unnaturally" produced types that one loses the possible posttranslational variants of some proteins. To address this issue, Madoz-Gurpide *et al.*^[82] have separated proteins from lung adenocarcinoma (a common lung cancer) cell lysates and dispensed them on a microarray. Dispensed proteins were identified with antibodies with good correlation to existing techniques. Proteins from cell and tissue lysates can be arrayed and screened against patient samples, thereby providing an invaluable tool for *e.g.* early cancer diagnosis. In a following study, the microarray has been tested for the diagnosis of lung cancer patients with promising results.^[94] The technique is new and the heterogenous nature of cancer between different patients requires better statistical methodology to be able to completely differentiate cancer patients from healthy controls, as was clearly shown with the study. However, their approach is very interesting as it provides a virtually limitless number of available proteins to dispense and the proteins are fully "natural" and represent the true proteome of a cell/tissue. It does, however, require that the proteins are separated with good efficiency and that they are properly identified for meaningful analyses.

In a final, high-lighted example, de Wildt *et al.*^[95] have constructed arrays of (recombinant) antibodies for HT screening of antibody-antigen interactions. The arrays consisted of more than 10.000 unique antibodies and were successfully screened against several antigens in complex solutions. Although the arrays might not technically fall within the micrometer-domain, their approach shows great potential for minituarisation and higher array density. Their microarrays can, in addition to the demonstrated antibody-antigen interaction screening, potentially be used for many applications within proteomics; ranging from detection of modified proteins to profiling protein expression.

3.4.2 Pharmaceutical Research and Drug Discovery

Biosensors and protein microarrays can be used in basic pharmaceutical research with similar approaches as discussed above. In addition to the basic research, biosensors can with great benefits be integrated into the drug discovery process at several stages in pharmaceutical research. In (drug) target discovery and characterization, the early stages of the process, they have been used for ligand fishing, hit confirmations, and to monitor antibody and cytokine production. As biosensors have become more sensitive they have also been successfully used in compound screenings, where they have been applied for the selection of *e.g.* thrombin inhibitors, HIV-protease inhibitors, and DNA-gyrase inhibitors. The ability to measure kinetics has also been important for the lead optimization stages.

The interaction of a drug with serum proteins/components (*e.g.* albumin) is an important aspect when determining drug pharmacokinetic and activity profiles. When screening a lot of potential drugs, it is beneficial to immobilize purified serum proteins on biosensor chips. This allows probing the interactions at an early stage of the drug discovery stage, and thereby quickly eliminating substances with poor ADME (absorption, distribution, metabolism, and excretion) properties. Biosensors are very good substitutions/complements to traditional assays like equilibrium dialysis or affinity chromatography. Mainly due to the low sample consumption, the low time consumption, and the high throughput possibilities (with automation). Excellent agreements have been found for biosensor data compared to the traditional methods. Two further examples of biosensors in drug discovery is for screening against membrane receptors in their native environment as briefly discussed in section 2.2.2 and in quality assurance and control.

So far, nothing has been said about microarrays. It is, however, apparent that by being able to use microarray formats in drug discovery the parallelization would further increase the throughput and efficiency of the biosensor assays. The economical profits to a pharmaceutical company successfully utilizing this technology could be very significant.

This section has been a summary of the excellent and very comprehensive review by Cooper,^[26] which the reader is encouraged to read for more information and references.

3.4.3 Clinical Diagnostics

Biosensors within clinical diagnostics are mainly used for the detection/quantification of (bio)molecules in patients. Clinical diagnostics was first to use biosensors with the development of the glucose sensor in 1962^[24, 28] with great importance to diabetes patients. Furthermore, it has been vital for the further development of biosensors since it has shown that there is an enormous potential market for good sensor systems within this (and other) areas.

The classic ELISA is a good method for detecting binding of biomolecules in patient samples. However, problems arise when there is a limited sample availability and when multiple analytes need to be measured (increasing time consumption). Microarrays are tools that can potentially fill the need for multiparametric analyses of biological samples with low sample consumption, higher throughput, convenience, and lower cost. Several publications have shown this potential in a variety of ways, discussed below. The possibility for diagnosis of cancer with protein microarrays has already been discussed above (section 3.4.1).

Vo-Dinh *et al.*^[96] have constructed a miniaturized biosensor based on DNA microarrays using a photoarray integrated circuit as a detection unit. This means that it could easily be manufactured as a portable unit and used in for example a physician's office. They illustrated its use with the probing of the human immunodeficiency virus 1 (HIV1).

Mezzasoma *et al.*^[83] produced a protein microarray of microbial antigens for the parallel detection of antibodies expressed in human sera against *Toxoplasma gondii*, rubella virus, cytomegalovirus, and type 1 and 2 herpes simplex virus. Excellent agreement between microarray and ELISA detection was demonstrated in human serum samples thereby showing the great potential for microarrays being used for the diagnosis of infectious diseases. Furthermore, they have summarized the sample and reagent consumptions together with time use and estimated costs. While ELISA shows an additive time consumption and cost for each additional analyte to be measured, microarrays are constant regardless analyte amount.

In a somewhat related study, Wiese *et al.*^[97] have designed the equivalent to an ELISA in a microarray format. Their micro-ELISA was able to

simultaneously measure prostate-specific antigen (PSA), antichymotrypsin-bound PSA, and interleukin-6. These proteins are serum markers of prostate health and indicators of prostatic cancer. They demonstrated the usefulness of their assay as a diagnostic tool by measuring the named proteins in human serum samples with similar results as normal ELISA.

Microarrays have also been shown to work for allergy testing by Wiltshire *et al.*^[98] In their interesting study they produced a microarray consisting of some common allergens (extracts of cat hair, house dust mites, and peanuts) and tested its performance on 30 allergy patients by quantifying their allergen-specific IgE antibody levels. Results were, again, in excellent agreement with the existing quantitative technique. In most cases of the study, the microarray even surpassed the performance of the existing technique in terms of sensitivity and specificity.

Despite all these promising results, the usage of microarrays in diagnostics has delayed. The natural reason for this is that it is still relatively new and unproven but also because of the low stability of proteins, complex coupling chemistries, and relatively weak detection signals. Protein microarrays are still difficult to manufacture and to validate for clinical use.^[83] Furthermore, following the discussion in section 3.1, complex samples like blood/serum have considerable non-specific interactions with surfaces, which needs to be addressed when designing the biosensors.

Nevertheless, the enormous potential benefits of using microarrays in diagnostics remain. The author is of firm belief that the technique will develop and that microarrays will be used in medical healthcare in the future. The ultimate goal is a microarray system that can diagnose any disease or medical disorder a patient is suffering from with a single drop of blood sample, or less.

3.4.4 Trace Detection of Harmful or Illegal Substances

Environmental monitoring of harmful or illegal substances is an area of application where protein microarrays also have great potential. There is a growing demand in rapid and dependable systems being able to detect trace levels of multiple harmful or illegal compounds, especially after the terrorist attacks on the World Trade Center in New York, USA. The dominating technique for field-detection of harmful or illegal compounds, ion mobility spectrometry, was discussed in the general introduction (chapter 1). Biosensors are also

suitable for this type of application with the possibility for improved selectivity due to biomolecular recognition. They, furthermore, also offer the possibility of on-site measurements and operation close to the field. So far, antibodies have been most extensively used as recognition elements because of their suitable features, see section 2.2.1. As discussed there, antibodies can (theoretically) be produced against virtually any compound. Therefore, protein microarrays could be made for the detection of for instance narcotics, explosives, toxins, microbial pathogens, and biological and chemical warfare agents. Antibody spots can be immobilized at high densities on one surface aimed against many different types of antigens (analytes). This means that complex mixtures (cocktails) of analytes can be analyzed with high selectivity with no requirements of pretreatments (e.g. separations/purifications). In theory, one (ultimate) chip could monitor all the compounds mentioned above simultaneously.

Possible areas of application include for example at customs, in airport and public transportation security, within the police force and at correctional facilities, drug interdiction, forensics, warning alerts on military equipment and personnel, and monitoring drinking water reservoirs. Protein (micro)arrays have already been shown to work for parallel detection of explosives,^[30] toxins,^[99] and bacterial microbial pathogens.^[99] Since many of the analytes of interest are low-molecular weight compounds, direct detection is normally difficult. In such cases, indirect detection can be used, which can increase the signals. Sapsford *et al.*^[30] demonstrate direct detection together with three variations of indirect detection (competitive, displacement, and sandwich) on the same type of microarray chip. In the mentioned areas of application, one is normally only interested in whether a harmful or illegal compound is present in a sample or not. Therefore, the analysis is more of a positive/negative type (hit/no hit), and important sensor features are then the lowest detectable analyte concentrations (limit of detection) and the selectivity.

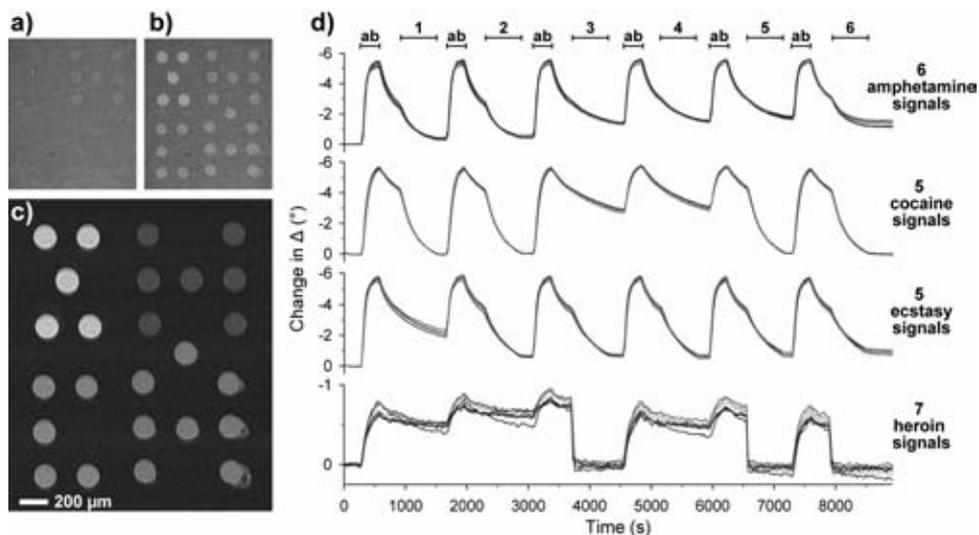


Figure 3.8. Imaging SPR for parallel detection of four narcotics. H=heroin, X=ecstasy, C=cocaine, and A=amphetamine. a) Live CCD-image of the narcotic array chip after docking in the instrument. Reflected (p-polarized) intensity is directly proportional to adsorbed mass on the chip. b) The chip after antibody loading. c) Difference image, *i.e.* image (b) subtracted by (a), showing the specific antibody adsorption to the surface. d) Analyte (narcotics) detection with the instrument in ellipsometric mode, Δ is inversely proportional to adsorbed mass. Injected cocktails consisted of 1: A+C, 2: A+C+X, 3: X+H, 4: X, 5: C+X+H, 6: A+C+X+H. For more details, see paper E.

In our work (paper E) we have constructed a protein microarray for the parallel detection of four narcotics, amphetamine, cocaine, ecstasy, and heroin. We have employed a displacement strategy for detection; antibodies are displaced from the sensor surfaces on an analyte injection and generate an indirect signal. In figure 3.8, the array is shown, together with signals generated from an experiment where cocktails of narcotics were injected. Continuous (real-time) measurement of binding events on the surface was undertaken with imaging surface plasmon resonance (SPR), which is a label-free technique (see sections 5.4 and 5.5). Detection limits of the narcotics were roughly determined to be 0.5 pg/μL for heroin and 5 pg/μL for the other three. In addition, the same microarray has also been shown to work together with the additional monitoring of the explosive trinitrotoluene (TNT). Our proof of concept chip could easily be upscaled to include parallel detection of more narcotics and other illegal and dangerous substances. Thus, making it a

potentially invaluable tool for public safety in the future, e.g. in the fight against crime and terrorism.

4 SURFACE MODIFICATIONS

An important aspect of most types of biosensors is the surface on which the biomolecular interactions will take place. A well-behaving surface is often the key to stable, efficient, and reliable biosensors. In this chapter a description of the surface modifications used in the included papers is given.

4.1 Self-Assembled Monolayers (SAMs) of Thiols

In 1983 Nuzzo and Allara discovered that organic disulfides spontaneously formed well-ordered monolayers on gold.^[100] They also showed that by choosing different functional tail-groups on their molecules, surfaces could be tailor-made with the physical and chemical characteristics of the respective tail-groups. This started a new era in the field of surface modifications. SAMs of many types of organosulfur compounds were found to be easily prepared on a range of different metal and even semiconductor surfaces, *e.g.* silver, copper, platinum, mercury, iron, GaAs, and InP.^[101] The preferred choice of substrate is in most cases gold. The reason for this is that it is easy to obtain in relatively pure form and thin films of it can be prepared easily on substrates by *e.g.* physical vapor deposition, ion sputtering, and electrodeposition. Gold is also a relatively inert metal, it does not oxidize under normal ambient conditions and is not reactive towards most chemicals.^[102]

The most common type of organosulfurs used for forming SAMs is alkanethiols, figure 4.1. They consist of a thiol head-group (HS-) coupled to a fatty alkane-chain of varying length $-(\text{CH}_2)_n-$ and terminated by a functional tail-group, *e.g.* a methyl, alcohol, or carboxyl. The binding energy of the thiolate bond with the gold surface is roughly 40 kcal/mol,^[101] which is very strong and corresponds to covalent binding energies. Thus, under normal conditions, the thiol adsorption is irreversible. The kinetics of alkanethiol (~ 1 mM) adsorption on gold usually reveal two steps,^[101] illustrated in figure 4.1. The first step is very fast and lasts ~ 1 min. During this step, which is mainly governed by the surface to head group reaction, the thickness of the monolayer

reaches roughly 80-90% of its final, maximum value. The second step takes several hours and can be described as a two-dimensional crystallization process as the alkane-chains go from a disordered phase (*gauche*) and form ordered unit cells (*all-trans*) as the monolayer density reaches its final value. In the monolayer, lateral van der Waal (vdW) interactions between neighboring alkane-chains will increase the stability and also effectively form a dense barrier, protecting the metal from the ambient. Normally alkanethiols will be tilted with respect to the normal on gold-surfaces. This is a result of a lattice mismatch between the adsorbed thiol on gold and a close-packed alkane layer. Hence, as the adsorbed molecules try to maximize their vdW interactions, a tilted layer is the consequence. Alkanethiols on Au(111) are normally tilted $\sim 26\text{-}28^\circ$ ^[101] from the surface normal. More details about the formation and structure of SAMs can be found in a text^[103] and review^[101] by Ulman. A more recent review is given by Whitesides *et al.*^[102].

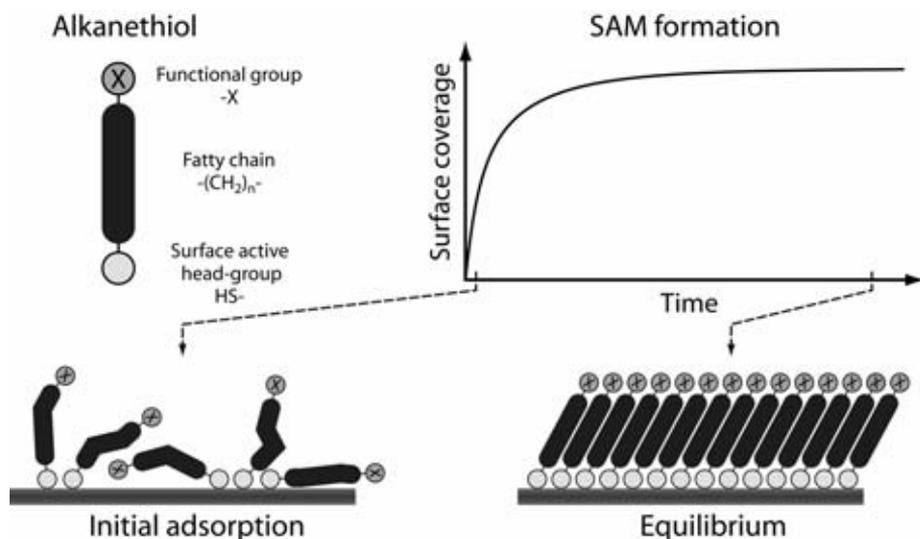


Figure 4.1. Schematic illustration of alkanethiols and their self-assembly on a gold substrate. In the first, rapid step of adsorption the thiol head group adsorb to the metal. The alkane chains are disordered on the surface and have a *gauche*-rich structure. In the second step the the alkane chains become highly ordered (*all-trans*) as the surface density increases and form a two-dimensional crystal lattice.

SAMs of alkanethiols have found great applications in the field of biosensors, as they serve as an excellent intermediate between a biomolecule

and the transducer surface. Their assembly on sensor surfaces provides a convenient way to control the surface composition, orientation, and presentation of biological macromolecules like proteins, peptides, DNA, and carbohydrates. Further modifications of the SAM layer can be performed with surface chemical reactions, *e.g.* Löfås and Johnsson^[75] have coupled a dextran hydrogel matrix to SAMs which has had great success in the biosensor field. Depending on the terminal functional tail-group of the alkanethiol, the SAM can have different interactions with the biomolecules. It can either attract biomolecules specifically or non-specifically, or it can repel them. An example of complex functional groups was shown in figure 3.5a. The interactions of SAMs with biomacromolecules, in particular proteins, has been extensively reviewed.^[35, 104] In paper A-C the specific adsorption of histidine-tagged proteins to nitrilotriacetic acid SAMs is reported. In paper E, however, a nonspecific interaction is used to immobilize proteins to a hydrophobic SAM.

4.2 Patterning

In this section a background will be given to the methods used for patterning the surfaces in the included papers. Naturally, many more ways to create structured patterns on surfaces exist. For a comprehensive introduction to microfabrication the reader is encouraged to see the text by Madou.^[81]

4.2.1 Microcontact Printing

Microcontact printing (μ CP) is a simple, cost-effective, and diverse soft-lithographic technique that can be used to form patterns of SAMs with linewidths ranging from 30 nm to 500 μ m.^[105, 106] It can also be used to form patterns of other types of molecules, *e.g.* proteins,^[107, 108] with very good results. For more details about μ CP see the excellent review by Xia and Whitesides.^[106]

In a simple example (figure 4.2), μ CP involves "inking" an elastomeric stamp with a solution of the molecule that is to pattern the surface. The stamp is then dried and put in contact with the substrate surface and the inking molecule is then transferred to the substrate as determined by the topographic pattern of the stamp. The non-stamped areas of the chip can be covered by another molecule by *e.g.* solution incubation, or be further processed if chosen.

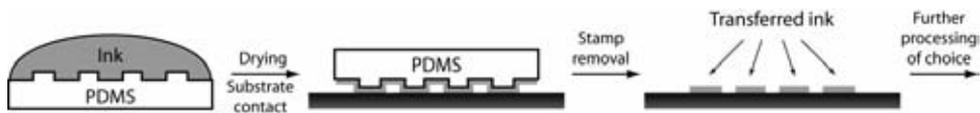


Figure 4.2. Schematic illustration of μ CP. The inked PDMS stamp is dried and brought in contact with the substrate. The ink is transferred to the substrate where there is contact with the stamp.

The most common material for the stamp is poly(dimethylsiloxane) (PDMS), also known as silicone. A PDMS prepolymer is cast and cured on a master, which has been produced by photolithography and contains the desired topographic structure in mirrored form.^[106] PDMS is a highly flexible material with a very low surface energy which makes it very suitable for printing alkanethiols. Printed alkanethiols form dense and well-ordered SAMs^[109] which have been shown to resist chemical etching.^[105] One of the known problems of μ CP is the possibility to transfer PDMS residues to the substrate and thus contaminating it.^[110] However, in the case of printing alkanethiols, Zhou *et al.* have shown that the PDMS contaminants can easily be removed by sonication in ethanol.^[109]

In the included papers μ CP has been used to form arrays of hydrophobic barriers, forming micro-wells, with methyl-terminated alkanethiols. The barriers were able to effectively contain dispensed solutions with water or ethanol as solvent in the wells.

4.2.2 Ink-jet Printing

Commercial ink-jet printers have been used with personal computers for many years (1980s). The potential use of the technology in microfabrication was discovered later on.^[81] An ink-jet printer is able to controllably dispense liquid droplets with sub-nanoliter volumes on a surface. The droplet is ejected from the printer head at a high velocity and traverses a distance in air to the underlying surface. On this micrometer scale, surface tension forces are dominant over the accelerating forces the droplet will experience, which means that the liquid droplet will remain intact after impact on the surface. The liquid is driven out from the printer by a pressure pulse. Thermal ink-jet printers, create the pulse by heating the ink and piezoelectric ink-jets produce the pulse by decreasing the volume of the liquid. Ink-jet printing has been used to

dispense e.g. alkanethiol SAMs,^[19, 111-113] DNA oligonucleotides,^[111] and proteins.^[112, 114, 115] It has been shown that even inexpensive commercial ink-jet printers with slight modifications can be used for dispensing with very good results.^[112, 113, 115]

The maximum spot-density on the dispensed array is determined by the wetting properties of the dispensed liquid on the surface, *i.e.* the difference in surface energy. Hydrophilic surfaces have low contact angles with hydrophilic liquids, thereby, the dispensed spots will spread over a large area. Hydrophobic surfaces, on the other hand, have high contact angles, giving more compact spots. To decrease the spreading of droplets on a surface, hydrophobic barriers made by μ CP of methyl-terminated alkanethiols can be used, illustrated in figure 4.3.

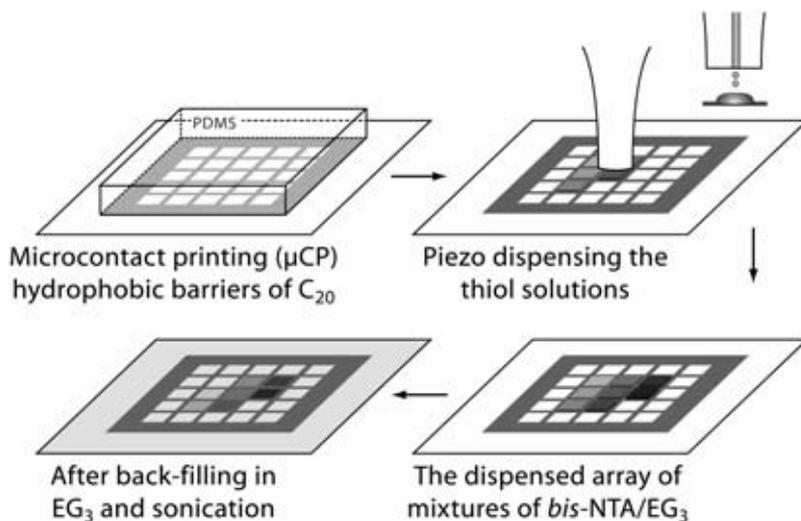


Figure 4.3. Using microcontact printed hydrophobic barriers of eicosanethiol (C_{20}) for dispensing ethanolic solutions of functionalized thiols (*bis*-NTA and EG₃). Figure from ^[19].

When dispensing liquids on surfaces, one has to be aware of the possibility of ring formations, or "coffee rings", at the periphery of the spot. The underlying mechanism, as investigated by Deegan *et al.*,^[116] is capillary flow of liquid towards the edges of the spot due to contact pinning, as the droplet evaporates. These imperfections in spot homogeneity can lead to possible complications when evaluating the function of the dispensed array. However,

they can often be avoided by hindering droplet evaporation and/or by using higher concentrations of the surface modifying molecule.

5 INSTRUMENTATION

In this chapter a presentation of the major instrumental techniques used in the papers will be given. They all have in common that they are optical and require no labelling of molecules. A brief theoretical background will be given together with applicational aspects of each technique. For a more elaborate description, the reader is encouraged to turn to the cited references.

5.1 Infrared Reflection Absorption Spectroscopy (IRAS)

Atoms in a molecule are connected to each other with bonds that can be approximated to be springs with characteristic spring constants. For two connected atoms in a molecule the vibration frequency is given by:^[117]

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{m_{\text{red}}}} \quad 5.1$$

where k is the force constant of the bond and m_{red} the reduced mass of the two atoms. By approximating the molecular vibrations with harmonic oscillations the allowed vibrational energy levels are:^[117]

$$E_v = \left(v + \frac{1}{2}\right) \cdot h\nu \quad v = 0, 1, 2, \dots \quad 5.2$$

The separation between two adjacent levels is $\Delta E = h\nu$, therefore the molecule is able to absorb light with the same frequency as its oscillation. Normally, molecular vibration frequencies lie in the infrared region of the spectrum. Therefore, infrared (IR) spectroscopy is suitable for studying molecular vibrations. The two atoms in a bond will have a characteristic spring constant and hence different atoms will have different vibration frequencies. IR spectroscopy can then identify molecules (or compositions of them) in a sample based on the observed absorption frequencies.

For a molecular vibration to be able to interact with the electromagnetic field of light, its dipole moment must change under the vibration. This condition can be formulated as:^[117]

$$M = \frac{d\mu}{dR} \neq 0 \quad 5.3$$

where μ is the dipole moment of the bonded atoms in the molecule and R is their separation and M is then the dipole moment change under the vibration. Molecular vibrations fulfilling equation 5.3 are called to be infrared active, since they can absorb IR light.

IR spectroscopy can be used to study molecular monolayers on surfaces in reflection mode, *i.e.* IRAS. However, since there are relatively few molecules able to absorb the light on the surface, mainly two strategies need to be employed to obtain good signals. The first is to measure many spectra and to average them, hence increasing the signal-to-noise ratio. Traditional spectrometers work with a monochromator that scans the wavelengths one at a time over the sample, which of course then takes an unreasonably long time. Fourier Transform (FT) IR spectrometers contain a Michelson-interferometer, which means that a continuous light source is used to irradiate the sample at once. The obtained interferogram is transformed into a spectrum by the mathematical FT operation. The speed at which an interferogram is recorded is given by the speed of the moving mirror in the interferometer. Typically, approximately 300 interferograms can be collected per minute. The second strategy is to measure at a grazing angle, *i.e.* at a very high angle of incidence. The reason for this is two-fold and is illustrated in figure 5.1.

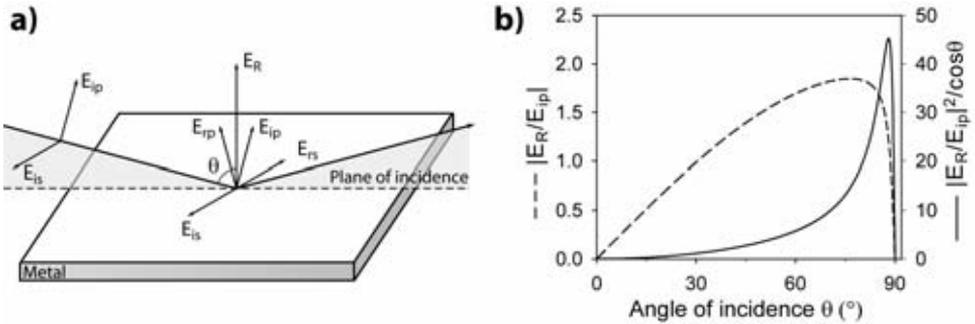


Figure 5.1. Illustration of IRAS. a) The incident (i) and reflected (r) electric fields on a metal surface. The plane of incidence is indicated in the figure and electric fields parallel to it are denoted with p and orthogonal with s. E_R is the net field close to the metal surface. b) Fresnel calculations showing $|E_R/E_{ip}|$ and the net relative driving force for exciting molecular vibrations. $N=9.05+28.20i$ was used as refractive index for gold.

In figure 5.1a the electric field on a metal (gold) surface is shown schematically. According to Fresnel reflection calculations the s-component (orthogonal to the plane of incidence) of the reflected light's electric vector (E_{rs}) will be phase-shifted $\sim 180^\circ$ with respect to the incident (E_{is}), which results in a net \sim zero electric s-field close to the surface. The p-component (parallel to the plane of incidence) of the electric field, on the other hand, will not phase-shift 180° upon reflection. Thus, at the surface the only electric field will be due to the superposition of the incident and reflected p-components (E_{ip} and E_{rp} , respectively). This resulting field at the metal surface, E_R , will then be normal to it. Figure 5.1b shows E_R quoted against E_{ip} for different angles of incident calculated with Fresnel's formula. It can be seen that there is a maximum in E_R at 76° where its amplitude is almost double the incident field, which is one of the benefits with measuring at near grazing angles. The other is that an incident angle above 0° will increase the area of the chip illuminated by the light beam as $1/\cos\theta$, thereby interacting with more molecules, which increases the signal. Also in figure 5.1b, this net relative intensity driving force for exciting molecular vibrations, $|E_R/E_{ip}|^2/\cos\theta$, is plotted. It can be seen that its maximum value results in a 45-fold effective increase in driving force at 88° (for the refractive index used in the calculations).

The absorption of light by a molecular vibration is proportional to the square of the scalar product of the electric field and the dipole moment change (equation 5.3), which in the case of IRAS is:

$$I_A \propto |\overline{\mathbf{M}} \cdot \overline{\mathbf{E}}_R|^2 = |\overline{\mathbf{M}}|^2 \cdot |\overline{\mathbf{E}}_R|^2 \cdot \cos^2 \phi \quad 5.4$$

where ϕ is the angle between the two vectors. Since E_R is directed normal to the surface, any off-normal molecular vibrations will decrease their absorption as $\cos^2\phi$. Equation 5.4 is called the surface selection rule, which adds the ability to IRAS to be able to determine the molecular orientation of adsorbed molecules.^[100, 118]

The above listed features of FT-IRAS allow characterization of *e.g.* ultrathin SAMs on Au with very good signals.^[100] Also, IR spectroscopy can be used to investigate vibrations in proteins and *e.g.* secondary structure (conformation) information can be obtained.^[37, 119]

5.2 Ellipsometry

Ellipsometry^[120] is a relatively old optical technique able to measure the refractive index of material surfaces and investigate adsorbed films on the same. The principle is that the polarization of incident light changes upon reflection on a surface, which the ellipsometer is able to measure. From the change in polarization, the refractive index and the thickness of a film on the surface can be determined by optical modeling.^[120] Since the calculations are quite tedious, it would take until the development of microcomputers before ellipsometry became a widespread technique. Many instrumental setups exist for measuring the polarization change of light after interacting with a sample. One common setup is called the null ellipsometer. The main components of a null ellipsometer are shown in figure 5.2. They are a light source, optical components that are used to manipulate the polarization of the light prior to and after reflection (polarizer, compensator and analyzer), and a detector. The polarizer (P) and analyzer (A) are simple linear polarizing filters, whereas the compensator (C) is a quarter-wavelength plate. Normally, the compensator is kept at $\pm 45^\circ$ and the polarizer and analyzer are rotated until a minimum in the detected signal is reached (null intensity). At this particular setting (null condition) the

light will be linearly polarized after reflection on the substrate and the analyzer will be rotated 90° with respect to it.

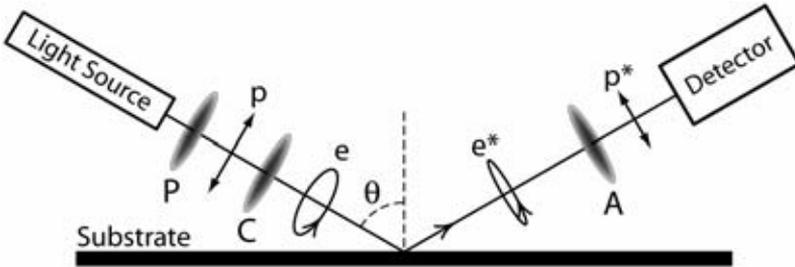


Figure 5.2. Schematic illustration of null-ellipsometry, P=polarizer, C=compensator and A=analyzer. The polarization of light is shown at different stages; lp =linearly polarized and e =elliptical. After reflection, e^* will be plane polarized for the correct P-C-A combination (null condition) and p^* will ideally have zero (null) intensity.

The optical modeling for null ellipsometry is rather complicated. The parameters Δ and Ψ for an isotropic material can be defined using the quote of the complex reflection coefficients R_p and R_s , which is actually measured by the ellipsometer:^[120]

$$\frac{R_p}{R_s} = \left| \frac{R_p}{R_s} \right| \cdot e^{i(\delta_p - \delta_s)} \equiv \tan \Psi \cdot e^{i\Delta} \quad 5.5$$

where Ψ is the angle of which tangent gives the ratio of amplitude change for the p and s vector components, and Δ represents the relative phase shift of the p and s components upon reflection. Together they are called the ellipsometric angles. After a successful null ellipsometry measurement of a substrate, one has a set of P , C , and A angles, which are converted to the ellipsometric angles by simple linear relations.^[120]

The ellipsometric angles can be used to obtain information of the measured system. For example, the film thickness of an organic adlayer can be calculated. In order to obtain such a parameter, one has to construct an optical model based on Fresnel's formulas that relates Δ and Ψ to the thickness, and then fit the model output to match the measured values. In other words, the

film thickness is varied until the resulting model angles of Δ and Ψ equal the observed data.

Ellipsometry has many positive features; it is fast and easy to perform, and no labeling of molecules (e.g. fluorescence) is required. The vertical resolution for measuring films is ~ 1 Å. The drawbacks are the fact that a reflecting surface has to be used and that a homogenous film has to be present on the surface. These limitations preclude the use of ordinary null ellipsometry for the evaluation of micro contact printed substrates. However, imaging null ellipsometry can be used for the characterization of these substrates (see below). Another disadvantage is the procedure of obtaining the substrate refractive index reference. Since it is normally performed in room atmosphere, the bare gold surface lowers its surface energy by adsorbing "pollutants", thus not really giving the refractive index of gold. After, for instance, a SAM formation, the "pollutants" will have been outcast by the competing alkanethiols, having a greater affinity to gold. However, the pollutants have already affected the determination of the substrate's refractive index. Therefore, a systematic error results, which varies unpredictable for different runs of gold substrates. In order to reduce this error, contaminants should be kept to a minimum.^[103]

5.3 Imaging Ellipsometry

Imaging ellipsometry^[121] is a natural development of ellipsometry. An imaging null-ellipsometer is obtained with the upgrade of the non-imaging setup by a charge-coupled device (CCD) camera as a detector and an objective to focus the reflected light on it. Pioneering work on imaging ellipsometry for biosensor applications has been done in Linköping.^[122] The camera resolves the observed surface in two dimensions and inhomogeneities can be seen as a contrast image due to differences in refractive index. Provided that the regions of interest are of reasonable homogeneity and have sizes that exceed the lateral resolution limit of the ellipsometer, regional thickness values can be calculated by finding the null condition for each region. In addition, thickness maps can be generated by stepping the polarizer (and/or the analyzer) over an interval encompassing the null conditions of the surface pattern and grabbing an image at each step. Through image processing the Ψ and/or Δ for each pixel of the surface seen with the CCD can be calculated. This Psi/Delta 2D-map can

for instance be transformed into a film thickness map with optical modeling. An example is shown from paper A in figure 5.3.^[19] The lateral resolution in imaging ellipsometry is determined by the wavelength of the light, the CCD resolution, and chosen objective; typically above $\sim 1 \mu\text{m}$. The vertical resolution is in principle the same as for non-imaging.

Ellipsometry can also be used with an SPR-setup, which is highly suitable in biosensing applications. See section 5.5 for more information.

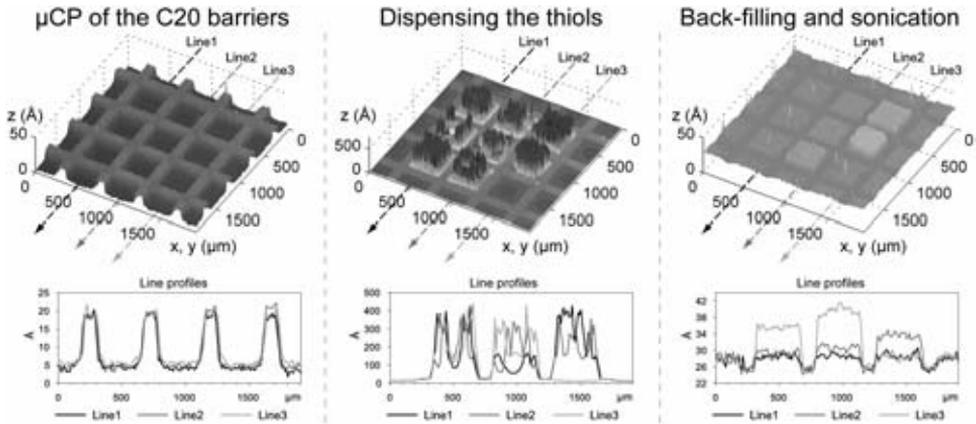


Figure 5.3. Example of thickness maps obtained with imaging ellipsometry. From paper A.^[19]

5.4 Surface Plasmon Resonance (SPR)

The SPR phenomenon has been known for a long time. However, its application in biosensing is relatively new (early 1980s).^[123]

The surface plasmon is a p-polarized electromagnetic surface-bound wave of coherently fluctuating electron charges, propagating along the interface between a metal (high density plasma) and a dielectric.^[124] The bound wave has an evanescent field associated with it, decaying exponentially perpendicular to the surface, figure 5.4b. It is the presence of this field that makes SPR a surface sensitive technique. The surface plasmon has a propagation constant, k_{sp} , which can be determined by solving Maxwell's equations for an interface separating a metal from a dielectric. Equation 5.6 is known as the surface plasmon dispersion relation.^[124]

$$k_{sp}^{\infty} = \frac{2\pi}{\lambda} \sqrt{\frac{\epsilon_m(\lambda) \cdot \epsilon_a}{\epsilon_m(\lambda) + \epsilon_a}} \quad 5.6$$

In the equation, the ∞ -symbol denotes that a semi-infinite interface is considered, λ is the wavelength of light, $\epsilon_m(\lambda)$ is the wavelength-dependent dielectric function of the metal, and ϵ_a is the dielectric function of the ambient/dielectric (considered constant in the relevant wavelength region).

Under normal circumstances, incident light cannot excite a surface plasmon, *i.e.* resonance or matching of the incident light's propagation vector with the surface plasmon's vector (k_{sp}^{∞}) cannot be achieved. However, the resonance condition can be met with the implementation of an ATR coupler, in which a thin metal film is attached to *e.g.* a prism with a relatively high refractive index. This setup is known as the Kretschmann configuration, figure 5.4a. The light then passes through the prism, at an angle of incidence greater than the critical for the prism, which means that total reflection occurs at the prism-metal interface.

At total reflection, an evanescent field is induced, which is able to penetrate the thin metal film and excite a surface plasmon at the metal-ambient interface. The horizontal propagation vector of light using an ATR coupler is:^[124]

$$k_x = \frac{2\pi}{\lambda} \sqrt{\epsilon_{prism}} \cdot \sin \theta \quad 5.7$$

where θ is the angle of incident light, being greater than the critical angle for the glass prism (total reflection condition), and ϵ_{prism} is the dielectric function of the prism. Since a thin (*i.e.* finite) metal film is used in the ATR coupler the surface plasmon propagation vector in equation 5.6 has to be adjusted to:^[124]

$$k_{sp} = k_{sp}^{\infty} + \Delta k_{sp} \quad 5.8$$

where Δk_{sp} is a complex function of λ , θ , ϵ_g , ϵ_m , ϵ_a , and the metal thickness, d_m . When $\text{Re}(k_x) = \text{Re}(k_{sp})$ surface plasmons resonance occurs and there is a drop in reflected intensity. By examining equations 5.6 - 5.8 one finds that the resonance condition can be met by varying the wavelength or the angle of the incident light. The most common choice is to vary the latter parameter.

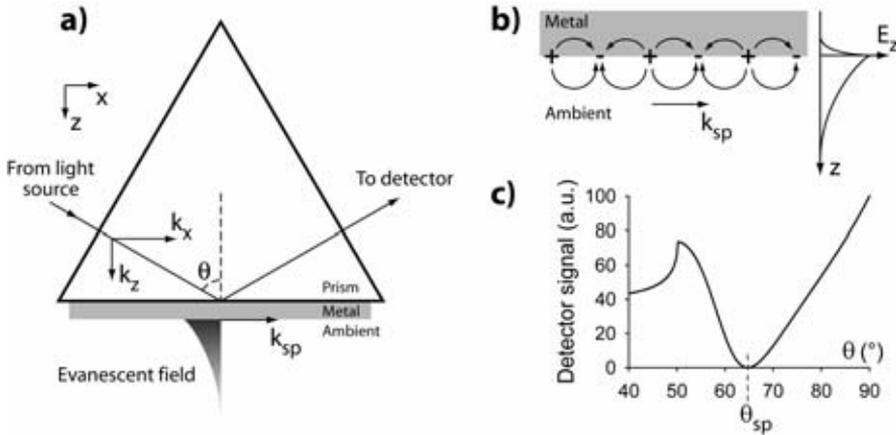


Figure 5.4 The surface plasmon phenomenon as a sensor. a) Schematic figure of the Kretschmann configuration, which is used to excite surface plasmons optically. b) Close-up of a surface plasmon wave at the semi-infinite interface between a metal and a dielectric. The exponentially decaying electric field in the z-direction is illustrated to the right. c) Fresnel modeled detector signal versus angle of incidence with prism=SF10 glass ($N=1.736$), metal=Au ($N=0.414+2.272i$, $d=340 \text{ \AA}$), and ambient=water ($N=1.333$) for p-polarized light ($\lambda=532 \text{ nm}$). The resonance angle θ_{sp} has been marked in the figure. Figure adapted from ^[124, 125].

In the angular case, monochromatic light is used and the intensity of reflected light is measured as a function of the angle θ , figure 5.4c. At a specific angle, $\theta=\theta_{sp}$, an intensity drop occurs, which indicates surface plasmon resonance. Since k_{sp} depends on the dielectric function of the ambient (ϵ_a), a change in ϵ_a will induce a shift in the resonance angle, θ_{sp} . This dependence facilitates the use of the SPR-phenomenon in measurements of the dielectric property (and consequently the refractive index) of the ambient near the metal surface. Of great importance to SPR measurements is the distance at which the exponentially decaying electric field penetrating the ambient reaches an intensity of $1/e$. This parameter is called the (intensity) probe depth and is given by: ^[124, 125]

$$\delta_z = \frac{1}{2 \cdot \text{Im}(k_{za})} \quad 5.9$$

where k_{za} is the propagation vector in z-direction in the ambient at the metal/ambient interface. For gold at $\lambda=532 \text{ nm}$, the probe depth is roughly 50 nm with water as ambient.

By modification of the metal surface with organic films (e.g. a SAM) followed by coupling of bioactive compounds (e.g. antibodies, receptors, or DNA) a biosensor is obtained.^[123] A change in the molecular concentration near the surface will induce a proportional shift in the refractive index of the ambient, thereby generating a signal. Hence, no labeling, e.g. fluorescence or radioactivity, of molecules is required. By monitoring this signal over time, a sensorgram is obtained, which gives real-time information about the molecular interactions on the surface of the sensor (metal) chip. Examples of SPR applications are the epitope mapping of antibodies, the determination of concentration, and the gathering of kinetic information regarding biomolecular interactions.^[27]

5.5 Imaging SPR

Imaging SPR (also known as SPR microscopy) is similar to ordinary non-imaging SPR, with the major exception that the detector is a CCD-camera, which is able to image the surface in two dimensions.^[126] One obtains a contrast image when different regions on the surface have different indices of refraction. Different instrumental setups exist for monitoring binding events on the surface. The simplest one is to lock one particular angle of incidence and to observe shifts in the SPR-curve as changes in the reflected intensity (or phase if one has the ability to measure it). This allows for the analysis of array surfaces that have a variety of biomolecules immobilized in different locations, and interactions over the surface will be seen by alterations in reflected intensity. An extensive review of imaging SPR and its applications has been written by Corn *et al.*^[127]

The Kretschmann SPR-configuration can with great benefits be used in an ellipsometer.^[19, 128-130] The greatest benefits are then the added capacity to measure phase-changes upon reflection (Δ) and an optical measurement system that is independent on fluctuations of light source intensity. Simulated SPR data are shown in figure 5.5.

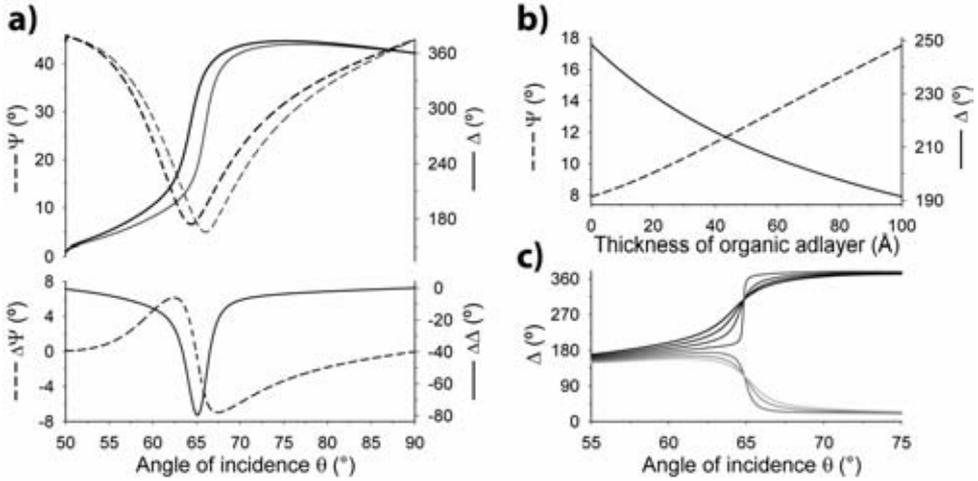


Figure 5.5. Simulated ellipsometry data with an SPR-setup. The setup and optical constants are identical to that of figure 5.4. a) Ψ and Δ angular data before (thicker lines) and after (thinner lines) adsorption of a 60 Å organic adlayer on a 315 Å gold film. The lower graph shows the differential data. b) Signal response to thickness of adsorbed organic layer on 315 Å gold at an angle of incidence of 63.5°. c) Angular Δ curves for different gold film thicknesses, from 310 (darkest) to 370 Å (brightest) in steps of 10 Å.

In figure 5.5a the angular response is seen for a gold film of 315 Å with and without an adsorbed organic film of 60 Å thickness. Also, the differential curves are shown to illustrate at what angle the sensitivity of the setup is greatest. The SPR-resonance angles are approximately 64.4° (gold) and 66.0° (gold+film). For Ψ (~intensity) the maximum absolute signal is roughly 6-7° for an angle of incidence of 62.4 and 67.4° whereas the maximum absolute signal is nearly 80° for Δ at an angle of 65.1°. Thus, Δ is more than 10 times more sensitive than Ψ under respective optimal angles of incidence.

For a maximum in sensitivity, the angle of incidence should therefore be 65.1°. However, this angle is very close to the SPR resonance angle, which means that the intensity of reflected light is very low and will make the determination of the ellipsometric angles difficult. Instead, an incident angle roughly 1° under the SPR resonance angle is usually chosen. In figure 5.5b the angle of incidence has been chosen to 63.5°, a commonly chosen angle in the included papers, and the simulated response is shown for the adsorption of an organic film ($n=1.5$)^[103]. Under these parameters Ψ is directly proportional to the adsorbed thickness with a high degree of linearity. The Δ -curve shows a

negative slope, with a higher sensitivity, but the degree of linearity is worse. However, for film thickness changes of up to ~ 40 Å the linearity is good.

In figure 5.5c Δ versus incident angle is shown for different choices of gold film thickness (without organic adlayer). The optimum gold thickness for SPR-resonance is close to 340 Å (zero intensity of reflected light). For this thickness Δ changes dramatically close to the SPR-angle, meaning that it will be extremely sensitive to changes in refractive index close to the surface. However, in addition to the above mentioned difficulty of measuring close to the resonance angle, the dynamic range of the sensor will be very small for this thickness. To be able to draw benefit of the extreme sensitivity under optimum conditions to its fullest, one would have to be able to keep ambient refractive index disturbances such as temperature and pressure/flow fluctuations to a minimum. As a compromise to the above discussed features of SPR together with imperfections in the gold deposition process the author has chosen to work with a (target) gold thickness of 315 Å and an angle of incidence of 63.5° . This gives a good theoretic sensitivity and dynamic range according to figure 5.5a-b and was also shown to be appropriate for most of the experiments described in the included papers.

The lateral resolution of imaging SPR is, in addition to the optical components, governed by the characteristic propagation length of the surface plasmons. The propagation length, L_x , is defined as the distance for which the intensity of the electric field decreases to $1/e$ and is given by:^[124, 125]

$$L_x = \frac{1}{2 \cdot \text{Im}(k_{sp})} = \text{/ at resonance /} = \frac{1}{4 \cdot \text{Im}(k_{sp}^\infty)} \quad 5.10$$

which is then, according to equation 5.6, dependent on the wavelength of light and the dielectric functions of the metal and ambient. For gold as metal film, water as ambient, and a wavelength of 532 nm the propagation length is negligible (< 1 μm) in comparison to the instrument's optical resolution.

The greatest benefit of imaging SPR is that many biomolecular interactions on one surface can be studied in parallel and in real time. In figure 3.8 (page 43) an example of using imaging SPR for parallel narcotics detection is shown. Figure 3.8a-c show images taken in reflected (p-polarized) intensity mode of the antibody loading of a sensor array consisting of 7 duplicate spots for measuring heroin (H), 5 for ecstasy (X), 5 for cocaine (C), and 8 for

amphetamine (A). The analyte (narcotics) detection is shown in figure 3.8d where the instrument is run in ellipsometric mode, Δ is measured over time for each spot. In total, 23 signals are monitored in parallel (excluding the reference signals).

An additional feature is that the inverted optical setup of the Kretschmann SPR setup, i.e. measuring on the back-side, allows the use of complex solvents that absorb or scatter light, e.g. blood and milk. In addition, the small probing depth allows flow channels of low height and thus low dead-volumes, leading to lower sample consumptions.

6 INCLUDED PAPERS

Paper A

Title

Piezo Dispensed Microarray of Multivalent Chelating Thiols for Dissecting Complex Protein-protein Interactions

Authors

Klenkar, G.; Valiokas, R.; Lundström, I.; Tinazli, A.; Tampe, R.; Piehler, J.; and Liedberg, B.

Journal

Anal. Chem. 2006, 78, 3643-3650.

Author's contribution

Performed majority of the experiments and writing.

Short description of paper

Microfabrication of chips with an array of piezo dispensed multivalent chelating thiols on hydrophobic micro contact printed barriers. Also, demonstration of the chip's function with a model protein system. Characterization with imaging ellipsometry and functional evaluation with imaging SPR.

Paper B

Title

Differential Protein Assembly on Micropatterned Surfaces with Tailored Molecular and Surface Multivalency

Authors

Valiokas, R.; Klenkar, G.; Tinazli, A.; Tampe, R.; Liedberg, B.; and Piehler, J.

Journal

ChemBioChem 2006, 7, 1325-1329.

Author's contribution

Design and production of chips, active participation in the rest of the experiments. Participation in the writing process.

Short description of paper

Differential immobilization and arraying of histidine-tagged proteins through combination of molecular and surface multivalency. Arrays of mono and multi-valent NTA chelators were produced and their functional aspects were investigated with a protein model system, monitored with imaging SPR.

Paper C

Title

Multivalent Self-Assembled Monolayers with Terminal Mono-, Bis-, and Tris-nitrilotriacetic Acid Groups: Characterization and Application

Authors

Valiokas, R.; Klenkar, G.; Tinazli, A.; Reichel, A.; Tampe, R.; Liedberg, B.; and Piehler, J.

Journal

In manuscript

Author's contribution

Ellipsometry and IRAS of tris-NTA thiol. Performed functional testing with SPR (Biacore). Designed and produced arrayed chips and participated in the protein adsorption and elution experiments. Writing functional evaluation parts of manuscript and active participation in other parts.

Short description of paper

Structural characterization of mono-, bis-, and tris-NTA thiols with mainly IRAS and ellipsometry. Functional evaluation with SPR of protein immobilization and ligand binding, as well as elution dependent on chelator multivalency and surface coverage. In addition, demonstration of a microarray with all thiols at varying surface concentrations where monitoring of protein immobilization and elution was done with imaging SPR.

Paper D

Title

A Microarray for Addressable Adsorption of Lipid Vesicles and Subsequent Protein Interaction Studies

Authors

Klenkar, G.; Brian, B.; Ederth, T.; Höök, F.; Piehler, J.; and Liedberg, B.

Journal

In manuscript

Author's contribution

Did initial experiments to verify concept. Experimental planning and supervision. Manuscript writing.

Short description of paper

The production and evaluation of a microarray for the DNA-mediated, addressable adsorption of lipid vesicles is reported. Four DNA-vesicle systems are used to show the specificity of the surface self-sorting concept. The vesicles contain, in addition, bis-NTA groups, which are able to capture histidine tagged receptor proteins to the lipid bilayer. The functionality of the captured receptors is validated by interactions with the complementary ligand. The formation of a ternary complex between two receptor subunits and the ligand is also shown to occur on the fluid lipid bilayer in agreement with previous studies, e.g. paper A.

Paper E

Title

A Microarray Chip for Label-free Detection of Narcotics

Authors

Klenkar, G. and Liedberg, B.

Journal

Submitted

Author's contribution

All experimental work. Manuscript writing.

Short description of paper

The production of a protein-microarray for the detection of four narcotics is reported. The detection mechanism is displacement of antibodies, which are loosely adsorbed to the chip via narcotic-analog conjugated proteins, upon injection of narcotic analytes.

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