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# Interaction Studies in Complex Fluids with Optical Biosensors

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During the course of the research underlying this thesis, Jenny Carlsson was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden

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*To my close ones*



## Abstract

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In this thesis interactions in complex fluids, such as serum and meat juice, were analysed with optical biosensor techniques.

Panels of lectins immobilised on gold surfaces were used for investigation of differences in protein glycosylation pattern in sera and meat juices between various species. The present panel was also used for investigation of global glycosylation changes of serum proteins in type 1 diabetes patients. Biorecognition was evaluated with null ellipsometry and scanning ellipsometry combined with multivariate data analysis techniques (MVDA). Principal component analysis (PCA) showed that the lectin panel enabled discrimination between sera from the different species as well as for the different meat juices. The results also indicate that there is a measurable global alteration in glycosylation pattern of serum proteins in type 1 diabetic patients compared to healthy subjects. Using an artificial neuronal net (ANN), it was also possible to correctly categorise unknown serum samples into their respective class or group. The analytical potential of combining information from lectin panels with multivariate data analysis was thereby demonstrated.

Also, a sensitive and specific method based on surface plasmon resonance (SPR) for detection of insulin autoantibodies (IAA) in serum samples from individuals at high risk of developing type 1 diabetes (T1D) has been developed. When measuring trace molecules, such as autoantibodies, in undiluted sera with label-free techniques like SPR, non-specific adsorption of matrix proteins to the sensor surface is often a problem, since it causes a signal that masks the analyte response. The developed method is an indirect competitive immunoassay designed to overcome these problems. Today, IAA is mainly measured in radio immunoassays (RIAs), which are time consuming and require radioactively labelled antigen. With our SPR-based immunoassay the overall assay time is reduced by a factor of >100 (from 4 days to 50 min), while sensitivity is maintained at a level comparable to that offered by RIA.

Finally, the assay was used in a screening study of newly diagnosed type 1 diabetes patients and non-diabetic subjects.

# Populärvetenskaplig sammanfattning

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Kunskap om biomolekylära interaktioner utnyttjas och används idag inom en rad olika områden, till exempel vid läkemedelsframställning, för att detektera miljöfarliga ämnen och för att följa och kontrollera olika processer inom den bioteknologiska industrin. Inom sjukvården kan biomolekyler användas som markörer. Närvaro av markören, ökad eller minskad halt av markören eller en förändring i markörens glykosyleringsmönster är kopplat till olika sjukdomstillstånd. (Glykosylerade proteiner är mycket vanliga och har olika kolhydrater fastsatta på sin yta.)

Arbetet i den här avhandlingen har innefattat analys av biomolekylära interaktioner i komplexa vätskor såsom serum och köttsaft (komplexa i det avseendet att de innehåller mer än 1000 olika slags proteiner). Vi började med att studera skillnader i glykosyleringsmönstret hos proteiner i serum eller köttsaft från olika djurarter. Inget specifikt protein studerades, utan det var mönstret hos den samlade serumproteinfloran som var intressant. Detta gjordes med hjälp av en array med olika lektiner fastsatta på en guldyta. Lektiner är proteiner som binder till olika kolhydratsstrukturer som finns på många proteins yta. Inbindningsmönstret studerades med hjälp den optiska tekniken ellipsometri och analyserades därefter med multivariata metoder. Multivariata metoder är matematiska/statistiska metoder som används för att gruppera och finna mönster i stora datamängder. Vi kunde då konstatera att glykosyleringsmönstret skiljer sig åt mellan olika arter och att vissa arter har mer lika mönster och är mer närbesläktade än andra. Till exempel visade det sig föga förvånande att vårt eget glykosyleringsmönster mer liknar grisens än marsvinets.

Det är känt att det vid olika sjukdomstillstånd, som exempelvis halsinfektioner, sker förändringar av glykosyleringsmönstret hos diverse serumproteiner. Vi ville undersöka om det med vår lektinarray gick att finna några sådana förändringar hos typ 1 diabetes patienter. Typ 1 diabetes är en autoimmun kronisk sjukdom och cirka 50000 personer lever med sjukdomen i Sverige idag. (Autoimmuna sjukdomar

innebär att det egna immunförsvaret felaktigt angriper kroppens egen vävnad.) Det visade sig då att det utifrån glykosyleringsmönstret gick att skilja patienter med sjukdomen från personer som inte hade sjukdomen. Idag finns inget botemedel för typ 1 diabetes, men när behandlingstekniker för sjukdomen blir tillgängliga är det av stort intresse att kunna identifiera personer som ligger i riskzonen för att utveckla diabetes. Då skulle denna teknik kunna vara intressant för att tidigt kunna upptäcka tecken på diabetes och sätta in behandling, kanske redan innan sjukdomen brutit ut.

En annan, redan etablerad, strategi för att tidigt upptäcka tecken på gynnande typ 1 diabetes är att mäta förekomsten av autoantikroppar i blodet, vilka är markörer för sjukdomen. Autoantikroppar mot patientens kroppsegna insulin är en sådan markör och mäts idag främst med radioimmunologiska metoder. Vi har utvecklat ett nytt sätt att påvisa närvaro av insulinautoantikroppar i serum med hjälp av en etablerad optisk teknik, ytplasmonresonanstekniken. Vår metod visade sig framför allt vara snabbare än de befintliga metoderna, men med bibehållen mät känslighet. Den utvecklade metoden testades sedan med gott resultat i en studie där insulinautoantikroppshalten i serumprover från nyligen insjuknade typ 1 diabetespatienter och friska kontrollpersoner undersöktes.

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# 1. Introduction

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Humankind has performed bioanalysis since the beginning of time, using the sensory nerve cells of the nose to detect scents and the receptors on the tongue to taste food. As time has progressed our understanding of different bioanalytical interactions has increased and scientists have sought to copy the high recognition ability of biochemical systems of complex organisms for various purposes.

A biosensor, Figure 1.1, is a device incorporating a biological recognition system, a bioelement, connected to a sensor element.<sup>[1]</sup> The bioelement recognises the target analyte and the sensor element directly or indirectly converts the recognition event into a measurable signal. Typical bioelements include biomolecules (*e.g.* antibodies, enzymes and nucleic acids) and living biological systems (cells, tissues or whole organisms), which utilises a biochemical mechanism for recognition. Common sensor element principles are based on for example optical, electrochemical or mass-sensitive phenomena.

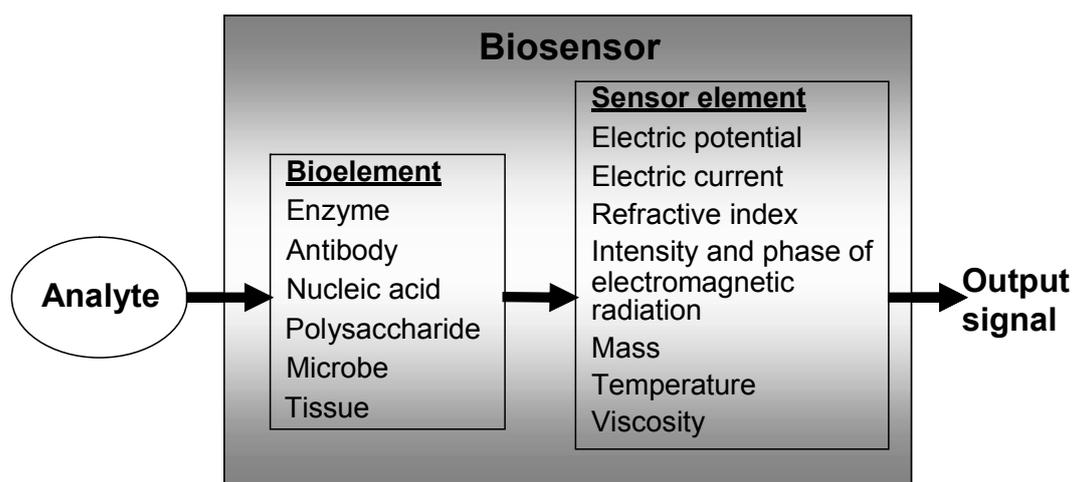


Figure 1.1. Schematic representation of a biosensor

## *1. Introduction*

Since the development of the first biosensor in the 1960s,<sup>[2]</sup> which was designed to detect glucose, there has been an explosive growth of biosensor-related research activities and biosensors have been used within many areas, for example in

- Clinical diagnostics and biomedicine
- Farm, garden and veterinary analysis
- Monitoring and control of processes in the biotechnology industry
- Pharmaceutical and drug analysis
- Environmental monitoring (toxins, pollutants etc)
- Military applications

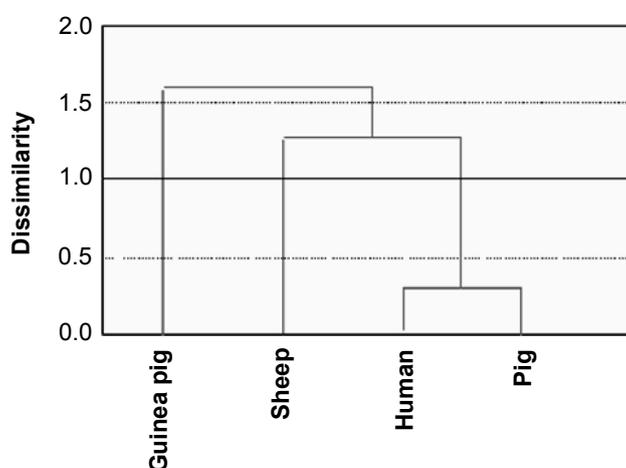
In this thesis, surface-based biosensors together with different optical sensor element principles were used for detecting analytes in complex fluids such as sera and meat juices. A key component in surface-based biosensors is the surface where the biologically active bioelement is immobilised. When analysing complex fluids, non-specific binding of sample components to the surface is frequently observed and may cause a signal that masks the analyte response of interest. To avoid this problem the surface chemistry is typically designed to minimise non-specific binding. Proper surface chemistry also allows for the bioelement to be immobilised to the surface without losing its function.

Optical biosensors have a good signal/noise ratio and are robust compared to for example electrical biosensors.<sup>[3]</sup> A vast number of optical sensor element techniques can be used in biosensors, some of which require labelling of the analyte of interest. In this thesis optical techniques that do not require labelling of the molecules being detected have been used. They all depend upon properties of light interacting with a solid/liquid interface. In papers I-III ellipsometry was used, which is based on analysis of polarisation changes occurring upon reflection of a light beam at a reflecting surface. In papers IV-V the optical phenomenon of surface plasmon resonance (SPR) was utilised for monitoring biochemical interactions by means of refractive index changes.

## 1. Introduction

Man-made biosensors are very crude and simplistic when compared to our natural ones (nose, eyes, tongue). The bioelements in the natural sensors are not necessarily very specific but the signal transduction via the biomolecules is highly sophisticated. The specificity arises from data processing and pattern recognition via a continuous learning process. Electronic tongues<sup>[4]</sup> and noses<sup>[5]</sup> have been developed trying to mimic our senses using arrays of many different non-specific sensors. Responses are then evaluated with multivariate data analysis techniques (MVDA) in order to classify samples or to find patterns in the large data set obtained. Arrays of bioelements immobilised on the sensor surface allow for simultaneous screening of multiple analytes to be carried out, reducing analysis time and sample consumption.

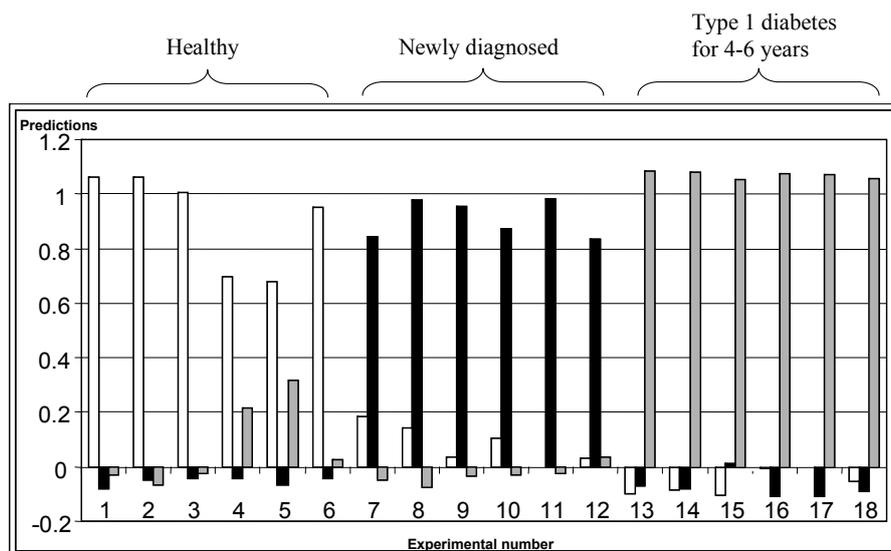
In this thesis interactions in complex fluids such as serum and meat juice were analysed with optical biosensor techniques. In papers I-III MVDA was utilised for classification of samples and for finding patterns in data obtained from lectin arrays. Lectins with different carbohydrate binding specificity were used in the arrays and the biorecognition of glycoproteins in serum and meat juice was evaluated using optical techniques based on ellipsometry together with MVDA. Differences in protein glycosylation pattern between different species were investigated in serum, paper I, and meat juice, paper II. The dendrogram in Figure 1.1 shows the relation of different species according to the results in paper I. In accordance with the literature, human and pig sera seem to be more closely related than for example human and guinea pig sera.



**Figure 1.1.** Dendrogram showing how sera from different species are related.

## 1. Introduction

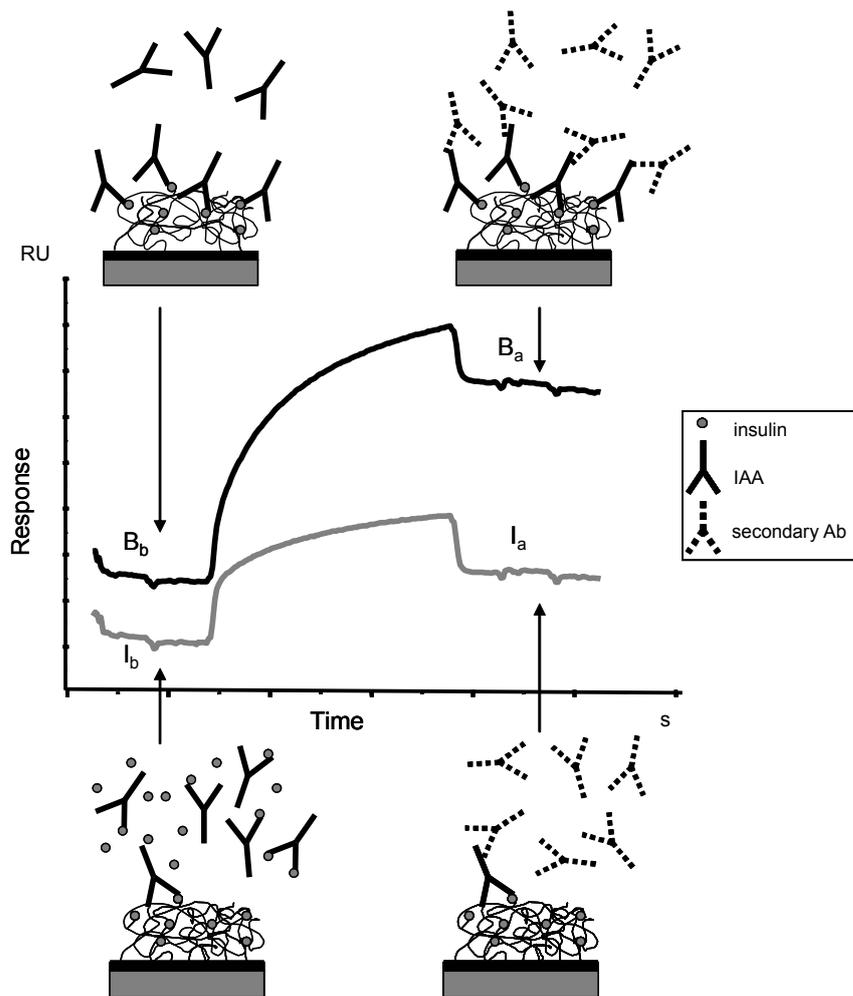
In paper III global changes in glycosylation pattern of serum proteins in type 1 diabetic patients compared to non-diabetics were analysed. It was shown that discrimination between sera from the three different groups; healthy, newly diagnosed and having suffered from the disease for 4-6 years was possible and that correct categorisation of unknown serum samples into one of the three groups could be done using a model based on an artificial neuronal net (ANN), Figure 1.2.



**Figure 1.2.** An ANN model was used for prediction of classification of sera into the three different serum groups; healthy, newly diagnosed and having suffered from the disease for 4-6 years.

In papers IV-V one particular analyte was in focus, insulin autoantibodies (IAA). IAA together with a few other autoantibodies can be used as markers to predict type 1 diabetes and assist in the diagnosis of the disease. For monitoring of the biorecognition event when IAA binds to immobilised insulin on the sensor surface SPR was used. IAA is present at very low concentration in sera. When measuring trace molecules such as IAA in serum, non-specific adsorption of matrix proteins to the sensor surface is often a problem. Also, the amount of non-specifically bound proteins differs substantially between serum samples from different individuals. Therefore, an indirect competitive immunoassay (Figure. 1.3) was developed to circumvent this problem (paper IV). Using this assay a screening study of newly diagnosed type 1 diabetes patients was made (paper V).

1. Introduction



**Figure 1.3.** The difference in SPR response from a pair of serum samples incubated in buffer (B) and insulin (I), gives a measure of the amount of IAA in the original sample.



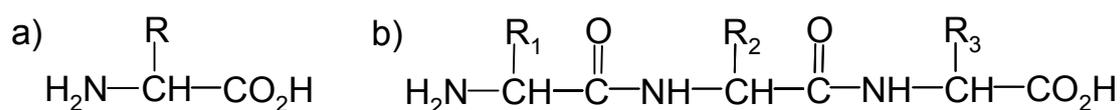
## 2. Biomolecules and biomolecular interactions

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All known forms of life are composed of naturally occurring biomolecules, which consist primarily of carbon and hydrogen along with mainly nitrogen, oxygen, phosphorus and sulphur. Amino acids are some of the most important building blocks used in nature to construct larger molecules, proteins. Another important type of building block is the nucleotides, which form our DNA. Biomolecules interact with each other in an intricate manner, which defines the living organism.

### 2.1 Proteins

The properties of proteins, the major functional molecules of life, are so useful that we employ them as therapeutic agents, catalysts, and materials.<sup>[6]</sup> Proteins are composed of 20 different naturally occurring amino acids of which 19 have the general structure shown in Figure 2.1 a), only differing in their side chain (R). The amino acids are linked together by peptide bonds, building up the linear sequence characteristic for proteins, Figure 2.1 b).



**Figure 2.1.** General structure of a) an amino acid and b) a peptide consisting of three linked amino acids.

The sequence of a protein is determined by the gene that encodes it. Protein folding, *i.e.* the arrangement of the polypeptide chain into two- and three-dimensional structures, is a consequence of the primary composition. Secondary structure (mainly  $\alpha$ -helices and  $\beta$ -sheets) is stabilised by hydrogen bonds within the peptide backbone. Tertiary structure is the "global" folding of a single polypeptide chain, where a major driving force is the hydrophobic effect, resulting in non-polar amino acid side chains becoming hidden in the interior of the protein, while polar residues are exposed on the

## 2. Biomolecules and biomolecular interactions

protein surface. Quaternary structure, not exhibited by all proteins, involves the association of two or more polypeptide chains into a multi-subunit structure and is mainly stabilised by non-covalent interactions (hydrogen bonds, van der Waals interactions and salt bridges). The native, folded, state of a protein does not differ substantially in free energy from the unfolded, denatured, state ( $\Delta\Delta G \sim 5-15$  kcal/mol), making proteins very sensitive to their environment.<sup>[6]</sup>

A characteristic property of many proteins is their ability to combine specifically and reversibly with other molecules, such as other proteins, nucleic acids, polysaccharides and lipids. Every aspect of the structure, growth and replication of an organism depends on such interactions, which take place on the surface of the protein, or in grooves or pockets on the surface, and are governed by many relatively weak non-covalent forces including hydrophobic effect and hydrogen bonds, van der Waals forces and salt bridges. For the reversible monovalent interaction between a protein (P), and its binding partner (A),



the association constant  $K_a$  is defined as

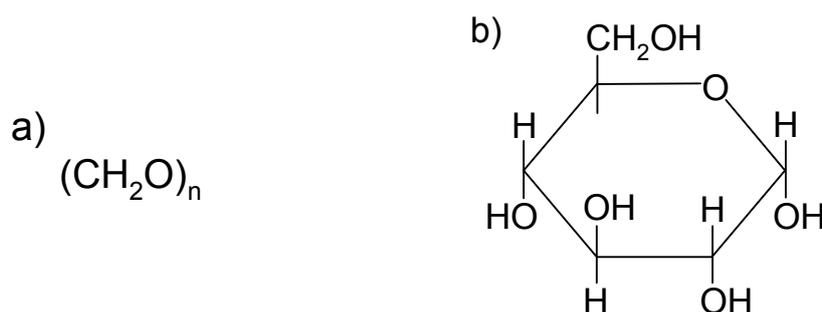
$$K_a = \frac{[PA]}{[P][A]} \quad (\text{M}^{-1}) \quad (2.2)$$

High affinity is characterised by a high value of  $K_a$  and typical  $K_a$  values range between  $10^4 \text{ M}^{-1}$  to  $10^{11} \text{ M}^{-1}$ .

Protein glycosylation is a covalent, enzyme-directed, site-specific association of carbohydrate moieties (oligosaccharides) to proteins in the ER (endoplasmic reticulum) and Golgi apparatus. An oligosaccharide (or glycan) is a saccharide polymer containing a small number of component sugars, Figure 2.2. Two types of glycosylation exist: *N*-linked glycosylation to the amide nitrogen of asparagine side chains and *O*-linked glycosylation to the hydroxyl oxygen of serine and threonine side chains of the protein. Enzymatic glycosylation of proteins is a common and complex form of posttranslational modification. The glycans perform important biological

## 2. Biomolecules and biomolecular interactions

roles, including stabilisation of the protein structure, protection from degradation and control of protein solubility, protein transport in cells and protein half-life in blood.<sup>[7]</sup> They also mediate the recognition and interaction with other macromolecules (*e.g.* enzymes and lectines) and the recognition and association with viruses. On the other hand, non-enzymatic glycosylation (glycation) is the result of a saccharide, such as fructose and glucose, being attached to a protein without the controlling action of an enzyme and is a haphazard process that impairs the function of the protein. Microbial infections, inflammations and autoimmune diseases, for example rheumatoid arthritis, result in glycosylation changes of serum proteins,<sup>[8, 9]</sup> which can be utilised in discriminative clinical tests. In cancer research much effort has been put into the study of glycosylation changes for diagnosis, prognosis and evaluation of effectiveness of treatment.<sup>[10]</sup> In paper III glycosylation patterns in type 1 diabetes patients are compared to those in non-diabetics. Also, in paper I and paper II differences in glycosylation pattern of serum proteins between different species were investigated.



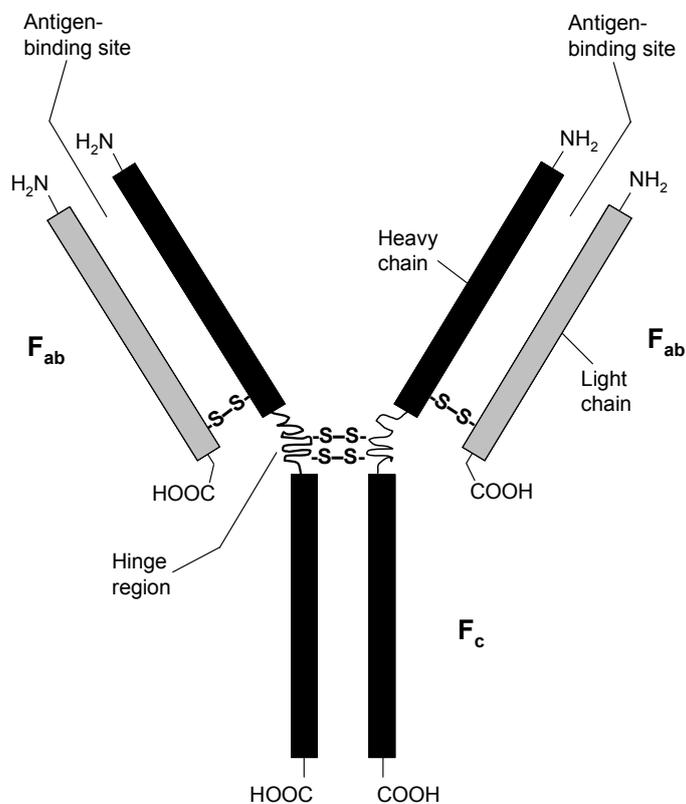
**Figure 2.2.** a) Fundamental composition of carbohydrates. b)  $\alpha$ -D-glucose, a common saccharide unit attached to proteins during glycosylation.

### 2.1.1 Antibodies

Antibodies are part of our immune system, where their main functions are to bind and neutralise pathogens or to recruit cells and molecules to destroy the pathogen once the antibody is bound to it.<sup>[11]</sup> Five different isotypes of antibodies exist in higher vertebrates; IgA, IgD, IgE, IgG and IgM. The basic structure of an intact antibody is a Y-shaped molecule composed of two identical heavy chains and two identical light chains.<sup>[12]</sup> In Figure 2.3, the structure of an IgG molecule is shown. The heavy and light chains are held together by disulfide bonds at the flexible hinge region, which separates the protein in an F<sub>c</sub> stem and two F<sub>ab</sub> arms. The major part of an antibody

## 2. Biomolecules and biomolecular interactions

consists of domains that are identical within each isotype. At the end of the  $F_{ab}$  arms, the antigen-binding sites are formed. Each antibody has two identical binding sites, which are highly variable between different antibodies.



**Figure 2.3.** Schematic illustration of an IgG antibody.

One antibody is usually capable of reversibly binding an antigen very specifically, but all antibodies in the body are collectively capable of recognising almost any molecule.<sup>[13]</sup> Antibodies with a  $K_a$  value lower than  $10^4 \text{ M}^{-1}$  would probably not be biologically efficient.<sup>[12]</sup>

Antibodies are highly attractive as recognition elements in bioarrays and biosensors thanks to their individual specificity and their collective capability to bind virtually any molecule. The introduction of antibody engineering by cloning and expression has permitted the design of antibodies or antibody fragments with improved qualities such as increased sensitivity and decreased antigen cross-reactivity.<sup>[14]</sup> The stability of immobilised antibodies is often reduced, but by using recombinant Fv fragments (composed of the variable part of a light chain tethered to the variable part of a heavy

chain) higher stability is obtained.<sup>[15]</sup> The most stable form of Fv is scFv (sc = single chain), in which the domains are associated by a peptide.

### 2.1.2 Lectins

Lectins are a class of proteins and glycoproteins found in all kinds of organisms, which agglutinate cells and/or precipitate glycoconjugates. They bind carbohydrates specifically and reversibly and are selective towards one or two saccharide residues, why their ability to discriminate polysaccharide structures is limited. Lectins typically play a role in biological recognition phenomena involving cells and proteins and are important as decoders of the complex saccharide language produced on the cell surface, acting as cell recognisers, in agglutination, in tissue regulation and in the development of organisms. Most lectins studied are homo- or heteromultimeric proteins. They contain at least two sugar-binding sites (sugar-binding proteins with a single site will not agglutinate or precipitate structures containing sugar residues, and are therefore not classified as lectins), recognising and adjusting to the ligand carbohydrate by induced fit, involving complex networks of hydrogen bonds and hydrophobic interactions.<sup>[16]</sup> The affinity of lectins for sugars ( $K_a=10^2-10^6 \text{ M}^{-1}$ ) is typically lower than the affinity between carbohydrates and specific antibodies ( $K_a=10^4-10^8 \text{ M}^{-1}$ ).<sup>[17, 18]</sup>

Lectins are very interesting for use in biosensor technology due to their broad diversity and specificity and have been utilised for blood typing,<sup>[19]</sup> for purification of cells and glycoproteins<sup>[17]</sup> and for distinguishing between microbial species.<sup>[20, 21]</sup> Arrays of lectins with different carbohydrate residue specificity have been used to differentiate between human serum samples from healthy individuals and patients with a throat infection.<sup>[22]</sup> Lectin arrays have also been used for the identification of microorganisms<sup>[20]</sup> and to identify viable *Escherichia coli* subspecies.<sup>[21]</sup>

In papers I-III arrays of lectins with different carbohydrate specificity were immobilised on a biosensor surface. Seven different plant lectins used in papers II-III are shown in Table 2.1 together with their carbohydrate specificities. The chosen lectins had different carbohydrate binding specificity and were also readily available

## 2. Biomolecules and biomolecular interactions

at reasonable cost. Some of the lectins in the table have specificity towards the same carbohydrates, but in such cases their association constants,  $K_a$ , differ. In papers I and II the lectin arrays were used to investigate serum protein glycosylation differences between species and in paper III differences in serum protein glycosylation pattern between non-diabetics, newly diagnosed type 1 diabetes patients, and diabetics having suffered from the disease for several years were investigated.

**Table 2.1.** Carbohydrate residue specificity for the lectins used in papers II-III.

Source of lectin	Specificity
<i>Canavalia ensiformis</i>	$\alpha$ -Man, $\alpha$ -Glc
<i>Triticum vulgare</i>	$\beta$ -GlcNAc, sialic acid
<i>Arachis hypogaea</i>	$\beta$ -Gal, GalNAc
<i>Griffonia simplicifolia</i>	$\alpha$ -Gal, $\alpha$ -GalNAc
<i>Phaseolus vulgaris agglutinin</i>	Complex structures
<i>Ulex europaeus agglutinin</i>	$\alpha$ -Fuc
<i>Lens culinaris agglutinin</i>	$\alpha$ -Glc, $\alpha$ -Man

## 2.2 Serum

Blood is composed of blood cells and plasma. Plasma mainly contains water and large amounts of proteins (6-8%), the concentrations of which are tightly controlled to balance their physiological roles in immunity, coagulation, small molecule transport, inflammation and lipid metabolism.<sup>[23]</sup> Serum is obtained by removing the clotting factors (mostly fibrinogen) from plasma and contains 60-80 mg protein per ml. Numerous 2D gel electrophoresis and mass-spectrometric analyses of plasma have resulted in the identification of more than 1000 different proteins:<sup>[24]</sup>

- Immunoglobulins (8%)
- Cellular proteins like cytokines, nuclear proteins, membrane-associated proteins etc (58%).

## *2. Biomolecules and biomolecular interactions*

- Classic plasma proteins in circulation (13%). These proteins are higher abundance proteins with concentrations above 1 µg/ml and some examples are enzymes, coagulation and complement factors.
- Unknown proteins (21%)

The most abundant proteins in serum are albumin (65 kDa), 40 mg/ml, which is important for regulating blood volume and also serves as a carrier for molecules of low water solubility, and IgG (150 kDa), 8-18 mg/ml. When measuring an analyte, such as a disease marker, in complex liquids like serum or plasma, the protein of interest is very rarely a high abundant classic plasma protein, but is present at a much lower concentration, making analysis without any pre-treatment of the sample complicated.

The proteome reflects the functional state of an organism at a given time. Lack of function and out-of-balance concentrations of plasma proteins can cause or result from disease processes.<sup>[23]</sup> By using a lectin array global glycosylation differences in the serum proteome due to the chronic autoimmune disease type 1 diabetes were investigated in paper III. In papers I and II proteome glycosylation differences between species were investigated based on differential binding to a lectin panel. A specific protein, a type 1 diabetes marker (present at low concentration in sera), was quantified in paper IV and V.



### 3. The immune system in short

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The immune system is a collection of mechanisms within an organism, which protects against disease through identification and killing of pathogens and tumour cells.<sup>[11]</sup> The immune system is composed of the innate and the adaptive immune responses together with the complement system. The innate and adaptive immune responses consist of a wide range of white blood cells. These cells carry receptors, which enable them to perform different tasks in the immune response, all with the purpose to eliminate pathogens and control inflammation. Activated cells produce signalling molecules, cytokines, which are of major importance in the regulation of the immune response.

Early phases of a host response to an infection depend on innate immunity, in which the phagocytic cells granulocytes and macrophages play an important role (phagocytosis is the internalisation and destruction of particulate matter such as bacteria). Adaptive immunity consists of lymphocytes (B-cells and T-cells), which interact with different antigen presenting cells (APCs) through their antigen-specific receptors, become activated and start to proliferate. T-cells evolve into antigen-specific effector cells and B-cells develop into antibody-secreting cells.

The precursors of T-cells mature in thymus, where the T-cell receptors (TCR) are generated and tested. Only lymphocytes that respond to antigen bound to the individuals own major histocompatibility complex (MHC) molecules will survive (positive selection). If the TCR binds strongly to self-antigen, the lymphocyte will die (negative selection). In this way tolerance to self-antigens is established. When the T-cells have left thymus they circulate continually from the blood to the lymph system and when presented to its specific antigen it will start to proliferate and differentiate into an effector T-cell. T-cells are divided in TH1, TH2 and cytotoxic T-cells. Cytotoxic T-cells recognise peptides from intracellular pathogens presented together with MHC I molecules, and kill the pathogen-infected cell. TH1- and TH2-cells both

### *3. The immune system in short*

recognise peptides derived from pathogens that are presented by APCs together with MHC II molecules. TH1-cells activate and attract macrophages and cytotoxic cells to the site of infection, while TH2-cells induce antibody production by B-cells.

When a B-cell is activated by an antigen via its B-cell receptor it differentiates into a plasma cell and produces antibodies, which are the secreted form of the B-cell receptor. The main functions of antibodies are to bind and neutralise pathogens or to prepare pathogens for uptake and destruction by phagocytes.

Following activation, B cells and T cells leave a lasting remembrance of the antigens they have encountered, in the form of memory cells. Throughout the lifetime of an animal these memory cells will “remember” each specific pathogen encountered, and are able to mount a strong response if the pathogen is detected again.

The complement system is complementary to the antibody-mediated specific immunity, but can also be initiated as part of innate immunity. Complement is a system of more than 20 different plasma proteins involving three separate but interacting pathways: classical, alternative and lectin mediated. One of the principal functions of complement is the non-specific recognition and elimination of foreign elements from the body, which is accomplished by coating the foreign material with complement fragments that permit phagocytosis by granulocytes (opsonisation). The classical pathway is triggered by antibody complexed with pathogen or directly by the pathogen. The alternative pathway is triggered directly on the pathogen surface and its non-specific and spontaneous nature permits activation by various biomaterial surfaces. The lectin pathway is triggered by mannose-binding lectin (a serum component that binds some encapsulated bacteria). All pathways generate a crucial enzymatic activity that, in turn, generates the effector molecules of complement. The three main consequences of complement activation are the opsonisation of pathogens, the recruitment of inflammatory cells and the direct killing of pathogens.

### **3.1 Autoimmunity and autoantibodies**

Autoimmune diseases, such as type 1 diabetes, result from a hyperactive adaptive immune system directed at self antigens, cells and tissues. While such a high-level autoimmunity is unhealthy, a low-level autoimmune response is vital for the development and function of the immune system and is central for the development of self-tolerance. Autoimmunity resembles normal immune responses to pathogens in that it is specifically activated by antigens, autoantigens. Autoimmune diseases are broadly divided into organ-specific diseases (*e.g.* type 1 diabetes) and systemic diseases (*e.g.* rheumatoid arthritis). The etiology of autoimmune diseases is still not clear, but genetic, immunological, hormonal<sup>[25]</sup> and environmental factors (such as infections, vaccines etc) are considered to be important triggers.<sup>[26]</sup> There is generally not a direct genetic link, however. While families may be susceptible to autoimmune conditions, individual family members may have different autoimmune disorders, or may never develop an autoimmune condition.<sup>[18]</sup>

An autoantibody is an antibody that is directed towards one or more of the individual's own proteins. It is not always known whether the autoantibodies play an important role in the disease or are a secondary result of tissue damage caused by the disease process itself.<sup>[27]</sup>



## 4. Diabetes

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Diabetes mellitus is a heterogeneous group of metabolic disorders characterised by a dysregulated carbohydrate metabolism. The World Health Organization (WHO) classifies diabetes into three major groups: type 1 diabetes, type 2 diabetes and gestational diabetes.<sup>[28]</sup> The disease occurs when the  $\beta$ -cells in the pancreas do not produce enough insulin or alternatively, when the body cannot effectively use the insulin it produces. According to WHO, in 2007 more than 180 million people worldwide suffered from diabetes, of which 5-10% were type 1 diabetics. The diabetes incidence is rapidly increasing and it is estimated that by the year 2030, the number will have doubled.<sup>[28]</sup>

Papers III-V in this thesis are focused on the analysis of serum samples from type 1 diabetes patients. In paper III global glycosylation changes of serum proteins in type 1 diabetes patients compared to non-diabetics were measured with a lectin panel. Paper IV describes the development of an assay for the detection of a marker for the disease and in paper V the screening potential of the developed assay is demonstrated.

### **4.1 Type 1 diabetes**

Type 1 diabetes (T1D) is a chronic autoimmune disease characterised by destruction of the insulin producing  $\beta$ -cells in the pancreas. In T1D patients there is no efficient glucose metabolism due to the lack of insulin and therefore life-long insulin therapy is necessary. Symptoms of the disease may occur suddenly and include excessive excretion of urine, increased thirst, constant hunger, weight loss, vision changes and fatigue. T1D occurs worldwide and can appear at any age, but the peak incidence is in children and young adults. At the time of diagnosis only 10-20% of the  $\beta$ -cells are functioning.<sup>[29]</sup> The clinical presentation of the disease is preceded by an asymptomatic period of highly variable duration. The first detectable sign of emerging  $\beta$ -cell autoimmunity is the appearance of diabetes-related autoantibodies.

#### 4. Diabetes

Autoantibodies of predictional and diagnostic value include islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), and autoantibodies to tyrosine phosphatase-like protein IA-2 (IA-2A).<sup>[27, 30-</sup>

<sup>32]</sup> In newly diagnosed type 1 diabetes patients ICA is detected in 71-86%, GADA in 69-80%, IA-2A in 60-70% and IAA in 43-70%, respectively.<sup>[27, 33-35]</sup>

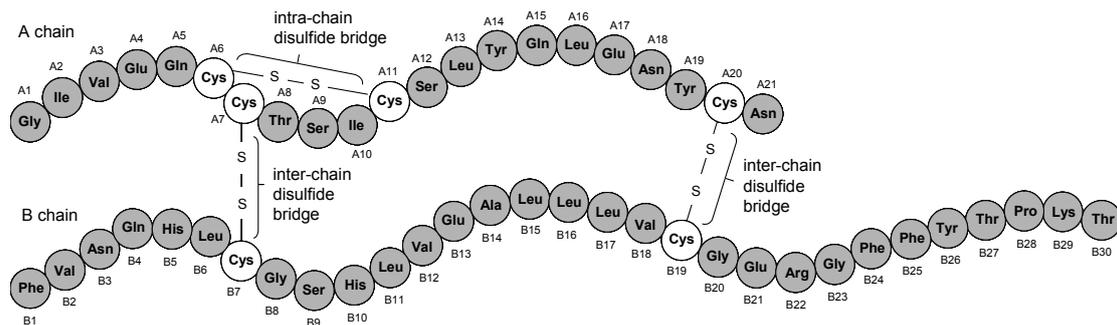
The series of events associated with the formation of autoantibodies to islet antigens in T1D is not yet clear and there is uncertainty about their pathogenic importance.<sup>[27,</sup>

<sup>31]</sup> The presence of different types of autoantibodies is related to the risk of progression to T1D.<sup>[31, 36-38]</sup> However, not everyone with multiple autoantibodies develops T1D and not all individuals that develop the disease have detectable autoantibodies.<sup>[39]</sup> The different autoantibodies may emerge in any order, but IAA is usually the first marker to appear in children. IAA can also be detected in other autoimmune diseases such as Grave's disease and rheumatoid arthritis.<sup>[27]</sup> In other types of diabetes autoantibodies do not occur. Most cases of T1D occur sporadically and only 15% of T1D patients have an affected first-degree relative.<sup>[30-32]</sup> Apart from genetics, the role of environmental factors such as viral infections, diet, breast feeding and early exposure to cow's milk as well as inflammatory mediators have been discussed in the context of triggering the  $\beta$ -cell autoimmunity.<sup>[29, 32, 40, 41]</sup>

At present there is no established therapy to delay or prevent T1D. When preventive therapies become available, autoantibody screening of the general population should be considered to identify at-risk individuals. Such a screening would enable swift preventive measures and will be of major importance, but there is also a clinical benefit in earlier diagnosis. By combining assays for IAA, GADA and IA-2A in the general population screening a high positive predictive value for disease is possible.<sup>[42, 43]</sup> In this thesis a novel indirect competitive assay for detecting IAA in sera from newly diagnosed T1D patients has been developed (paper IV), see section 7.4.1, and its screening potential was demonstrated (paper V).

## 4.2 IAA and insulin

IAA is the only  $\beta$ -cell specific autoantibody to date and is directed towards insulin. Insulin, Figure 4.1, is a polypeptide hormone consisting of an A and a B chain, 21 and 30 amino acids respectively, which are linked by two disulphide bridges. Insulin was the first protein sequence to be determined and the British molecular biologist Frederick Sanger was awarded the Nobel Price in Chemistry in 1958 for this work.



**Figure 4.1.** The insulin molecule.

Insulin is produced by  $\beta$ -cells in the islets of Langerhans in the pancreas. It is very important in the metabolism of carbohydrates, lipids, proteins and minerals. Insulin also increases the permeability of many cells to potassium, magnesium and phosphate ions. Consequently, derangements in insulin signalling have widespread and devastating effects on many organs and tissues. In healthy individuals, elevated concentrations of glucose in blood stimulate release of insulin, which binds to specific insulin receptors on cells throughout the body and stimulates uptake, utilisation and storage of glucose in the form of glycogen. As blood glucose concentrations fall, insulin secretion ceases. In the absence of insulin many cells become unable to take up glucose, and switch to using alternative energy sources like fatty acids.

In newly diagnosed T1D patients IAA should be measured within 5-7 days after the first insulin injection. Thereafter, antibodies towards exogenous insulin (IA) will have developed, which often are 10-fold or greater in concentration than IAA in serum.<sup>[27, 44-46]</sup> IAA of different affinities have been identified. In individuals at risk for T1D, manifestation of high-affinity IAA ( $K_a > 10^9 \text{ M}^{-1}$ ) is often followed by multiple autoantibodies and T1D, while in those with low-affinity IAA multiple autoantibodies and T1D are rare.<sup>[47, 48]</sup> Insulin epitopes are not well-characterised. The major binding

#### *4. Diabetes*

site for the high-affinity IAA is probably a conformational epitope requiring both the A- and B-chain and conservation of region A8-A13 on the insulin molecule, while low-affinity IAA is dependent on the B28-B30 region for binding.<sup>[48]</sup>

IAA is usually quantified in a radioimmunoassay (RIA), which requires radioactively labelled antigen and takes four days to carry out. For a description of RIA, see section 7.5.

## 5. Sensor surfaces: protein immobilisation and non-specific binding

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The performance of a biosensor is largely dependent on the nature of the bioelements and the sensor element, but the characteristics of the biosensor surface and the mode of immobilisation of the bioelements are equally important. Within biomaterial and biosensor research much effort has been put into the development of surfaces and surface coatings to which proteins do not adsorb spontaneously, *i.e.* that resist non-specific binding. In interaction analysis using biosensors, the first step is always the immobilisation of the bioelement (the ligand) onto the sensor surface. The ideal sensor surface allows for a rapid, straightforward and reproducible immobilisation of the ligand in a way that allows its function to be preserved. Moreover, non-specific binding of the analyte or other compounds in the sample solution to the sensor surface must be minimised in order to ensure that the interaction of interest is the one being monitored.

### 5.1 Immobilisation methods

There are many different strategies for immobilisation of biomolecules to surfaces and these can be divided in adsorption, covalent binding and bioaffinity binding (capturing).

#### 5.1.1 Adsorption

Adsorption of biomolecules on a surface is the most straightforward immobilisation method, as it only requires the incubation of the sensor surface in a solution of the compound to be immobilised. Adsorption is a complex process that is not fully understood.<sup>[49-54]</sup> This complexity is a consequence of the virtually infinite number of variations in physical and chemical properties of different proteins and surfaces. Furthermore, their interaction with solvent molecules (mainly water and ions) is

## *5. Sensor surfaces: protein immobilisation and non-specific binding*

critical, which complicates matters further.<sup>[49, 50]</sup> Protein adsorption to surfaces involves electrostatic interactions, van der Waals forces, hydrophobic interactions and protein structure rearrangements. The adsorption is largely irreversible unless some drastic change in the solvent is made, such as the introduction of a detergent that binds strongly to the adsorbed protein as well as to the underlying substrate.<sup>[51]</sup> In general proteins are not denatured upon adsorption onto a solid surface but they may undergo conformational changes that can influence their shape, specificity or activity.<sup>[49, 50]</sup> For monolayer formation there is a limited number of available binding sites on the surface for the proteins present in the bulk phase, resulting in competition. Depending on the relative bulk concentration, the intrinsic surface activity of each protein and their diffusion velocity, the outcome of the competitive process is an adsorbed layer that is richer in some proteins than others, which means that the surface composition will differ from the bulk composition. In complex protein mixtures, such as blood, adsorption to a sensor surface is a dynamic process in which proteins may compete for the adsorption sites and can displace each other. Furthermore, because the proteins have different affinities for each type of surface the outcome of the competition varies for each type of surface.

### **5.1.2 Controlled protein immobilisation**

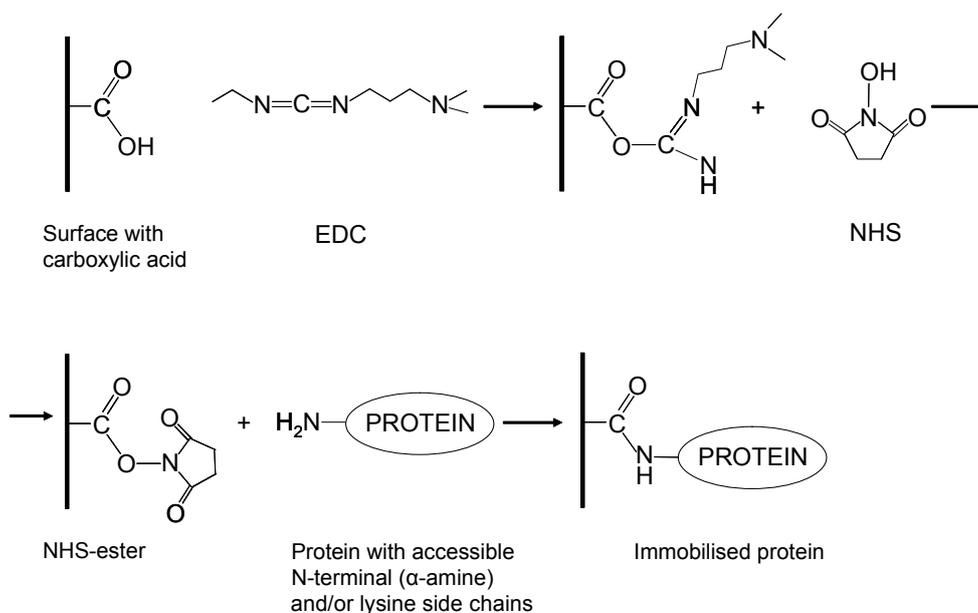
Adsorption onto surfaces may influence protein activity through conformational changes. The development of biosensor interfaces that allow for control of the amount and orientation of the biomolecules without damaging their structure and function is therefore a crucial issue. Consequently a variety of strategies, of which some are presented in sections 5.1.2.1-5.1.2.2, have been introduced for controlled immobilisation of ligands onto the sensor surface.

#### **5.1.2.1 Covalent binding**

Compared to adsorption, covalent immobilisation offers the possibility to control the orientation of ligands while maintaining their function. Further, the immobilisation

## 5. Sensor surfaces: protein immobilisation and non-specific binding

can be controlled and ligand consumption minimised. The choice of coupling chemistry is largely dependent on the nature of the reactive groups available on the ligand, such as  $\text{-NH}_2$ ,  $\text{-COOH}$  and  $\text{-SH}$ . Similarly, the surface must provide reactive groups ( $\text{-OH}$ ,  $\text{-NH}_2$ ,  $\text{-COOH}$ ,  $\text{-SH}$ ). Often a biomolecule may be covalently immobilised to a surface by many different strategies. One of the most commonly used coupling chemistries is shown in Figure 5.1, through which accessible amino groups of the protein reacts with pre-activated carboxyl groups on the surface. Most macromolecules contain amino groups, which can be used in amine coupling, making it a highly generally applicable strategy. However, often many attachment points (*e.g.* many lysine side chains in proteins) are available, resulting in heterogeneously immobilised ligands with this method. For a more homogeneously oriented ligand population, other coupling methods with more defined attachment sites might be desired.



**Figure 5.1.** Example of covalent immobilisation of a protein to a surface. The protein amino group is coupled to carboxyl groups on the surface via the activation agents 1-ethyl-3(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS).

### 5.1.2.2 Bioaffinity binding (capturing)

Adsorption and covalent immobilisation often lead to randomly oriented proteins, and also the analyte binding site of the protein might be obscured. Therefore, different bioaffinity-based methods have been developed, which allow for an oriented and

## 5. Sensor surfaces: protein immobilisation and non-specific binding

gentle immobilisation of proteins, even if suitable chemical groups are absent for covalent immobilisation.<sup>[55]</sup> Examples are the oriented immobilisation of antibodies via protein A or protein G, the use of capturing antibodies, the immobilisation of biotinylated ligands via biotin-binding proteins such as avidin, streptavidin or NeutrAvidin<sup>TM</sup>, or the immobilisation of recombinant proteins via tags such as polyhistidine.

### 5.1.2.2.1 Immobilisation via protein A or protein G

Protein A<sup>[56, 57]</sup> and protein G<sup>[58]</sup> are recombinant forms of bacterial cell wall proteins found in *Staphylococcus aureus* and in most species of *Streptococci*, respectively, which specifically bind polysaccharides at the Fc region of antibodies. Thereby, they orient the antibodies so that their antigen binding sites, the Fab regions, are available for binding. Protein A or G is first deposited on the sensor surface by adsorption or covalent immobilisation and then the antibody to be immobilised is added<sup>[59]</sup> (Figure 5.2). Each protein A or protein G molecule has more than one Fc-binding site and thereby the orientation of the capture protein on the surface is of minor importance. Protein G layers have for example been used for detection of the pathogen *Yersinia enterocolitica* by means of an immobilised monoclonal antibody.<sup>[60]</sup>

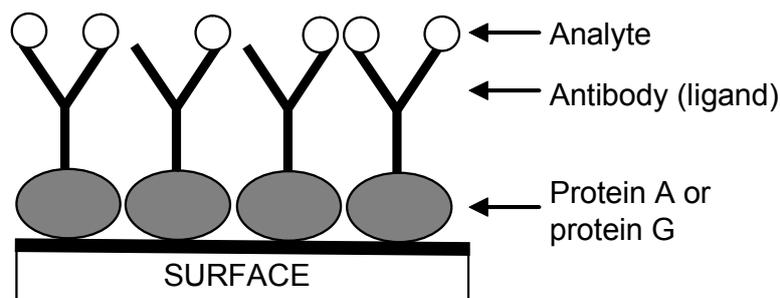


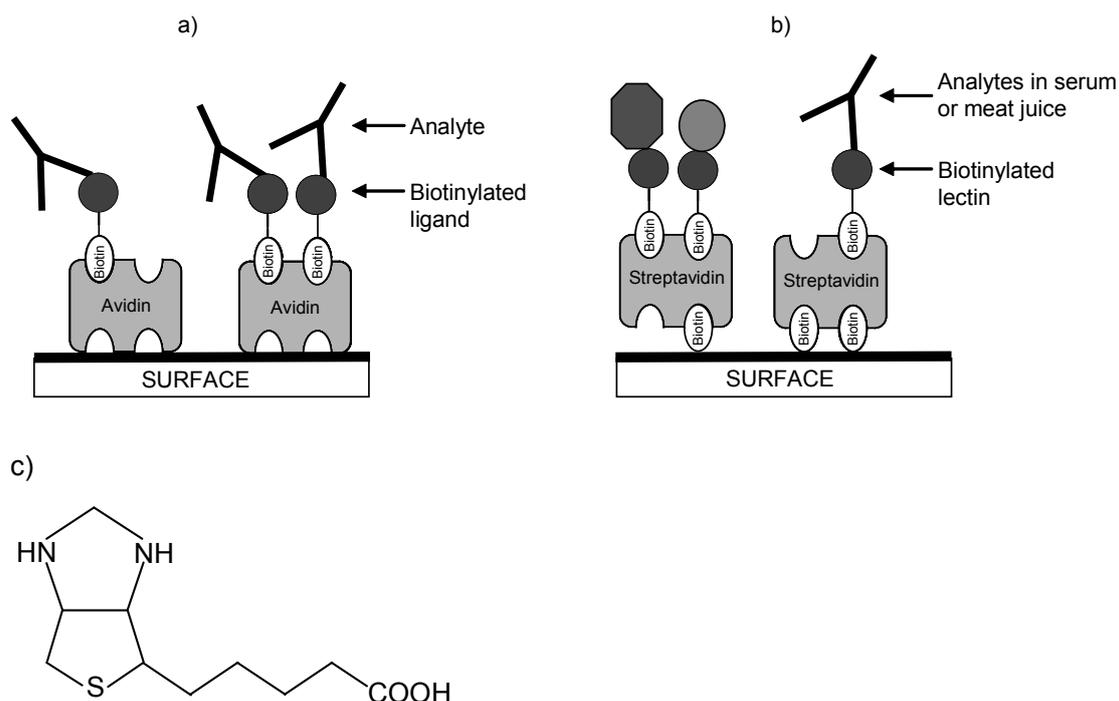
Figure 5.2. Immobilisation via protein A or G

### 5.1.2.2.2 Immobilisation via biotin-avidin coupling.

Biotin-avidin coupling, Figure 5.3, is often chosen when direct covalent coupling of a protein to a surface is unsatisfactory or unsuitable.<sup>[61]</sup> Biotin, Figure 5.3 c), is a small, hydrophobic vitamin with extremely high affinity ( $K_a \sim 10^{15} \text{ M}^{-1}$ ) for avidin, streptavidin and NeutrAvidin<sup>TM</sup> (a chemically modified avidin), which all offer four

## 5. Sensor surfaces: protein immobilisation and non-specific binding

binding sites for biotin. The complex is highly insensitive to harsh regeneration conditions. Biotin contains a carboxylic acid group which can be used for covalent coupling of proteins or for coupling to the sensor surface. Also, biotin has been derivatised for conjugation to amines, thiols or aldehydes on proteins, glycoproteins or other polymers. Attachment of spacers to the biotin carboxyl group greatly enhances the efficiency of formation of the complex between a biotinylated protein and the avidin molecule.



**Figure 5.3.** Coupling of a biotinylated ligand to a surface via avidin or streptavidin. a) A direct covalent coupling of avidin to the surface. b) Streptavidin is coupled to the surface via a biotin molecule. This strategy was adopted in papers I-III, where arrays of lectins with different carbohydrate specificity were immobilised and glycosylation patterns in serum or meat juice samples investigated. c) Chemical structure of biotin.

### 5.1.2.2.3 Immobilisation via capturing antibodies

Immobilisation via capturing antibodies, Figure 5.4, results in attachment of ligands to the surface with a relatively well-defined orientation, but the affinity of the formed complex is not as high as that of biotin and avidin. The capturing antibody, which is immobilised to the surface by for example covalent coupling or via protein A or G, should have a sufficient affinity for the ligand in order to form a stable complex. The capturing antibody should not interfere with the analyte binding site of the ligand.

## 5. Sensor surfaces: protein immobilisation and non-specific binding

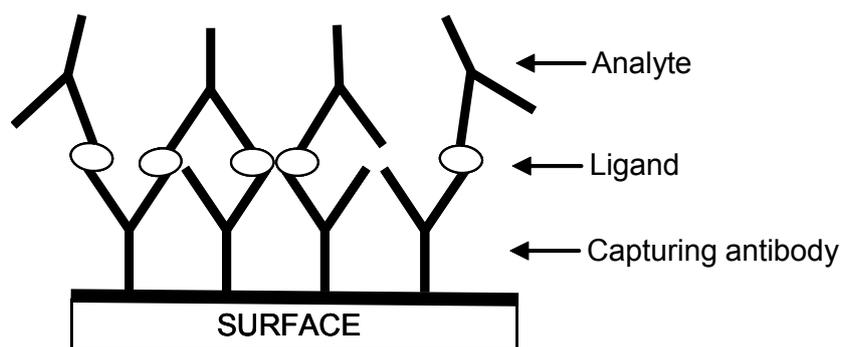


Figure 5.4. Immobilisation via capturing antibodies

### 5.1.2.2.4 Immobilisation of recombinant proteins with tags.

If the ligand is produced as a recombinant protein, tags for simplified purification or immobilisation to a surface can easily be introduced.<sup>[55]</sup> A common modification is the introduction of a hexahistidine tail, which binds to nitrilotriacetic acid (NTA) on the surface in the presence of  $\text{Ni}^{2+}$ . Chips with  $\text{Ni}^{2+}$  chelates are commercially available or can be generated by covalent binding of NTA to modified substrate surfaces. Immobilisation occurs when the tagged protein is added to the surface in a  $\text{Ni}^{2+}$ -containing buffer.

## 5.2 Strategies to combat non-specific binding

A low degree of non-specific binding of the analyte or other compounds present in the sample solution to the sensor surface is important in order to ensure that the interaction of interest is the one being monitored. The sensitivity of the biosensor also increases with a decreased non-specific signal. Especially when addressing analytes in complex media such as cell extracts and body fluids, problems with non-specific binding is often encountered. The analyte of interest in a serum sample may be present at a concentration that is a thousand to a million times lower than that of the most abundant proteins, complicating the measurements. All papers included in this thesis are focused on interaction studies in complex solutions such as sera and meat juices. Many different strategies were evaluated in order to reduce non-specific binding, including dilution of the sample solution, introduction of additives to the

## 5. Sensor surfaces: protein immobilisation and non-specific binding

sample solution and different designs of the sensor surface. In the following sections these and some other common strategies are presented.

### 5.2.1 Dilution

The most common strategy to reduce non-specific binding is to dilute the sample more than 1:20.<sup>[62]</sup> This approach was adopted in papers I-III, where global differences in glycosylation pattern of serum proteins in sera or meat juices were analysed. When quantifying trace molecules in serum, however, for example insulin autoantibodies (ng/ml) addressed in papers IV-V, such a dilution is not feasible and other strategies are needed to combat non-specific binding.

### 5.2.2 Pre-separation

Often some pre-separation steps, *e.g.* by means of affinity chromatography, are used for protein depletion or protein fractionation of complex solutions. Pre-separation of complex fluids might reduce non-specific binding, but also increases analysis time and complexity, while introducing uncertainty due to sample loss during the process.

### 5.2.3 Surface chemistry

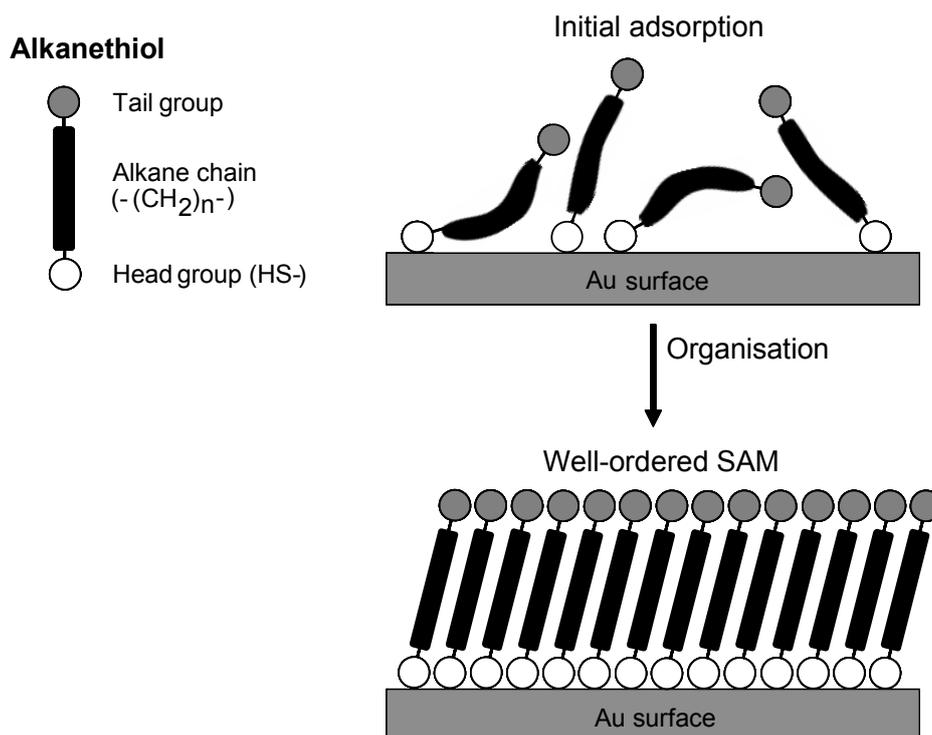
In most biosensor applications the ligand is not immobilised directly onto a flat surface. Instead, it is tethered to some kind of linker layer. Low levels of non-specific binding can be obtained by proper design of the surface chemistry. Numerous surface modifications exist and vary between different kinds of surfaces (metals, polymers etc). It has been suggested that proteins are competing with water for adsorption to the surface.<sup>[50-54]</sup> In general, proteins adsorb more readily onto hydrophobic surfaces (with poor wettability) than onto hydrophilic surfaces (with high wettability) because vicinal water is more readily displaced on hydrophobic surfaces. Also, proteins seem to be more deformed on hydrophobic than on hydrophilic surfaces.<sup>[50]</sup> In contrast, proteins cannot adsorb to fully wettable surfaces because it is not energetically

feasible to dehydrate the surface. This water-competition theory is far from universally accepted, however. Hydrophilicity alone does not guarantee that proteins do not adsorb. While surface wettability may be a good general indicator of the propensity of a surface to adsorb proteins, it is also necessary to consider specific structural features, for example group dipole moment, hydrogen bonding and conformational disorder for each surface.<sup>[51]</sup> In papers II-III sensor surfaces were modified using microcontact printing to form self-assembled monolayers. In papers IV-V commercially available hydrogel surfaces were used. Despite extensive research efforts, there exist to date no completely non-fouling surfaces with respect to undiluted or low-diluted complex samples like serum.

### 5.2.3.1 Self-assembled monolayers

Self assembled monolayers (SAMs) are two dimensional ordered monomolecular films frequently used as linker layers between a surface and the ligand to be immobilised. SAMs form spontaneously through the adsorption of organic compounds to an inorganic or metal surface. The formation of SAMs is extremely versatile and allows remarkable flexibility with respect to terminal functionality, size of the organic compound and orientation of the immobilised ligand. There are many different systems of SAMs based on different organic components and surfaces. In particular, adsorption of alkanethiols on gold is frequently used. An alkanethiol, Figure 5.5, has a thiol head group (HS-) coupled to an alkane chain of varying length  $-(\text{CH}_2)_n-$  and is terminated by a functional tail group (*e.g.* methyl, hydroxyl or carboxyl). Alkanethiolate self-assembled monolayers are straightforward to prepare, functionalise, pattern and are also stable<sup>[63, 64]</sup> SAMs form alkanethiolate films with a binding energy of approximately 40 kcal/mol and are irreversible under normal conditions.<sup>[63]</sup> Adsorption kinetics of SAM formation of alkanethiols of low concentrations (1mM) on gold is a two-step process<sup>[63, 65]</sup>: an initial fast step (within seconds) and a slow step (lasting several hours), Figure 5.5.

## 5. Sensor surfaces: protein immobilisation and non-specific binding



**Figure 5.5.** Illustration of the formation of a self-assembled monolayer of alkanethiolates on gold.

The fast step leads to anchoring of the molecule to the surface with tilt angles being close to their limiting values (alkanethiols on Au(111) are normally tilted  $\sim 26\text{-}28^\circ$  [63, 64]) and with a thickness reaching about 80-90% of its final value. In the end of the slow step, the final ordering of the monolayer takes place. van der Waals forces between the closely packed alkane chains stabilise the monolayer laterally and the stability of the layer is increased with increasing chain length of the molecules that it is composed of. The tilting of molecules is due to the maximisation of the van der Waals interactions between neighbouring alkane chains.<sup>[66]</sup> The SAM can be tailored to provide a wide variety of properties by varying the length of the organic chain and the identity of the tail group of the alkanethiol. SAM layers are not perfect and common defects are pinholes, low-density packing of molecules in the monolayer and large uncovered sites.<sup>[67]</sup>

The surface chemistry of SAMs can be controlled to enhance the ability to prevent non-specific adsorption. By using mixed SAMs, *i.e.* SAMs formed from alkanethiols with different tail group functionalities, this ability can be further improved, and also oriented immobilisation of biomolecules is possible. Mixed SAMs generally consist of one thiolate for immobilisation and another diluting dummy thiolate. The dummy

## 5. Sensor surfaces: protein immobilisation and non-specific binding

thiol, often terminated with  $-\text{CH}_3$  or  $-\text{OH}$ , is included to reduce the surface concentration of terminal functional groups, such as  $-\text{COOH}$ , for ligand immobilisation and thus minimises steric hindrance and partial denaturation of the protein. Also, patterned SAM surfaces are used for controlled surface chemistry and immobilisation of biomolecules.

By using SAMs composed of oligo- or poly(ethylene glycol) (OEG or PEG)-containing alkanethiols excellent protein resistance is achieved.<sup>[68]</sup> Thiolated alkyl chains with a small number ( $\leq 20$ ) of ethylene glycol units are commonly used. The ability to resist protein adsorption is mainly due to their high degree of hydrophilicity and appreciable chain flexibility inducing an exclusion volume effect. PEG polymers with high surface densities and long chains exhibit good protein resistance often explained by a steric repulsion model. The steric repulsion resulting from compression of PEG chains as proteins approach the surface is responsible for the prevention of protein adsorption. The protein resistance may also be explained by the water barrier theory,<sup>[50-52, 54]</sup> which suggests that proteins cannot adsorb on the PEG surface because of the presence of a layer of tightly bound water molecules around PEG chains.

A limitation with SAM surfaces is the low ligand binding capacity compared to surfaces with 3D layers, such as dextran and other polymer matrices.

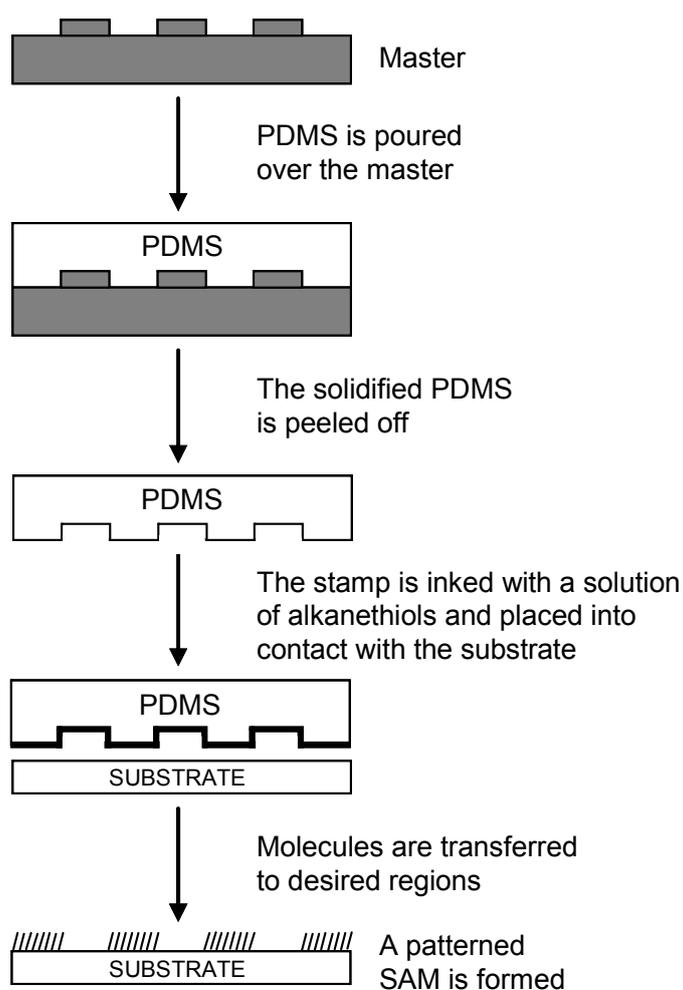
### 5.2.3.2 Microcontact printing

Microcontact printing ( $\mu\text{CP}$ ) is an efficient method for pattern transfer and was first demonstrated for alkanethiolates on gold.<sup>[69]</sup> The combination of SAMs and  $\mu\text{CP}$  provides a remarkably convenient technology for the preparation of patterned surfaces with well-defined regions of different chemical functionality.<sup>[70]</sup> In contrast to *e.g.* photolithography  $\mu\text{CP}$  is straightforward, and once a mask has been produced it can be performed in an ordinary laboratory and working in a clean room is not necessary. The technique is conceptually simple: a stamp impregnated with thiols is placed in contact with a bare gold surface, and the thiols diffuse from the stamp onto the surface where they assemble into ordered structures. However, the process is

## 5. Sensor surfaces: protein immobilisation and non-specific binding

complex and depends on a number of different factors, including the choice and concentration of the SAM-forming molecule, the contact time and the pressure applied to the stamp. With  $\mu$ CP highly ordered SAMs on gold can be formed that are indistinguishable from ordinary SAMs [71-73]. The method can produce patterns with dimensions ranging from nm to several cm.

The stamp material is usually polydimethylsiloxane (PDMS), which is an elastic and inert polymer.<sup>[69]</sup> The stamp is fabricated by casting PDMS on a master having the desired pattern. After curing, the stamp is peeled off from the master and inked in a thiol solution. There are different inking techniques like wet inking, contact inking and reservoir inking.<sup>[74]</sup> Wet inking is easy to perform and was used in papers II-III (Figure 5.6).



**Figure 5.6.** Schematic illustration of microcontact printing by wet inking.

## *5. Sensor surfaces: protein immobilisation and non-specific binding*

The stamp is inked, dried and brought into contact with the surface. Alkanethiols are transferred to the surface at those regions where the stamp contacts the surface, producing a pattern. After removal of the stamp, the surface can be used as it is or be exposed to a solution of a different alkanethiol to cover the remaining bare regions with a SAM with a different tail group or other functionality (backfilling).<sup>[73]</sup> A surface with regions of different hydrophobicity/hydrophilicity may be created in such a procedure. This could be utilised to direct an added sample to certain areas of the chip, which is interesting for many biosensor applications.<sup>[75, 76]</sup>

It has been shown that loosely bound PDMS residues are being transferred to the surface during the stamping procedure,<sup>[75, 76]</sup> which could contaminate the printed surface. UV/ozone treatment of the PDMS stamp lowers the transfer of PDMS residues.<sup>[75]</sup> Ultrasonication in ethanol or exposure of the printed surface to a second thiol removes PDMS residues and loosely bound ink molecules.

### **5.2.3.3. Hydrogels**

Hydrogels are three dimensional polymeric networks capable of swelling in water or biological fluids.<sup>[77]</sup> They can absorb large amounts of water (due to the presence of hydrophilic groups) but remain insoluble because of the presence of cross-links, entanglements, or crystalline regions. The water content in hydrogels affect different properties like permeability, mechanical properties, surface properties, and biocompatibility. Hydrogels are appealing for use as biosensor surface coatings and in biomaterials due to their high water content, their biocompatibility and their high ligand binding capacity compared to two dimensional surfaces. The novelty of hydrogel coatings on biosensor surfaces was first presented by Löfås and Johnsson.<sup>[78]</sup>

Carboxymethylated (CM) dextran is a hydrophilic surface matrix, which is negatively charged at neutral pH and exhibits very low non-specific adsorption of biomolecules. CM dextran consists of linear polymers of glucose units covalently attached to the surface through an inert linker layer (a self-assembled monolayer of a hydroxyl-terminated alkanethiolate) and is swollen in aqueous media, providing an extensively solvated hydrogel. The flexible CM dextran layer provides carboxylic acid groups for

## *5. Sensor surfaces: protein immobilisation and non-specific binding*

site-directed, covalent attachment of ligands. With this matrix a hydrophilic environment around the immobilised ligands is provided. In contrast, when using 2D surfaces a considerable part of the immobilised molecule may be sterically hindered by the surface or by neighbouring molecules.

More recently, polymer matrices of PEG tethered chains have shown to be very protein resistant.<sup>[59, 79-81]</sup>

### **5.2.4 Additives**

Introduction of additives to the sample solution is another frequently used strategy to reduce biofouling. Addition of a salt at high concentration reduces non-specific electrostatic effects.<sup>[82]</sup> If a CM dextran surface is used, addition of CM dextran to the sample reduces non-specific binding with up to 75% via a competition effect.<sup>[62]</sup> Finally, surfactants like P20, Tween 80 and Tween 20 are often added to prevent non-specific adsorption.

### **5.2.5 Blocking**

Another strategy to prevent non-specific adsorption is to use reagents such as non-fat dry milk, bovine serum albumin, PEG and casein to block sites for non-specific binding on the surface where the ligand has been immobilised before addition of the analyte-containing sample. This approach was used by Masson et al., who quantified cardiac markers in undiluted serum. The sensor surface was incubated with analyte-free serum prior to analysis to block sites prone to non-specific adsorption.<sup>[83]</sup> Each blocker may behave differently depending on the nature of the ligand-analyte interaction and the chemistry of the surface. In addition to long incubation times and cumbersome procedures, it is well documented that blocking proteins can reduce binding efficiency and interfere with specific interactions.<sup>[84]</sup> Also, if the biosensor is used in a constant flow set-up, the blocking strategy may not be feasible.

## *5. Sensor surfaces: protein immobilisation and non-specific binding*

Another approach, still complicated, is to use supported membranes. Phillips et al. used a membrane cloaking technique in which the sensor surface with immobilised ligand was incubated with lipid vesicles, creating a membrane.<sup>[85]</sup> The analyte in serum was then introduced for binding and non-specifically adsorbed proteins were thereafter removed together with the membrane using a surfactant, leaving the analyte bound to the ligand.

### **5.2.6 Heat treatment**

Sometimes heat treatment of the sample may be applicable before analysis to reduce non-specific binding.<sup>[86]</sup> This might however affect the properties of the analyte and its interaction with the surface-bound ligand.

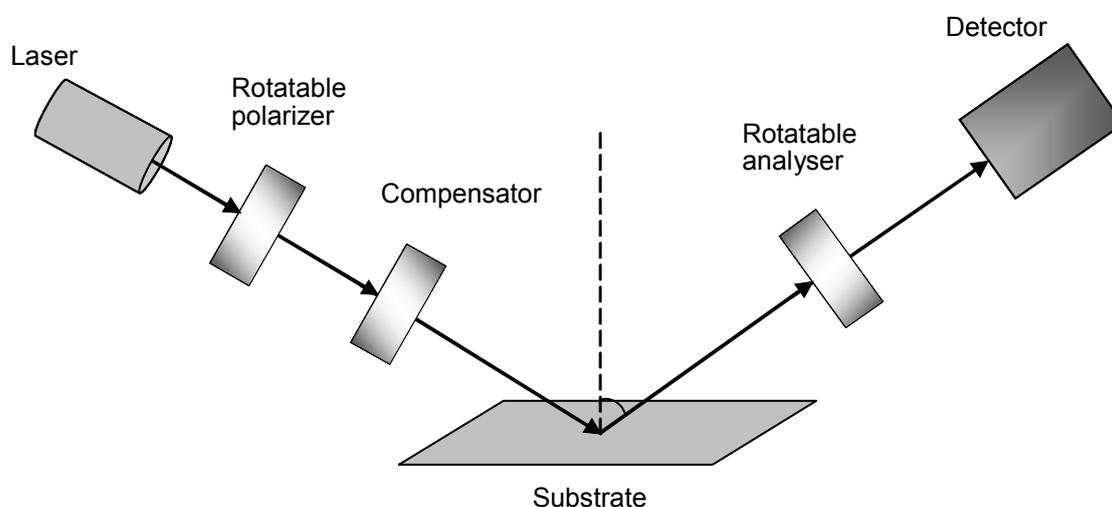
## 6. Optical biosensor techniques

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In this chapter the optical biosensor techniques used for the interaction studies in this work are briefly described. The common denominator of these techniques is that there is no need for labelling of the interacting biomolecules.

### 6.1 Ellipsometry

Ellipsometry is an attractive optical technique for surface and thin film characterisation and has been widely used for studying biomolecules at surfaces. Ellipsometry is based on analysis of polarisation changes occurring upon reflection of a light beam of known polarisation at a reflecting surface. When a film is deposited on a surface the refractive index and thickness of the film can be calculated from such polarisation changes if the optical properties of the bare surface are known. A protein layer thickness of 1-1000 Å can be accurately and reproducibly determined.<sup>[87]</sup> A common ellipsometric setup is null ellipsometry, Figure 6.1, in which the laser beam becomes elliptically polarised after having passed through a linear polarizer and a quarter-wave plate (compensator). The elliptically polarised light is then reflected off the sample onto an analyser and finally to the detector.



**Figure 6.1.** Setup for a null ellipsometer.

In this configuration, the orientations of the polarizer and the compensator are chosen in such a way that the elliptically polarised light becomes completely linearly polarised after reflection off the sample. The analyser is aligned at an angle so that the light reaching the detector has reached a minimum (null) intensity. Normally the compensator is kept at  $\pm 45^\circ$  and the polarizer and analyser are rotated until the nulling condition is fulfilled. The optical parameters can then be deduced from the angular positions of the polarizer and analyser. By using different models, like the McCrackin algorithm,<sup>[88]</sup> with different assumptions about the optical properties of a surface/film/ambient, it is possible to calculate a value of the thickness of the material on flat surfaces from the ellipsometric angles  $\Delta$  and  $\psi$ . These angles can be defined using the complex reflection coefficients  $R_p$  and  $R_s$ , which are the parameters actually measured by the ellipsometer, as:

$$\frac{R_p}{R_s} = \left| \frac{R_p}{R_s} \right| \cdot e^{i(\delta_p - \delta_s)} = \tan \Psi \cdot e^{i\Delta} \quad (6.1)$$

The lateral resolution in null ellipsometry is determined by the cross section and the angle of the incident beam and is often of the order of  $\sim 1 \text{ mm}^2$ .

### 6.1.1 Scanning ellipsometry

Scanning ellipsometry is a three-dimensional imaging method. An in-house built instrument was used for visualisation of surface bound material in paper I and II and the instrumental setup was similar to that in Figure 6.1. The instrument was used in off-null mode, where the analyser and polarizer are fixed and set close to extinction of the reflected light. The amount of adsorbed material on the surface is measured as the increase in intensity of the reflected light, which is proportional to the square of the film thickness  $d$  (for small  $d$ ). (An absolute value of  $d$  cannot be obtained, since it depends on the refractive index of the adsorbed layer, which is not possible to obtain from off-null measurements.) Images of protein distributions were obtained by placing the sample on an X-Y-table. The intensity of reflected light was measured with a photodiode and registered together with the X- and Y-coordinates of the

incident light beam. Raw data was treated with signal and imaging analysis. The scanning instrument enabled measurements of a large surface and made it possible to visualise various areas with immobilised material. The total distribution and not only smaller patches of the surface could thus be investigated.

## 6.2 Surface plasmon resonance

Surface plasmon resonance (SPR) is a powerful technique for monitoring biomolecular interactions in real time in the near surface region of noble metals (often gold).<sup>[89]</sup> Information about the interaction such as specificity, affinity and binding kinetics can be obtained. A surface plasmon is a p-polarized, longitudinal, charge density surface-bound wave propagating along the interface between a metal and a dielectric.<sup>[90]</sup> The SPR setup is often based on the Kretschmann configuration,<sup>[91]</sup> shown in Figure 6.2.

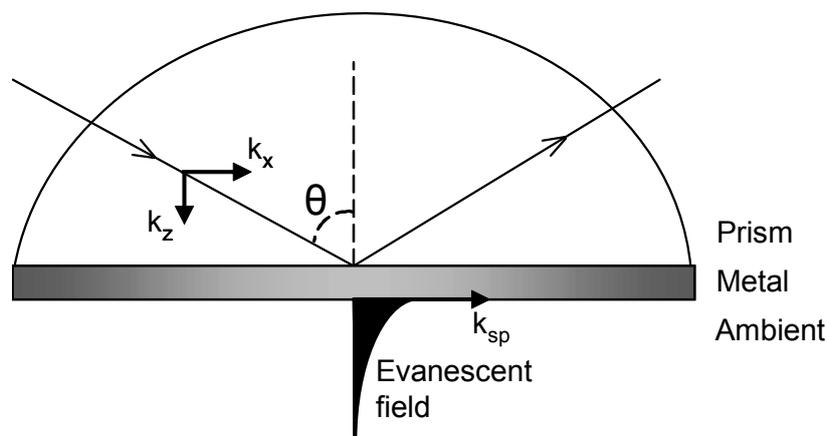


Figure 6.2. Illustration of the Kretschmann setup.

A thin metal film is attached to *e.g.* a prism and light is allowed to pass through the prism at an angle of incidence,  $\theta$ , where it is totally internally reflected at the prism-metal interface. However, part of the light penetrates outside the film as an evanescent field, the intensity of which decreases exponentially with the distance from the surface. If the metal film is sufficiently thin ( $\sim 50$  nm) the evanescent field can excite the surface plasmon. The phenomenon of surface plasmon resonance occurs when the parallel component of the propagation vector of the incident light,  $k_x$ , equals the propagation vector of the surface plasmon,  $k_{sp}$ .  $k_x$  is dependent on the wavelength of

## 6. Optical biosensor techniques

the incident light,  $\lambda$ , the angle of incidence,  $\theta$ , and the dielectric functions of the metal,  $\varepsilon_m(\lambda)$ , glass prism,  $\varepsilon_g$ , and ambient,  $\varepsilon_a$ , according to

$$k_x = \frac{2\pi}{\lambda} \cdot \sqrt{\varepsilon_g} \cdot \sin \theta \quad (6.2)$$

The propagation vector of the surface plasmon,  $k_{sp}$ , can be expressed as

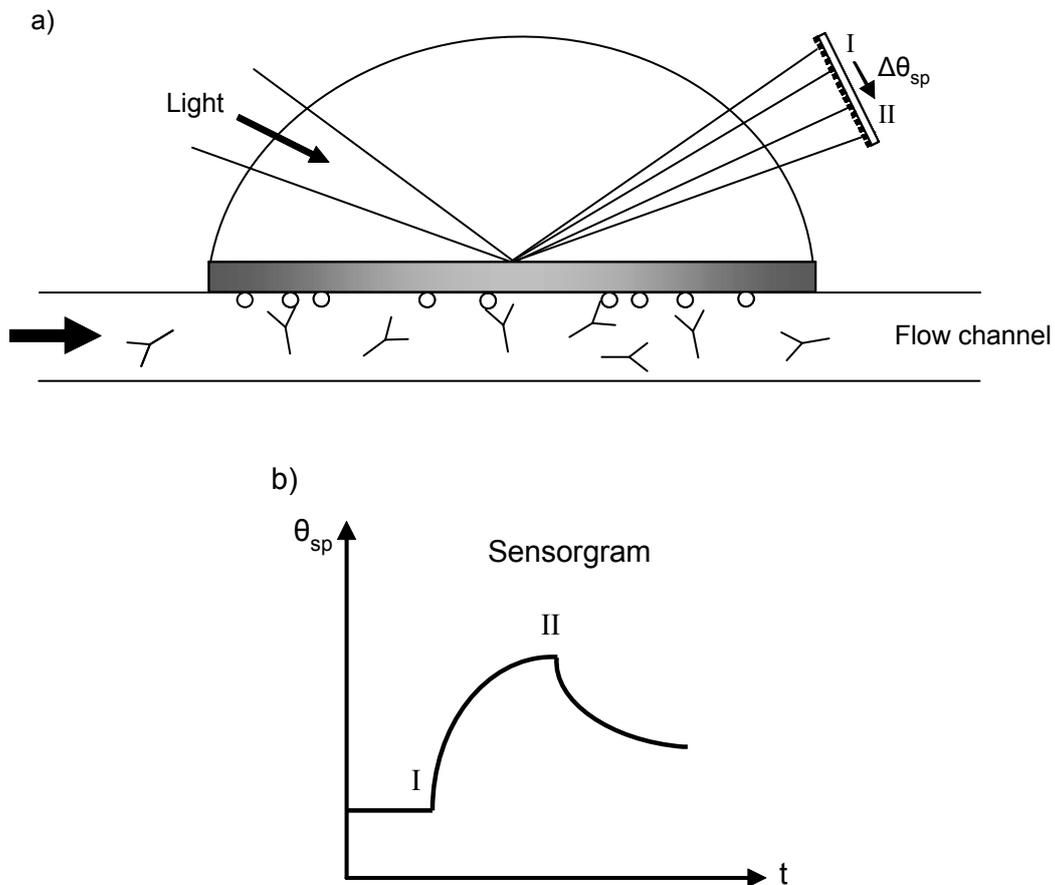
$$k_{sp} \approx \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon_m(\lambda) \cdot \varepsilon_a}{(\varepsilon_m(\lambda) + \varepsilon_a)}} \quad (6.3)$$

where  $\varepsilon_m(\lambda)$  is the wavelength-dependent dielectric function of the metal, and  $\varepsilon_a$  is the dielectric function of the ambient. The condition for surface plasmon resonance,  $k_x = k_{sp}$ , then becomes

$$\sin \theta_{sp} = \frac{1}{\sqrt{\varepsilon_g}} \cdot \operatorname{Re} \left( \sqrt{\frac{\varepsilon_m(\lambda) \cdot \varepsilon_a}{(\varepsilon_m(\lambda) + \varepsilon_a)}} \right) \quad (6.4)$$

The metal, the glass prism and the wavelength of the incident light are usually constant, while any change at the metal-ambient interface caused by formation of the ligand-analyte complex will cause a change of the refractive index  $n_a$  close to the surface, Figure 6.3 a). This will affect  $\varepsilon_a$  ( $\varepsilon_a^{1/2} = n_a$ ), which gives rise to a shift in the resonance angle,  $\Delta\theta_{sp}$ .  $\Delta\theta_{sp}$  is monitored as a function of time in a so-called sensorgram, Figure 6.3 b), which provides record of the progress of association and dissociation between the ligand and analyte. The response signal given from the SPR instrument used in this thesis is expressed in resonance units RU (1 RU  $\sim$  1 pg/mm<sup>2</sup> of a medium sized protein), where 1 RU corresponds to  $\Delta\theta_{sp} = 0.0001^\circ$ .

## 6. Optical biosensor techniques



**Figure 6.3.** a) SPR setup for monitoring biomolecular interactions in a flow channel. An incoming light cone is passed through a prism with a deposited metal film. I shows  $\theta_{sp}$  before addition of analyte. Changes in the refractive index at the metal-ambient interface caused by formation of ligand-analyte complexes, II, give rise to a shift in the resonance angle,  $\Delta\theta_{sp}$ . b) A shift in the resonance angle,  $\Delta\theta_{sp}$ , is monitored as a function of time in a sensorgram.

The penetration depth of the evanescent field is defined as the distance from the interface where the evanescent field strength has decayed to  $1/e$  of that close to the interface. Within this distance from the surface, refractive index changes are detectable. For a setup with gold and water and a wavelength of 632.8 nm the penetration depth is  $\sim 190$  nm.<sup>[89]</sup>



## 7. Immunoassays

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Antibody-based immunoassays are the most commonly used types of diagnostic assays and are still one of the fastest growing technologies for the analysis of biomolecules. Immunoassays have for example an important role in the diagnosis of HIV through the HIV test. Although antibodies are not the only molecule that can be used for quantification of antigens, bioassays based on whole cells, receptors and enzymes do not, in many instances, offer the same unlimited applicability and specificity.

The specificity and sensitivity of antibody assays are important parameters that can vary significantly, depending on the method and the performance characteristics of the assay. The specificity of the assay reflects how well a test correctly identifies the negative samples, *e.g.* the probability that the test indicates negative if a person does not have the antibody. The higher the threshold for antibody positivity, the more specifically the assay identifies persons with antibodies, but at the cost of excluding those with low antibody concentrations. The sensitivity reflects the probability that all individuals with antibodies are identified as antibody positive. Low sensitivity means that not all individuals with the specific antibody are identified with the test

General assay formats for analysing intermolecular interactions are direct,<sup>[92, 93]</sup> sandwich,<sup>[94]</sup> displacement<sup>[95, 96]</sup> and indirect competitive assays<sup>[97]</sup>, most of which are often used together with SPR sensors. Each approach has its own advantages and limitations with respect to sensitivity, assay time, availability of suitable reagents, required sample volume, complexity, nature of the target analyte and application. In paper IV an SPR-based indirect competitive assay for quantification of IAA in serum was developed (described in section 7.4.1) and a radioimmunoassay was used as reference method (described in section 7.5).

### 7.1 Direct binding

Direct binding (Figure 7.1) is the simplest assay format, in which a ligand is immobilised on the sensor surface and binding of the corresponding analyte (target molecule) is monitored directly. This detection principle is generally used for detection of large molecules (>10 kDa) and direct measurements of analytes smaller than 2 kDa are seldom feasible.<sup>[82]</sup>

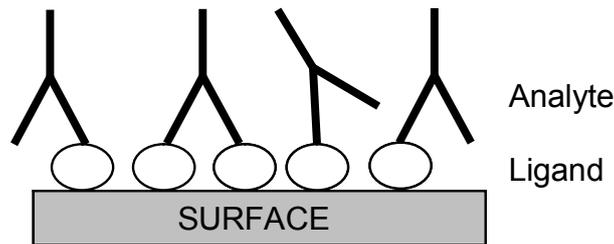


Figure 7.1. Principle of the direct binding assay

### 7.2 Sandwich assay

In sandwich assays (Figure 7.2) the analyte is captured between two binding partners, of which one is immobilised and the other added either together with the analyte or in a second step after binding of the analyte to the immobilised ligand. Two specific binding partners with non-overlapping binding sites are required, which may be a problem to find for small proteins. The best established sandwich assays are based on two specific antibodies as binding partners. The instrument signal increases with increasing concentration of analyte.

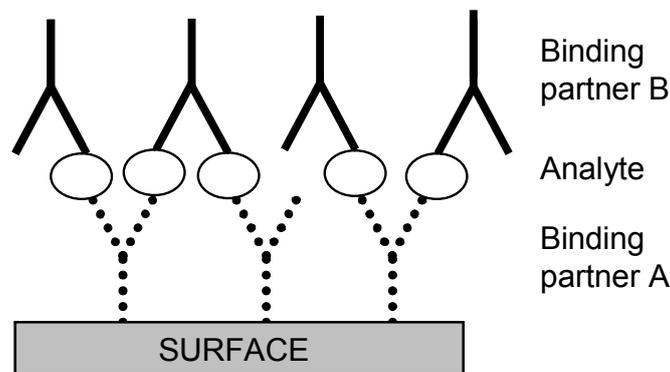
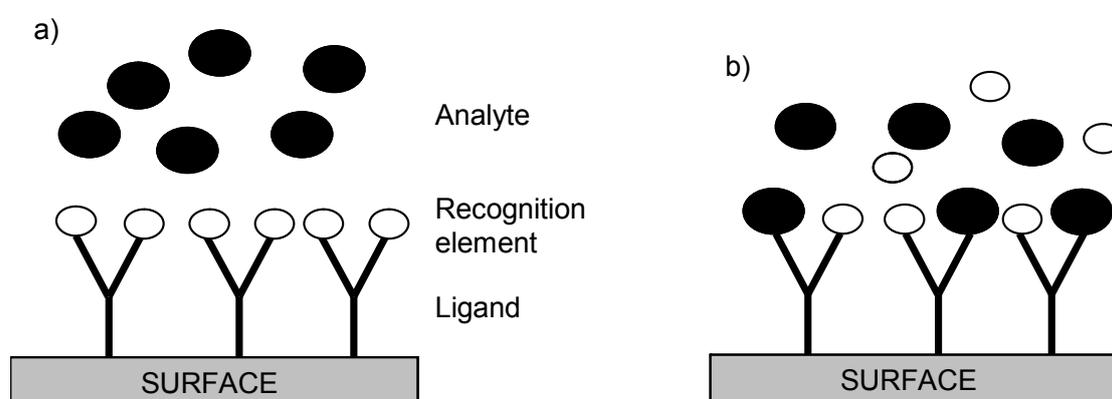


Figure 7.2. Principle of the sandwich assay

### 7.3 Displacement assay

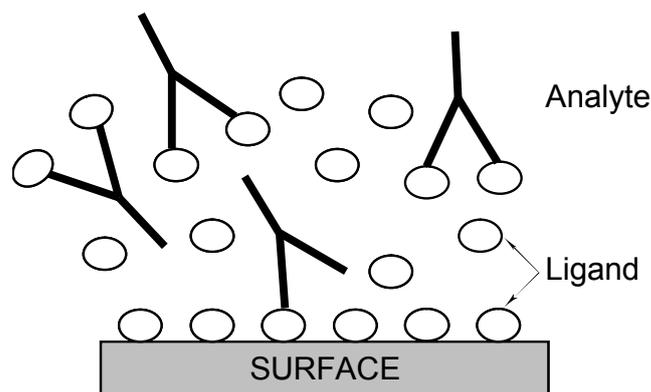
In displacement assay (Figure 7.3) formats, an excess of a recognition element is introduced to the immobilised ligand to occupy all the binding sites. Upon introduction of the analyte, displacement of the first recognition molecule occurs. When using SPR-based biosensors the recognition element and the analyte must differ substantially in size. This format however, is generally used with labelled antibodies and measured by fluorescence or chemiluminescence methods.



**Figure 7.3.** Principle of the displacement assay. a) Excess of a recognition molecule occupies all binding sites on the ligand. b) When the analyte is introduced displacement of the recognition molecule takes place.

### 7.4 Indirect competitive inhibition

The principle of indirect competitive inhibition (Figure 7.4) generally involves immobilisation of the purified ligand or a ligand analogue on the surface. The analyte containing sample is then mixed with ligand and introduced to the surface, resulting in competition between immobilised ligand and ligand in solution for binding of the analyte. The concentration of added ligand is kept constant so that the response variations are monotonically related to the amount of analyte molecules in the sample. An increased ligand concentration in solution results in a lower response, due to less binding of analyte to the immobilised molecules. This assay provides remarkable sensitivity for detection of low-molecular weight analytes without the need for labelling.



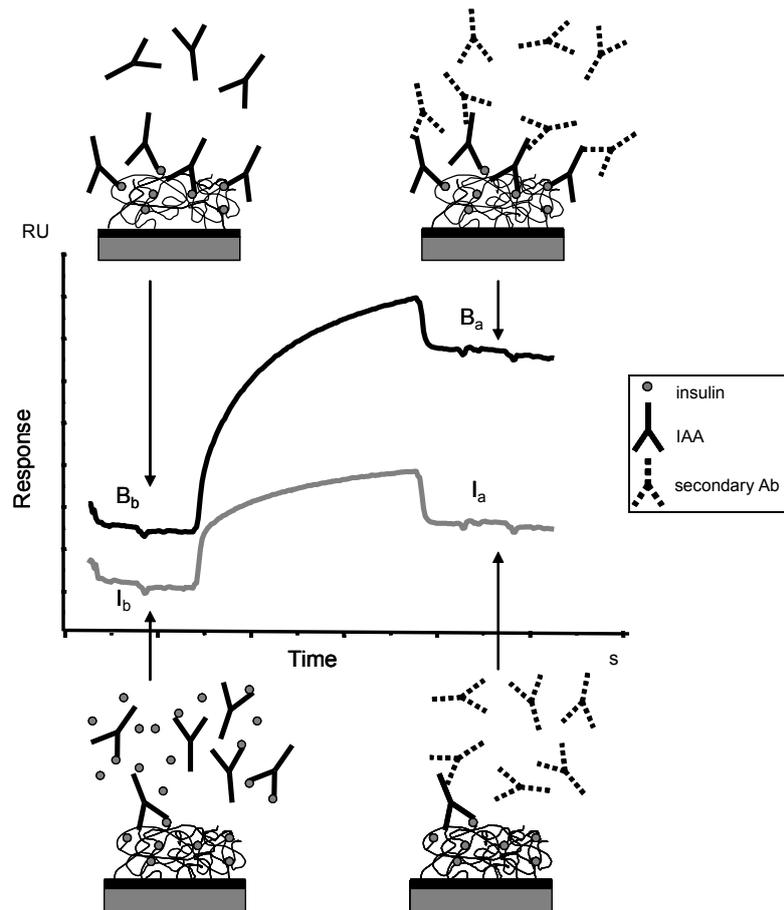
**Figure 7.4.** Principle of the indirect competitive inhibition assay. The ligand is immobilised on the surface. Analyte mixed with ligand is then introduced over the surface and competition between immobilised ligand and ligand in solution for binding of the analyte occurs.

#### 7.4.1 An indirect competitive immunoassay for IAA based on SPR

In papers IV-V a variant of the indirect competitive assay was used together with SPR for detection of IAA in serum. When measuring trace molecules in undiluted sera with label-free techniques like SPR, non-specific adsorption of matrix proteins to the sensor surface is often a problem, since it causes a signal that masks the analyte response. Also, the amount of non-specifically bound proteins differs substantially between serum samples from different individuals. In order to reduce non-specific binding of serum components to the surface many different strategies were tried, for example different surface coatings and different additives. Although some strategies reduced non-specific binding in low-diluted sera to a certain extent, the problem with fluctuations in non-specific binding between sera from different individuals still persisted and was difficult to overcome. Therefore, an indirect competitive immunoassay for IAA was developed in order to circumvent this problem.

In Figure 7.5 the assay principle is shown. Human insulin was immobilised on the surface and a serum sample was divided in two equal aliquots, where one was mixed with buffer and the other with an excess amount of insulin. When the serum portion mixed with buffer was introduced to the surface, IAA in the sample was free to bind immobilised insulin on the surface. When premixed with insulin competition for binding of IAA between insulin in solution and insulin immobilised on the surface

took place. However, due to the large excess of free insulin molecules in solution, very little binding of IAA to the surface-bound ligands would occur. In this assay the analyte-ligand complex was detected by a secondary antibody. By subtracting the response from serum mixed with excess insulin from the response received from serum mixed with buffer, IAA in the particular sample can be quantified. Using this assay a screening study of newly diagnosed type 1 diabetes patients was made (paper V).



**Figure 7.5.** Principle of the indirect competitive immunoassay for IAA. For a pair of samples incubated with either insulin (I) or buffer (B), the relative amount of IAA in the original sample (R) is calculated from the baseline before ( $B_b$ ,  $I_b$ ) and after ( $B_a$ ,  $I_a$ ) injection of the secondary antibody, according to  $R = (B_a - B_b) - (I_a - I_b)$ .

### 7.5 Radioimmunoassay (RIA)

A radioimmunoassay (RIA) is a method for quantifying antigen-antibody interactions utilising radioactive labelling. The technique was introduced in 1960 by Berson and Yelow as an assay for measuring the concentration of insulin in plasma.<sup>[98]</sup> RIA is

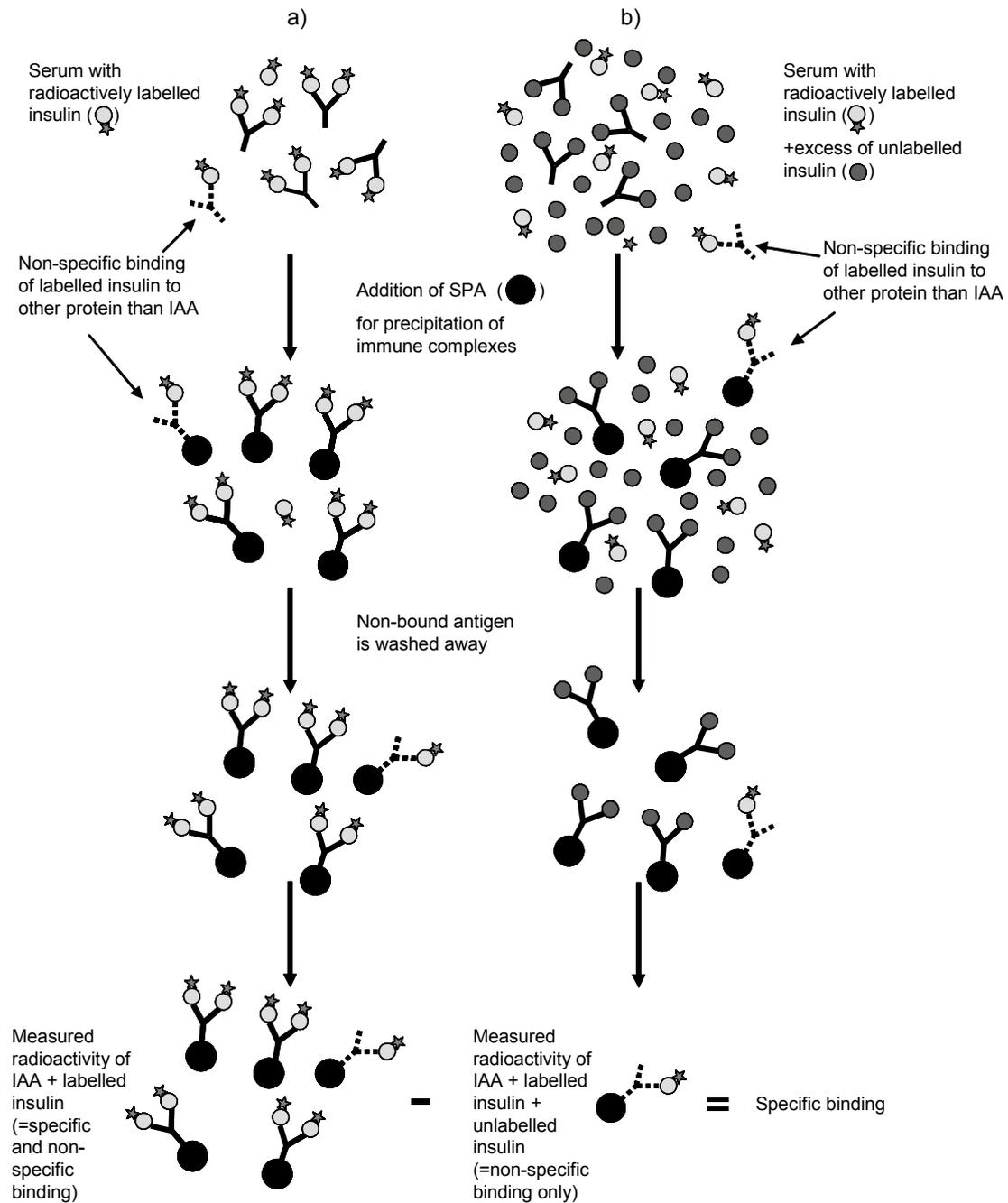
## 7. Immunoassays

now an important research tool, as well as being frequently used in hospitals for diagnostic purposes. The technique is both sensitive and specific, but requires special precautions (because radioactive substances are used), sophisticated apparatus, and is expensive. RIA has been largely replaced by the Enzyme-Linked ImmunoSorbent Assay (ELISA), where the detection principle relies on colorimetric changes.

To measure IAA in serum, a microassay RIA based on Williams et al. is often used.<sup>[99]</sup> The RIA used for IAA quantification in this thesis is a competitive assay based on Williams et al. with some modifications,<sup>[100]</sup> Figure 7.6. In short, a serum sample is added to four separate wells in a microtiter plate. To the first duplicate wells <sup>125</sup>I-labelled human insulin is added and to the second set of duplicate wells labelled insulin mixed with a known concentration of excess unlabelled, “cold”, human insulin is added. The mixtures are agitated for 72h at 4°C and subsequently incubated with protein A-sepharose (SPA) for 90 min at 4°C during agitation to precipitate the formed immune complexes (protein A binds with high affinity to human IgG1 and IgG2). Much of the added SPA binds other IgGs in the sera than IAA, since IgG is present at mg/ml concentrations and IAA at ng/ml. After a number of washing steps the activity of the precipitate is measured in a gamma counter

Cold insulin and radioactively labelled insulin compete for the binding sites on the antibodies, but due to the large excess of unlabelled insulin added to the second set of duplicate wells, all IAA in the serum sample will bind predominantly unlabelled insulin. The reason for using unlabelled insulin is to be able to subtract responses from non-specific binding of labelled insulin to other molecules than IAA in the serum sample (big proteins or other IgGs than IAA, which are not removed during the washing steps but stay with the precipitate). Relative quantification is obtained after subtraction of counts for the sample incubated with labelled + unlabelled insulin from counts of the sample incubated with only labelled insulin. Results are expressed as arbitrary units (U/ml) and are derived from a standard curve, which is constructed from a pooled IAA-positive sample diluted to different concentrations in a non-diabetic serum sample.

## 7. Immunoassays



**Figure 7.6.** Serum is a) mixed with radioactively labelled insulin and b) mixed with radioactively labelled insulin + excess of unlabelled insulin. Immune complexes are precipitated by addition of protein A-sepharose (SPA). After washing, radioactivity is measured in a gamma counter. The signal corresponding to specific binding of IAA in the sample is obtained from subtraction of counts for the sample incubated with radioactively labelled insulin + unlabelled insulin from counts of the sample incubated with only labelled insulin.

## *7. Immunoassays*

International workshops have shown that there is poor agreement between results from different laboratories performing IAA RIAs and that the IAA sensitivity is rather low.<sup>[44, 46]</sup> In such a workshop from 2003 where 46 laboratories participated, the median adjusted sensitivity was 58 % while the specificity was 99%.<sup>[44]</sup>

ELISA is not recommended for measuring IAA, since it has been shown that in contrast to RIA, ELISA does not measure IAA correlated to T1D.<sup>[30, 101]</sup> In a solution-based technique such as RIA most insulin epitopes would be available for binding, while in ELISA epitopes may be hidden and sterically unavailable.

## 8. Data analysis

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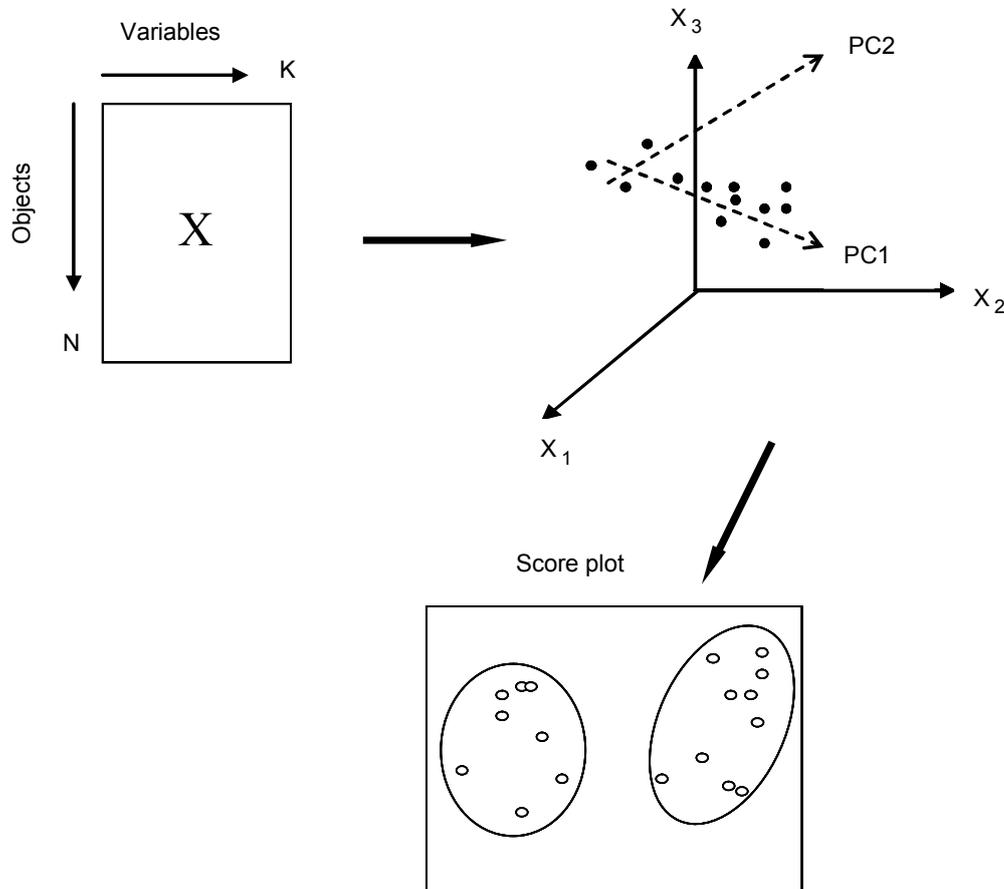
Multivariate data analysis (MVDA) or chemometrics is a way to extract information and recognise patterns in large amounts of data using statistical and mathematical methods. MVDA is also used for making calibration models that can predict groupings of data. The techniques are invaluable tools when evaluating the huge amounts of information obtained from for example electronic noses<sup>[5]</sup> and tongues<sup>[4]</sup>. MVDA has also been applied in other areas, such as in the evaluation of data from lectin arrays.<sup>[20-22]</sup> There are different ways to perform MVDA depending on the information desired. Some of the most common MVDA methods; principal component analysis and artificial neuronal networks, were used for analysis of the data received from the lectin arrays used in papers I-III and are presented below.

### **8.1 Principal component analysis**

With principal component analysis (PCA) an overview of a data set is given and patterns in the data can be revealed.<sup>[102]</sup> In Figure 8.1 an illustration is given. PCA is a mathematical transform used to explain the variance in a matrix ( $X$ ) with  $N$  number of objects (measurements) and  $K$  number of variables (sensor outputs). This creates a multidimensional space of  $K$  dimensions containing  $N$  points. Each object is represented in the variable space. With PCA a vector called the first principal component (PC1) is calculated by fitting the data points to a line using partial least squares in order to find the direction that maximises the variance, *i.e.* the direction that describes the largest difference between observations. A second principal component (PC2) is orthogonal to PC1 and contains the second largest variance, which describes as much as possible of the remaining information in the data set. More PCs are calculated until all the information has been accounted for. With PCA the  $K$  dimensions are reduced to a smaller number of dimensions defined by the principal components and can be represented in score and loading plots. The score plot shows the objects placed in variable space consisting of two principal

## 8. Data analysis

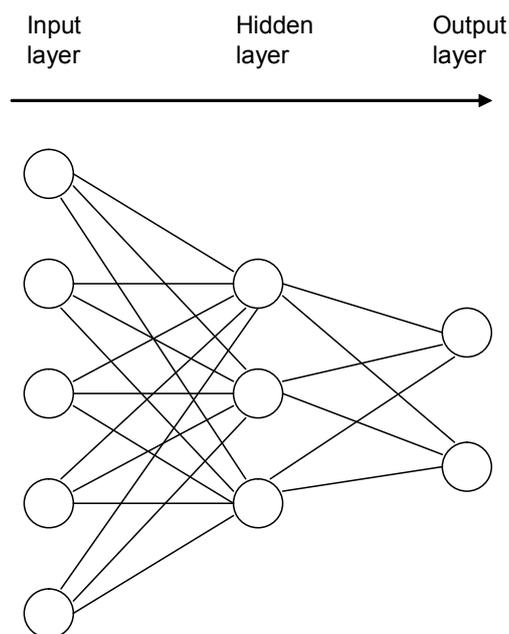
components and the relations between different objects can be studied, such as clustering. In a loading plot variables are plotted in object space, showing how much each variable is contributing to each PC.



**Figure 8.1.** Principle of principal component analysis (PCA).

## 8.2 Artificial neuronal networks

An artificial neuronal network (ANN) is a non-linear model tolerant to noise and faults, which can be used for modelling of complicated patterns and to predict new samples.<sup>[103]</sup> It is built to resemble the biological nervous system with signal transfer between neurons connected by synapses in a complicated network. In ANN the neurons have been replaced by nodes that receive information from other nodes, perform a simple calculation of the information obtained and forward this new information to other nodes. ANN is a layered structure with an input layer, at least one hidden layer and an output layer, Figure 8.2.



**Figure 8.2.** Schematic illustration of an ANN with three layers

The input variables enter through the nodes in the input layer, which compared to the hidden and the output layer is inactive in the modelling process. The number of nodes in the layers and the number of hidden layers are dependent on the problem and decided by the user. Modelling of neural networks consists of training and optimisation and in the training step the number of layers and nodes in each layer is determined. There are different algorithms available for training of the net, for example cross-validation. During the training, output values are compared to true values and the coupling constants are adjusted to minimise the differences by minimising the sum of square errors. ANN-modelling is complex and there is a risk of over-fitting. The number of variables that can be used in the model is limited and also sets the training time of the ANN architecture.

### **8.3 Bioanalytical methods and MVDA**

In spite of its great analytical potential and capability to enhance biorecognition, MVDA has not yet been widely used together with bioanalytical methods. Some different bioanalytical methods have however been combined with MVDA techniques to analyse different interaction patterns. For example, surface plasmon resonance has been combined with PCA to differentiate between human serum

## 8. Data analysis

samples from healthy individuals and patients with a throat infection using a lectin panel.<sup>[22]</sup> An electrochemical lectin array, also combined with PCA, has been used for the identification of microorganisms<sup>[20]</sup> and to identify viable *Escherichia coli* subspecies.<sup>[21]</sup>

## 9. Included papers

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### ***Paper I***

#### **Investigation of sera from various species by using lectin affinity arrays and scanning ellipsometry**

*Jenny Carlsson, Michael Mecklenburg, Ingemar Lundström, Bengt Danielsson, Fredrik Winqvist*

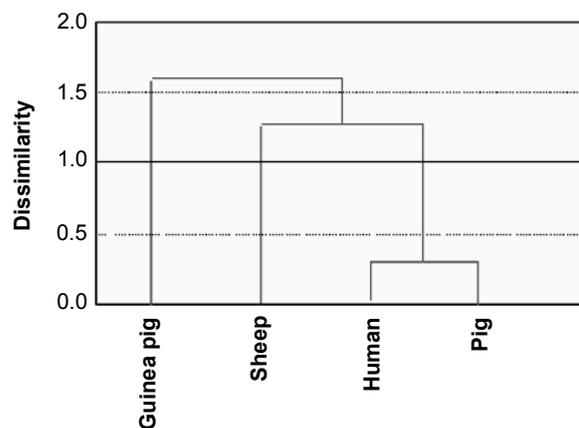
Anal. Chim. Acta **2005**, 530: 167-171

#### **My contribution**

Most evaluation and analysis of data and all writing.

#### **Short description**

Sera from human, pig, sheep and guinea pig were applied to an array of different lectins immobilised on a gold surface. The biorecognition of glycosylated serum proteins binding to the lectins was evaluated with scanning ellipsometry and multivariate data analysis. The results showed a significant difference in serum protein glycosylation pattern between the different species investigated. The dendrogram in Figure 9.1 shows the relation of different species according to the results. In accordance with the literature, human and pig sera seem to be more closely related than for example human and guinea pig sera.



**Figure 9.1.** Dendrogram showing how sera from different species are related.

## Paper II

### Biosensor discrimination of meat juice from various animals using a lectin panel and ellipsometry

Jenny Carlsson, Fredrik Winquist, Bengt Danielsson, Ingemar Lundström

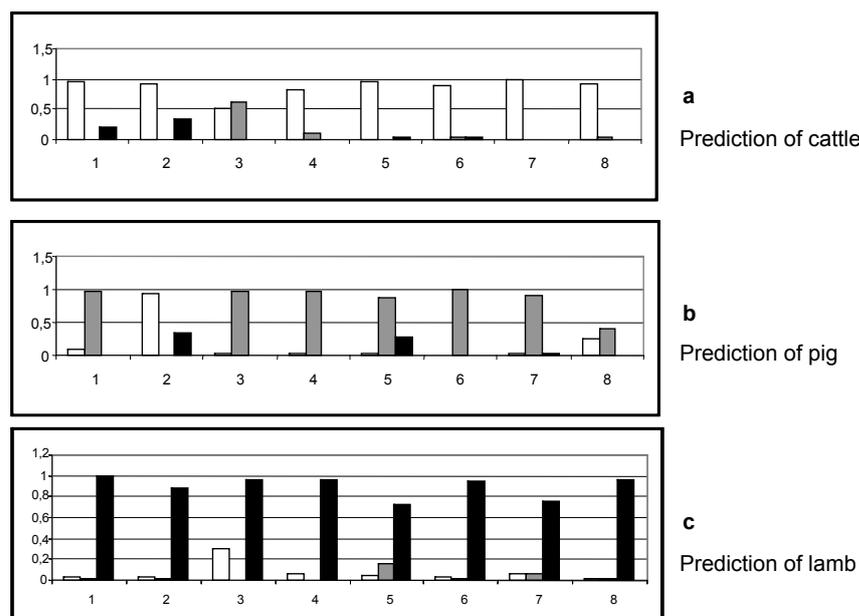
Anal. Chim. Acta **2005**, 547: 229-236

#### My contribution

All experimental work, most analysis and all writing.

#### Short description

A lectin array was used for investigation and discrimination of different meat juices from fresh meat of cattle, chicken, pig, cod, turkey and lamb and the biorecognition was detected with ellipsometry. The data obtained was evaluated with multivariate data analysis techniques and showed that the different meat juices could be separated from each other due to their different serum protein glycosylation patterns. A model based on an ANN was made to classify meat juices from the mammals. Its prediction ability is shown in Figure 9.2, and worked generally well.



**Figure 9.2.** Prediction of classification of meat juices from mammals: cattle = □ pig = ■ and lamb = ■ On the ordinate, the predictions are shown, where 1 corresponds to 100%. The corresponding experiments are shown on the abscissa.

**Paper III****Detection of global glycosylation changes of serum proteins in type 1 diabetes using a lectin panel and multivariate data analysis**

*Jenny Carlsson, Camilla Gullstrand, Johnny Ludvigsson, Ingemar Lundström, Fredrik Winqvist*

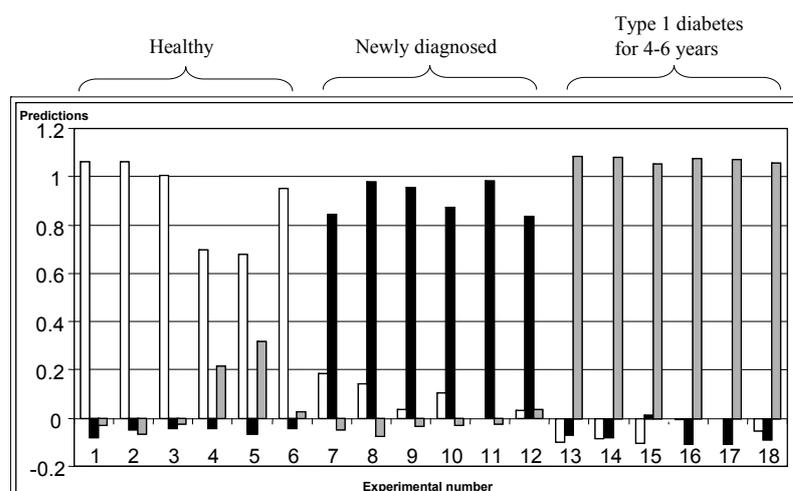
Talanta **2008**, 76(2): 333-337

**My contribution**

All experimental work, most analysis and all writing.

**Short description**

Global glycosylation changes of serum proteins in type 1 diabetic patients were investigated with a lectin array. Sera from healthy individuals, newly diagnosed type 1 diabetes patients and type 1 diabetes patients having suffered from the disease for several years were compared. The biorecognition was evaluated with null ellipsometry and MVDA techniques were used to analyse data. It was shown that discrimination between sera from the three different groups was possible and that correct categorisation of unknown serum samples into one of the three groups could be made using a model based on an ANN, Figure 9.3.



**Figure 9.3.** Prediction of classification of sera into the three different serum groups; healthy, newly diagnosed and having suffered from the disease for 4-6 years.

## Paper IV

### An indirect competitive immunoassay for insulin autoantibodies based on surface plasmon resonance

Jenny Carlsson, Camilla Gullstrand, Gunilla T. Westermarck, Johnny Ludvigsson, Karin Enander, Bo Liedberg

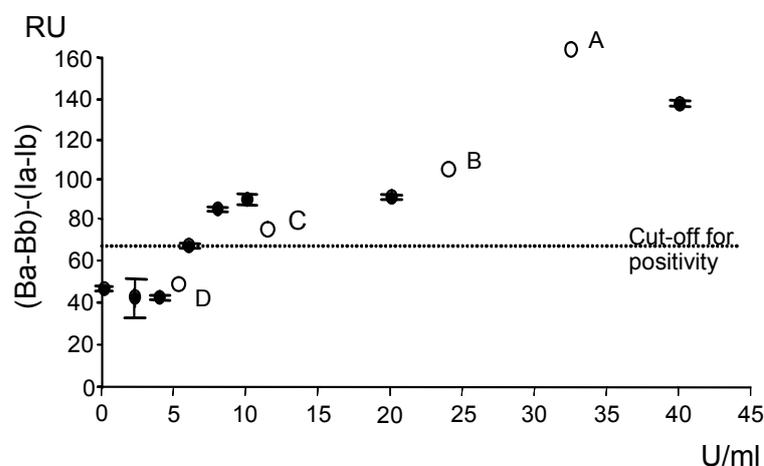
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#### My contribution

Experimental work including assay development together with Camilla Gullstrand, except from the RIA measurements which were performed by Camilla Gullstrand and Ingela Johansson. Most analysis and writing.

#### Short description

An indirect competitive immunoassay based on SPR for detection of IAA in serum samples from individuals at high risk of developing type 1 diabetes was developed. With this immunoassay the overall assay time compared to the reference RIA was reduced, while sensitivity was maintained at a level comparable to that offered by RIA. In Figure 9.4, a standard curve from a pooled high IAA-positive serum diluted to different concentrations in different non-diabetic serum samples is shown together with four samples from newly diagnosed T1D children.



**Figure 9.4.** Standard curve with error bars from a high IAA-positive sample diluted in different non-diabetic serum samples. Sample A, B, C and D are non-pooled sera from T1D patients.

**Paper V****Determination of insulin autoantibodies using surface plasmon resonance:  
A screening study of newly diagnosed type 1 diabetes patients**

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In manuscript

**My contribution**

All experimental work except from RIA measurements, which were performed by Ingela Johansson. Most analysis and writing.

**Short description**

The screening potential of the indirect competitive immunoassay developed in paper IV was investigated for quantification of IAA in sera from newly diagnosed T1D patients using a RIA as reference technique. The two methods agreed well with respect to sample classification, Table 9.1. The sensitivity was higher in the SPR-based assay and the specificity was comparable to that of the RIA. Fourteen percent of the analysed samples gave rise to anomalously high and easily distinguishable responses with the SPR-based assay, precluding IAA-quantification, possibly revealing the presence of immune complexes.

**Table 9.1.** Eighty-six serum samples from newly diagnosed T1D patients (T1D) and non-diabetics (non-T1D) were classified as IAA-positive (+) or IAA-negative (-) with the SPR-based indirect immunoassay and the reference RIA. With SPR, 12 samples were classified as outliers.

	RIA		SPR		
	+	-	+	-	Outliers
T1D	24	30	27	20	7
Non-T1D	0	32	0	27	5



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