INSIGHTS INTO WEAK AFFINITY ANTIBODY-ANTIGEN INTERACTIONS

Studies using affinity chromatography
and optical biosensor

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Till mamma, pappa och Chanti

I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Sir Isaac Newton, 1642-1726

POPULÄRVETENSKAPLIG SAMMANFATTNING

Livets finurlighet är fascinerande att studera. När man undersöker biologiska samband i detalj visar det sig att allt styrs av biomolekylernas förmåga till igenkänning och växelverkan med varandra. Molekylernas speciella egenskaper bygger upp kroppens alla komplicerade funktioner, till exempel immunförsvar, celldelning och muskelarbete. Interaktionerna mellan biomolekylerna kan vara av olika karaktär; de beskrivs som starka eller svaga (affinitet), långsamma eller snabba (kinetik). Det har visat sig att många biologiska mekanismer styrs av svaga interaktioner som, flera tillsammans, skapar igenkänning och stringens. Tack vare att de enskilda interaktionerna är svaga skapas samtidigt en dynamik och förmåga för systemen att snabbt anpassa sig till omgivande förhållanden. Detta har samtidigt medfört att de varit har svåra att studera, just på grund av att de är så svaga.

Denna avhandling handlar om dessa svaga biomolekylära interaktioner. Den diskuterar vilken roll de har i biokemiska kommunikationer och hur man kan studera dem. Tonvikten ligger dock på hur man kan utnyttja svagt växelverkande biomolekyler som reagens i analytiska metoder. Som reagens använder man också antikroppar. Dessa proteiner är en viktig del i kroppens immunförsvar och kan på konstgjord väg fås att känna igen de speciella molekyler (antigen) man är intresserad av. Antikropparna används sedan i analyser av exempelvis läkemedel, diagnostik eller i biotekniska processer. I de arbeten som presenteras här har låg-affina antikroppar använts i olika modellsystem. Artikel I visar att svaga interaktioner lämpar sig väl för kromatografisk separation av strukturellt närbesläktade molekyler. Övriga arbeten handlar om hur svaga interaktioner kan tillämpas i biosensorer. I artiklarna II och III utforskas möjligheterna till mätning av verkligt svaga biomolekylinteraktioner. Den dynamik och specificitet som kännetecknar dessa gör att man kan använda dem till att övervaka föränderliga processer. Artiklarna IV och V introducerar den kontinuerliga immunsensorn som har stora möjligheter till att användas i områden där man vill mäta fluktuerande koncentrationer av intressanta molekyler.

CONTENTS

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Kalmar, October 2000

LIST OF SYMBOLS AND ABBREVIATIONS

Ab	antibody binding site
$[Ab]_t$	total Ab concntration, see also Q_{max}
Ag	antigen
ACE	affinity capillary electrophoresis
AFM	atomic force microscopy
APC	antigen-presenting cell
CAM	cell adhesion molecule
CD	cluster of differentiation
CDR	complementarity determining region
CE	capillary electrophoresis
Da	Dalton (g/mol)
ELISA	enzyme linked immuno-sorbent assay
Fab	fragment antigen binding
Fuc	fucose
Fv	fragment variable
Gal	galactose
Glc	glucose
HPLC	high performance LC
HPLAC	high performance liquid affinity chromatography
HSA	human serum albumin
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IS	immunological synapse
ITC	isothermal titration calorimetry
K_A	association constant
k_{ass}	association rate constant
K_D	dissociation constant
k_{diss}	dissociation rate constant
Le^x	Lewis X antigen (Galβ1-4[Fucα1-3]GlcNAc-β1-R)
lmw	low molecular weight
LC	liquid chromatography
MHC	major histocompatibility complex
MS	mass spectrometry
Mw	molecular weight
NMR	nuclear magnetic resonance
pnp	*p*-nitrophenyl
PEEK	polyetheretherketone
Q_{max}	maximum binding capacity, equivalent to $[Ab]_t$
RI	refractive index
RU	resonance unit
SPR	surface plasmon resonance
scFv	single chain Fv
$t_{½}$	half-life
TCR	T-cell receptor
WAC	weak affinity chromatography
WGA	wheat germ agglutinin

ORIGINAL PAPERS

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Strandh, M., Ohlin, M., Borrebaeck, C. A. K., and Ohlson, S. (1998). New approach to steroid separation based on a low affinity IgM antibody.
Journal of Immunological Methods *214*, 73-79.

II. Ohlson, S., Strandh, M., and Nilshans, H. (1997).
Detection and characterization of weak affinity antibody-antigen recognition with biomolecular interaction analysis.
Journal of Molecular Recognition *10*, 135-138.

III. Strandh, M., Persson, B., Roos, H., and Ohlson, S. (1998).
Studies of interactions with weak affinities and low molecular weight compounds using surface plasmon resonance technology.
Journal of Molecular Recognition *11*, 188-190.

IV. Ohlson, S., Jungar, C., Strandh, M., and Mandenius, C.-F. (2000).
Continuous weak-affinity immunosensing.
Trends in Biotechnology *18*, 55-58.

V. Jungar, C., Strandh, M., Ohlson, S., and Mandenius, C.-F. (2000).
Analysis of carbohydrates using liquid chromatography-surface plasmon resonance immunosensing systems.
Analytical Biochemistry *281*, 151-158.

1. INTRODUCTION

The wonders and mysteries of life have always intrigued mankind. Our innate curiosity has led us to dissect the mechanisms and processes that make us what we are. On every level that we have studied, from the macroscopic to the microscopic scale, the concept of recognition and interaction has been a striking feature. It is indeed hard to imagine something more fundamental in biological systems than molecular interactions. The properties of the interacting biomolecules dictate the type of forces involved and the strength and dynamics of the interaction. Many interactions are very strong whereas others are readily reversible and each interaction is evolving through time to be a refined part of Natures machinery.

Typically, weak interactions work in concert to trigger a biological response. The advantage with this approach, compared to the use of one or a few strong binders, is the inherent dynamics. It has been shown that this approach can be successful for *in vitro* applications as well. By exposing analytes to a multitude of specific, weak affinity interactions, which are governed by fast association and dissociation rates, separation based on small differences in affinity is possible. The same principles can also be used to characterize biological weak affinity binders and for analytical purposes.

This thesis will discuss biomolecular interactions in the weak affinity range (defined in this investigation as dissociation constants (K_D) larger than 10 μM) in general and focus on how they are studied and how they can be exploited *in vitro*. Weak affinity monoclonal antibodies have been used as model systems; (i) to explore how weak interactions can be employed in chromatographic separations of structurally related compounds (**paper I**); (ii) to explore the possibilities of studying weak interactions with a real-time optical biosensor (**papers II** and **III**); and finally (iii) to introduce continuous immunosensing for monitoring fluctuating concentrations of analytes in a flow (**papers IV** and **V**).

2. THEORY OF MOLECULAR INTERACTIONS

2.1. Mathematical musings

To describe a biomolecular interaction, terms describing affinity, kinetics and thermodynamics are used. The fundamental laws and equations are discussed below and exemplified with the antibody–antigen interaction.

2.1.1. Affinity

In a reversible biomolecular interaction in solution, Ab (e.g. antibody-binding site) and Ag (e.g. antigen) form the complex AbAg

$$\mathrm{Ab} + \mathrm{Ag} \leftrightarrows \mathrm{AbAg}$$

At equilibrium, the mass action law states

$$K_D = \frac{[\mathrm{Ab}][\mathrm{Ag}]}{[\mathrm{AbAg}]} \qquad \text{[eq. 1]}$$

where K_D (M) is the dissociation constant and the brackets denote molar concentrations. The magnitude of K_D describes the affinity, or "tightness", of the Ab–Ag binding. The closer K_D is to zero, the higher is the affinity that characterizes the complex. The association constant K_A (M^{-1}) is often used instead of K_D and they are related according to

$$K_A = \frac{1}{K_D} \qquad [2]$$

The mass action law can be rewritten

$$[\mathrm{AbAg}] = K_A([\mathrm{Ab}]_t - [\mathrm{AbAg}])[\mathrm{Ag}] \qquad [3]$$

or

$$[\mathrm{AbAg}] = \frac{K_A[\mathrm{Ab}]_t[\mathrm{Ag}]}{1 + \mathrm{K}_A[\mathrm{Ag}]} \qquad [3\ ]$$

where $[\mathrm{Ab}]_t$ is total concentration of antibody sites ($[\mathrm{Ab}]_t$ = [Ab] + [AbAg]). Figure 1 shows a saturation binding curve of [AbAg] *versus* free antigen [Ag]. The curve illustrates the effect of increased free antigen concentration [Ag] on the concentration of

formed complex [AbAg] at constant antibody concentration $[Ab]_t$. When $K_A[Ag] << 1$, there is a near linear relationship between [AbAg] and [Ag]. At higher [Ag], the complex concentration [AbAg] asymptotically approaches the plateau denoting total antibody concentration $[Ab]_t$. The antibody saturation, i.e. $[AbAg]/[Ab]_t$ is given by

$$\frac{[AbAg]}{[Ab]_t} = \frac{K_A[Ag]}{1 + K_A[Ag]} \qquad [3\ ]$$

For example, when $[Ag] = 1/K_A$ (that is K_D), then 50 % of the antibody binding sites are saturated, independent of $[Ab]_t$.

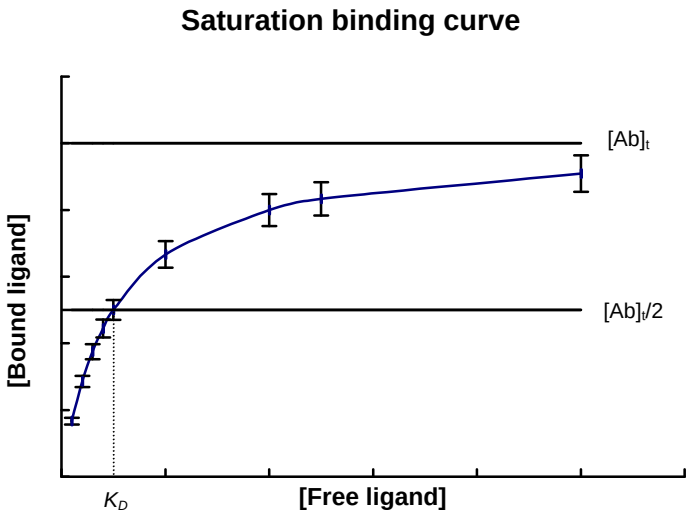

Figure 1. Binding curve for increasing antigen concentration (free ligand) at constant amount of binding sites ($[Ab]_t$). Graphical representations of K_D, $[Ab]_t$ and $[Ab]_t/2$ are indicated.

In many methods for measuring affinity of a biomolecular interaction, for example equilibrium analysis, another variant of [3] is used, also known as the Scatchard equation:

$$\frac{[AbAg]}{[Ag]} = K_A([Ab]_t - [AbAg]) \qquad [3\ ]$$

By plotting the ratio between bound and free antigen concentrations ([AbAg]/[Ag]) *versus* bound antigen concentration [AbAg], a straight line is obtained (fig. 2). The slope equals $-K_A$ and the intercept on the ordinate gives the concentration of antibody binding sites ($[Ab]_t$). Any heterogeneities are indicated by a curved Scatchard plot.

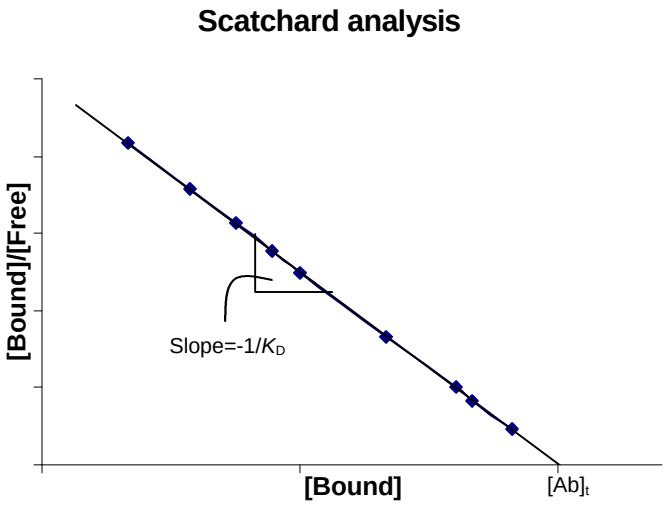

Figure 2. Scatchard plot for determination of dissociation constant (K_D) and $[Ab]_t$

2.1.2. Kinetics

The affinity can also be expressed as the ratio between the kinetic constants of the interaction

$$K_D = \frac{k_{diss}}{k_{ass}} \quad [4]$$

where k_{ass} ($M^{-1}s^{-1}$) is the association rate constant (also denoted k_1 or k_{on}) and k_{diss} (s^{-1}) is the dissociation rate constant (k_{-1} or k_{off}), describing the rate of formation and decay of the complex, respectively. Thus

$$\frac{d[AbAg]}{dt} = k_{ass}[Ab][Ag] - k_{diss}[AbAg] \quad [5]$$

describe the complex formation as a function of time. The association is determined mainly by the diffusion rate (theoretical upper limit 10^9 $M^{-1}s^{-1}$) and collision frequency, whereas k_{diss} depends mostly on the strengths of the participating bonds.

2.1.3. Thermodynamics

The laws of thermodynamic tell whether an interaction is energetically favorable or not. For an interaction to be spontaneous, the change in Gibb's free energy (ΔG, J/mol) must be negative. The equation

$$\Delta G = \Delta H - T\Delta S \quad [6]$$

consists of three factors; ΔH is the change in enthalpy (exo- or endothermic), T is the absolute temperature (Kelvin) and ΔS denotes the entropy change (degree of order). The entropy is decreased by the loss of configurational freedom in the molecules and is increased by expulsion of surface-bound water, hydrophobic effects in the binding interface and by induced conformational movements. The balance between the gain of new interactions and the loss of protein-solvent interaction upon binding generates the net enthalpy contribution to binding. Of the many interactions that occur in the Ab–Ag interface, almost as many are repulsive as attractive. The difference dictates the affinity that often corresponds to only one or a few (net) hydrogen bonds. Therefore, a small modification in the contact area can have a remarkable impact on the affinity.

The Gibb's free energy relates to affinity according to

$$\Delta G = -RT \ln K_A \qquad [7]$$

where R is the gas constant (8.314 J/K·mol).

2.2. Molecular basis of interactions: The antibody–antigen recognition

The knowledge about the relationship between structure and function has increased tremendously due to the technical achievements during the last decades, but still many problems remain to be solved. Structures of antibodies, with or without bound antigen (Davies and Cohen, 1996; Chen *et al.*, 1999), have been determined with x-ray crystallography and NMR. The results have confirmed many theories about molecular recognition, but they have also revealed new and surprising features.

Antibodies (or immunoglobulins, Ig) are glycoproteins coded by the immunoglobulin gene superfamily and all have the basic structure with two identical heavy chains and two identical light chains (Frazer and Capra, 1999). The chains are held together by disulfide bridges and non-covalent hydrophobic and hydrophilic interactions forming a flexible Y-shape. Humans have five different classes of antibodies; IgA, IgD, IgE, IgG and IgM. All antibodies are present in the serum with IgG being the most abundant (~11 mg/ml).

The N-terminal domains of one heavy and one light chain together make up the antigen-binding site, called paratope. The amino acids that take part in the direct antigen binding are divided on six loops called complementary determining regions (CDRs); three on the light chain (CDR L1-3) and three on the heavy chain (CDR H1-3). Binding

can also be affected by structural changes separate from the CDRs. This is most apparent in the framework regions that connect the loops (Panka *et al.*, 1988), but also in more remote positions of the antibody (Bazin *et al.*, 1992; Ito *et al.*, 1993; Rauffer-Bruyère *et al.*, 1997; Daugherty *et al.*, 2000). The CDR-sequences differ from one antibody to another, while the amino acid sequence for the rest of the molecule is more or less constant. CDR H3 is the most diverse region in both sequence and length and it appears to be the most important loop for antigen binding (Wilson and Stanfield, 1994).

The antibody can bind to many different molecular structures, called epitopes, such as carbohydrates, peptides, protein moieties, lipoproteins, and nucleic acids. Small antigens are called haptens and they usually enclose only one epitope. The binding affinity for antibodies isolated *in vivo* is typically in the range of 10 mM to 0.1 nM (K_D). The affinity is dictated by the type of interactions (hydrophobic, van der Waal and electrostatic forces, and hydrogen bonds, see below) involved, the "goodness" of structural and electrostatic fit and the surface area buried in the paratope-epitope interface (700-900 $Å^2$) (van Oss, 1995). There is a hyperbolic relationship between K_D values for monoclonal antibodies and the molecular weight of the epitope or hapten, with a minimum (maximum affinity) of approximately 0.1 nM for antigen determinants above 350 Da (Chappey *et al.*, 1994). A large variation in affinities for different antibodies elicited to the same antigen exists however. The clonal selection for affinity maturation *in vivo* lacks a mechanism to select higher affinity antibodies, i.e. there is no evolutionary advantage to increase the affinity further. However *in vitro*, the potential affinity is much higher; K_D = 48 fM and a dissociation rate corresponding to a half-time ($t_{½}$) of more than five days have recently been reported (Boder *et al.*, 2000; Foote and Eisen, 2000).

The antibody-antigen interaction is often discussed in terms of "domains". The CDR amino acids interact with other moieties, such as other amino acids as in the case of protein or peptide antigens or other defined molecular building blocks. It is, however, important to remember that the actual interaction is built by attractions on the atomic level. This is referred to as the "functional" epitope, which subsequently is much smaller than the structural epitope. Together the more or less favorable attracting forces result in the affinity between the two macromolecules. The non-covalent forces that determine the specific epitope-paratope interactions also act as repulsive forces between all molecules or cells on a macroscopic level. Only when the specific, local attraction is strong enough to overcome the general repulsion, binding can occur (van Oss, 1995). The actual epitope-paratope binding can be divided into primary and secondary interactions (which can be further subdivided in several overlapping steps). The primary attraction works at a distance of at least 3 nm and has to surmount the macroscopic repulsion. The secondary interaction happens when the distance decreases and the attractive forces may

modify the shape of the interacting species and thus optimizing the goodness of fit. During this step, water of hydration is expelled and short-range forces (van der Waals) takes over. As a final step, residues in the vicinity might now be closer together, rendering secondary bonding according to the steps above. Direct hydrogen bonding has it effects at very short distances (approximately 0.2 nm) and cannot initiate the attraction. Hydrogen bonds can be a part of the consolidation of the binding, but require precise angles and distances and are therefore not frequent. Remaining water molecules in the interface can form hydrogen bonds to the paratope and/or epitope, thus compensating for a sub-optimal fit and mediating interaction (Bhat *et al.*, 1994).

Two main types of antibody-antigen recognition have been described. One is the **induced fit** model, where the structures of free and antigen-bound antibodies are different in the backbone of the antibody-combining site, thus implicating a structural adaptation that improves binding (Rini *et al.*, 1992; Wilson and Stanfield, 1994; Davies and Cohen, 1996). The other model is the **lock-and-key** recognition, which presumes rather rigid structures of both antibody and antigen, where the epitope complementarity is preorganized in the paratope before binding. One can speculate if the flexibility (movement of side-chains and changes of angles) of the induced fit mechanism is a pre-requisite for the function of germline antibodies as broad range defenders. These antibodies usually exhibit low to moderate affinities and pronounced multispecificity, which could suggest a significant expansion of the diversity in the early response due to the adaptation ability. Examples show that antibodies exhibit an induced fit recognition in the first line of the immune response and then, as hypermutated and affinity maturated binders, assume a more rigid lock-and-key mechanism (Wedemayer *et al.*, 1997). Of course, it is impossible to draw any clear boundaries between the two types of binding mechanisms and the principle has to be determined on a case-to-case basis.

One individual cell can only encode a single antibody sequence, yet the expressed antibodies can have differences in fold and antigen-combining sites. Foote and Milstein (1994) reported of a structural diversity for monoclonal antibodies, where the different forms exist in an isomeric equilibrium and bind haptens with different kinetics and affinities. This isomerism for antibodies with identical structures may imply an enhanced diversity in the antibody repertoire, similar to the increased diversity originating from induced fit recognition; hence a way to cope with the countless foreign structures that challenge the immune system.

Different epitopes elicit antibodies of varying strengths; some antigen determinants are immunodominant due to a high antigenicity, which is increased by its accessibility, hydrophilicity and mobility. There are methods available to calculate the antigenicity

from the primary amino acid sequence of a protein or from a three-dimensional structure (Branden and Tooze, 1998), but a true prediction is often impossible, among other things since most protein epitopes are discontinuous (van Regenmortel, 1986; van Regenmortel, 1996).

2.3. Specificity and selectivity

When weak affinity biomolecular interactions are discussed, the issue of specificity *versus* non-specificity is inevitably raised. The term specificity is used in various contexts to denote a unique relation. When antibody-antigen interactions are described, specificity has to be used in a relative manner. Polyspecificity, arguably "non-specificity", is found, at least to some extent, for every antibody (Richards *et al.*, 1975). If a large number of target compounds are screened, there is a high probability that a cross-reacting antigen, which can be structurally related or unrelated to the immunogen used to raise the antibody, with similar or even higher affinity for the antibody is found. The phenomenon is called heterospecificity and originates in that the antigens bind to different functional paratopes (Keitel *et al.*, 1997). Therefore, the term specificity can be misleading and instead *selectivity* has been introduced (Berzofsky and Schechter, 1981). Selectivity is defined as the ratio of affinities for the binding to two molecules, i.e the antibody is $K_{A,X}/K_{A,Y}$ times more specific for X than for Y. A selectivity of $> 10^3$ is considered to be highly specific. As concluded from the discussion above about cross-reactivity and isomerism, there are no defined boundaries that separate one paratope or epitope from another, but more a sliding scale of overlapping interaction sites, which only can be defined in the presence of the two reactants together. Van Regenmortel (1998) describes this heterospecificity as an inherent "fuzziness" of the nature of molecular recognition.

Weak affinity interactions are less tight, i.e. more dynamic, but not automatically less specific, compared to stronger binding pairs. Instead, they tend to have a better "power of discrimination" than high affinity antibodies. If two molecules should be separated from a crude extract *and* from each other, a weak-affinity ligand that interacts with both molecules with a low, but detectable, selectivity and no affinity for irrelevant molecules would suffice your needs. The small selectivity factor in cross-reactive interactions can be amplified by a multitude of fast-kinetic interactions to obtain the desired separation. The advantages with weak affinity and this type of multispecificity in analytical methods are discussed in chapter 5.

3. WEAK AFFINITY BIOMOLECULAR INTERACTIONS

As previously discussed, human antibodies exhibit affinities up to 10^{10} M^{-1} (K_A) which make them powerful enough to initiate neutralization of infectious agents by various mechanisms in the immune system. Other events however, rely on low affinity interaction characteristics to meet special demands. The expression "low affinity" is often used in the literature to describe something with *lower* affinity, for example a receptor that is present in two (conformational) states can exhibit 10-100 fold difference in binding affinity between the forms (Schlessinger *et al.,* 1995). The examples below are all characterized by weak affinities, K_D 10 μM, for the one-to-one interaction, even though multivalency can increase the apparent affinity (avidity) 100-1000-fold.

3.1. Cell-adhesion molecules

The most well studied weak interaction events are the different cell-cell interactions, which characteristically have a pronounced polyvalency and a high degree of carbohydrate elements that function as recognition structures for numerous ligands (Gahmberg and Tolvanen, 1996). Adhesion of cells to other cells is essential in many biological processes, not only in the obvious maintenance of tissue structure, but also in areas as signal transduction and in the immune system (van der Merwe and Barclay, 1994). The mediators for these interactions are called cell adhesion molecules (CAMs). They are anchored in the cell membrane and are heavily glycosylated (Wagner and Wyss, 1994). Cell-cell adhesion is highly multimeric due to clustering of different complexes, which provide the cells with a functional stability. The monomeric CAMs interact with low affinity and fast kinetics, which has prevented the detection and quantification of these interactions until recently.

One example is the interaction between the CAM CD2 on the T cell surface and its ligand (CD48 in rat or CD58 in human) on the antigen presenting cell (APC) (van der Merwe *et al.,* 1993b; van der Merwe *et al.,* 1993a; Brown *et al.,* 1995; reviewed in van der Merwe and Barclay, 1996). Combined with site-directed mutagenesis, the experiments have explored the complex surface on a molecular level. It was revealed that the adhesion molecules interact with low affinities (50–100 μM for the CD2-CD48 interaction), which were governed by exceptionally fast dissociation (>1 s^{-1}) and average association rates (~10^5 $M^{-1}s^{-1}$).

Carbohydrate-binding CAMs (e.g. selectins and sialoadhesins) require correct glycosylation for unaffected interaction with the ligand (Crocker and Feizi, 1996). This have been studied by modifying the carbohydrates on the native, immobilized glycoproteins *in situ*, by treating them with glycosidases and/or sialidases (Hutchinson, 1994) or by direct lectin-oligosaccharide analysis (Shinohara *et al.*, 1994; Shinohara *et al.*, 1995). The leukocyte selectin (CD62L) mediates the tethering and rolling that initiates extravasation of leukocytes. The endothelium-attached leukocyte is subjected to a considerable mechanical stress from the flowing blood. The interactions that strap the cells and yet manage to permit a low-velocity rolling, without the leukocytes floating away, have special binding properties that cannot be described by affinity constants alone (van der Merwe, 1999). CD62L has been shown to bind preferentially to *O*-linked carbohydrates on glycosylation dependent CAMs (GlyCAM-1) with typical monomeric CAM interaction affinity; K_D ~ 100 μM and k_{diss} 10 s^{-1} (Nicholson *et al.*, 1998). Interestingly, soluble GlyCAM-1 cross-linked immobilized CD62L with a dramatically lower apparent dissociation rate ($k_{diss} < 0.001\ s^{-1}$) at serum level concentrations, due to the avidity effect. It has been proposed that this binding initiates CD62L mediated signal transduction and subsequent cell adhesion (Nicholson *et al.*, 1998). Another example of the features of weak CAM interactions was presented by Ganpule *et al.* (1997). Examination of the binding of ICAM-coated, cell-sized particles to activated T cells revealed that the selectivity for cell-cell adhesion was maintained by low affinity cell surface lymphocyte function-associated antigen-1 (LFA-1).

3.2. T-cell receptor–MHC/peptide interaction

Apart from the CD2-CD58 interaction discussed above, the T-cell – APC communication provides another intriguing weak receptor–counter receptor interaction. The binding between the αβ T-cell receptor (TCR) and the major histocompatibility class I and II (MHC I/II)-peptide complex on APC plays a central role in the immune system. There seems to be a difference in the affinity between the helper T-cell (to MHC II) and the cytotoxic T-cell (MHC I) with K_D of 10-100 μM for the former and 0.1-1 μM for the latter complexes. Both are characterized by relatively fast dissociation rate constants (~0.05 s^{-1}) (Matsui *et al.*, 1991; Corr *et al.*, 1994).

At a quick glance, these data (k_{diss}~0.05 s^{-1}, corresponding to a half-life of less than 30 s) seem to be in disagreement with the fact that only a few TCR molecules need to be engaged to activate the T-cell. Several solutions for this apparent mystery have been presented; (i) multivalency possibly followed by conformational changes to prolong residence time (Corr *et al.*, 1994); (ii) allosteric binding (Karjalainen, 1994); (iii) re-

current binding (Valitutti *et al.*, 1995; Valitutti and Lanzavecchia, 1997); or (iv) a kinetic model based on the dissociation rate of the ligand from TCR (Rabinowitz *et al.*, 1996; Rabinowitz *et al.*, 1997). Furthermore, Matsui *et al.* (1991) suggested that an antigen-independent adhesion precedes TCR engagement. The fast on/off binding has been shown to be physiologically relevant for T-cell repertoire selection *in vivo* and for certain immunological disorders (Brock *et al.*, 1996; Fairchild and Wraith, 1996). The concept of the "immunological synapse" (IS) has recently been proposed (Grakoui *et al.*, 1999; van der Merwe *et al.*, 2000). An IS is located in the cell membrane of a T-cell and consists of a ring of CAMs with TCRs positioned in the center. It was proposed that the synapse is formed in three stages, which involve, among other regulatory routes, discrimination steps in the screening for self *versus* non-self structures in the MHC-cleft.

3.3. Weak affinity antibodies *in vivo*

In case of infection by a foreign agent, there is an immediate need for an effective protection. The first (hours) active defense against for example a virus is the release of interferons. Within a couple of days, the first antibodies that neutralize virus and initiates further immune response mechanisms can be detected. The antibodies in the primary immune system are encoded by the germline genes, which provide a vast, but limited, number of antigen binding sites, and are therefore devoid of any somatic mutations. To compensate the lack of affinity maturation, germline antibodies rely on *multispecificity* (see chapter 2.3) and/or *polyvalence* (especially for the IgM decamer). These features are well adapted to repetitive epitopes that are found on bacteria and viruses. Kalinke *et al.* (2000) detected neutralizing capacity for all germline antibodies that were isolated shortly after a viral infection. The binding constants for the one-to-one interaction were approximately 300–fold lower for the initial antibodies than for the hypermutated one. However, the bivalent interaction for germline and mature antibodies (all IgG) differed only by a factor of 10-15. The suggested cause for this apparent inconsequence in affinity data was that the selection is based on the *monovalent* interaction between membrane bound Ig on B-cells and processed viral epitopes on APCs and not the functional, *multivalent* interaction. The lower avidity for the antibodies in the initial response is compensated by a high antibody titer so that the critical serum level for neutralization is reached. The low-affinity (K_D= 0.1-1 μM) antibodies *in vivo* cannot be regarded as weak in the context of this thesis (K_D> 10 μM), but the described principle displays a way to solve a complicated challenge (effective protection fast) using generous quantities of weak affinity, but sufficient avidity, interactions. The alternative, i.e. a set of genes coding for perfect matching antibodies for every imaginable intruder, would be impossible.

3.4. Carbohydrate-carbohydrate interactions

The surfaces of cells are covered with intricate carbohydrate structures. The importance of these in cell-cell adhesion mediated by protein-carbohydrate interactions has been previously described. Additionally, carbohydrate-carbohydrate interactions based on a multitude of very weak affinities have been suggested to play a central role in cell recognition (Bovin, 1997). There are several examples in the literature that can illustrate this type of interaction (Spillmann, 1994). The most studied interaction is the homotypic binding of Lewis X antigen (Le^x) to itself (Eggens *et al.*, 1989). Le^x is highly expressed during the mammalian embryogenesis and has been found to be crucial for the 8-32 cell stage. Carbohydrate structures offer many sites for interaction; ring structures, hydrophobic stretches and hydrophilic groups. The structure is also very flexible and facilitates complementarity. A "zipper-like" interaction model, constituted of highly ordered arrays of polyvalent carbohydrate moieties has been proposed (Spillmann, 1994). The forces involved are the same as for other interactions, which include ionic bonds, van der Waals forces and hydrogen bonds. The ionic attraction is stabilized in the presence of bivalent cations, such as Ca^{2+} or Mg^{2+}. Of course it is extremely difficult to quantify these individual interactions, but preliminary results using AFM and WAC, indicated an unbinding force of 4 pN and affinities of K_D = 0.1 M (Barrientos *et al.*, 2000; Martinez de la Fuente, 2000, *Personal communication*).

3.5. Applications of multivalent agents

The examples above illustrate the principles of how to achieve biological function by utilizing weak affinity interactions, mostly working together in ingenious arrangements. By mimicking this principle for biological recognition, a new approach for synthesis of medical drugs, inhibitors, antiviral- and antibacterial agents has been suggested (Ohlson, 1990; von Itzstein and Colman, 1996; Mammen *et al.*, 1998; Koeller and Wong, 2000). As the knowledge of these very weak interactions grows and the techniques for rational design of drug candidates improve, a number of new applications in the glycoscience will evolve.

4. HOW TO MEASURE WEAK BIOMOLECULAR INTERACTIONS

Characterization of the interaction between two or more biomolecules is often fundamental to elucidate biological mechanisms. It is also important in the development of new assays, for instance in the screening for antibodies for immunoassays or biotechnological applications (Hage, 1999). In the available methods for interaction analysis, affinity is measured either with both reactants in solution or with one (both in the case of AFM) immobilized to a solid phase. Some methods provide information on both affinity and binding kinetics, whereas in others only equilibrium constants can be obtained (Phizicky and Fileds, 1995; Neri *et al.*, 1996).

It is often difficult to obtain a reliable estimate of the kinetics and the affinity for a biomolecular interaction. Deviations, such as heterogeneities and mass transport limitations, make the evaluation of data a delicate task (van der Merwe and Barclay, 1996). For weak interactions the task is even more challenging and only a few methods are available, which will be discussed here.

4.1. Analytical affinity chromatography

Preparative affinity chromatography, for example high performance liquid affinity chromatography (HPLAC) (Ohlson *et al.*, 1978; Larsson *et al.*, 1983; Chaiken, 1990) or perfusion biochromatography (Katoh *et al.*, 1994; Whitney *et al.*, 1998), is used to effectively isolate compounds from various sources. Additionally, the same kind of affinity supports can be used in analytical affinity chromatography to analyze the binding of macromolecules to both immobilized ligands and to soluble competitors (Dunn and Chaiken, 1975; Jones *et al.*, 1995; Hage, 1998). Low-affinity reagents, such as weak affinity antibodies, have been utilized to extend traditional affinity chromatography to provide simultaneous multi-molecular analysis using under isocratic, non-competitive conditions (Ohlson *et al.*, 1988). The technique was named weak affinity chromatography (WAC) and can be used both for separations under non-denaturing conditions and for the study of weak interactions between any kinds of biomolecules. This method will be discussed in detail in chapter 5.1.

4.1.1. Determination of affinity constants

A representative example of competitive analytical affinity chromatography is the work by Inman (1983). Multispecificity and association constants for 24 hapten-sized compounds to an anti-2,4-dinitrophenyl monoclonal antibody were estimated. The compounds were used as competitive inhibitors to elute the radioactive labeled monoclonal antibody that was bound to immobilized antigen. The method is, for practical reasons, most suited for weak affinity (or avidity) interactions where the elution volumes are quite small. The affinities for the interactions with disparate hapten structures were in the range of K_D = 0.2–3 mM at 24 °C.

A commonly used method to determine K_D and maximum binding capacity (Q_{max}) with chromatography is frontal elution analysis (Kasai *et al.*, 1986; Hirabayashi *et al.*, 2000). This procedure involves saturation of the column by the analyte at various concentrations ([A]), which renders chromatograms describing elution profiles each composed of an elution front and a plateau, see Figure 3a. The elution volume (V) depends on [A] and the affinity (K_D) between the analyte and the immobilized ligand and is determined by the inflection point of the front. V_0 describes the front volume when no adsorption exists. Varying the flow rate (within reasonable limits) should not alter V or V_0. By plotting $1/([A](V-V_0))$ *versus* 1/[A], in analogy with the Lineweaver-Burk plot of enzyme kinetics, K_D can be calculated from the intercept on the abscissa (fig 3b). The intercept on the ordinate reflects $1/Q_{max}$. Figure 3c illustrates a saturation binding curve which is analogous to the graph in Figure 1. One example of frontal elution analysis is the report of Rosé *et al.* (1992) where the self-association of the capsid protein p24gag of human immunodeficiency virus was studied. Once the Q_{max} and K_D of the p24gag column were determined, zonal chromatography was used to estimate K_D at different pH values, using a simple mathematical expression (Strandh *et al.*, 2000). This method was reported to be less time consuming than many other equilibrium phase methods.

4.1.2. Determination of kinetic constants

Reliable binding rates are extremely difficult to estimate with affinity chromatography (Arnold and Blanch, 1986). Apparent association rate constants for antibody-protein antigen interactions have however been determined using successive pulse injections in a competitive (Hage *et al.*, 1993) or non-competitive (Renard *et al.*, 1995) HPLAC immunoassay. The adsorption rate for the binding of antibodies to column matrices with antibody binding proteins (e.g. protein A and G) has been measured using the split-peak phenomenon (Rollag and Hage, 1998). Different theoretical models were

used to evaluate data from these methods and to predict adsorption rates. However, the obtained data are system dependent (due to heterogeneities and multi-point interactions) and differ from the true one-to-one association rates. Furthermore, it remains to be seen how predictive these mathematical expressions are for weak interactions.

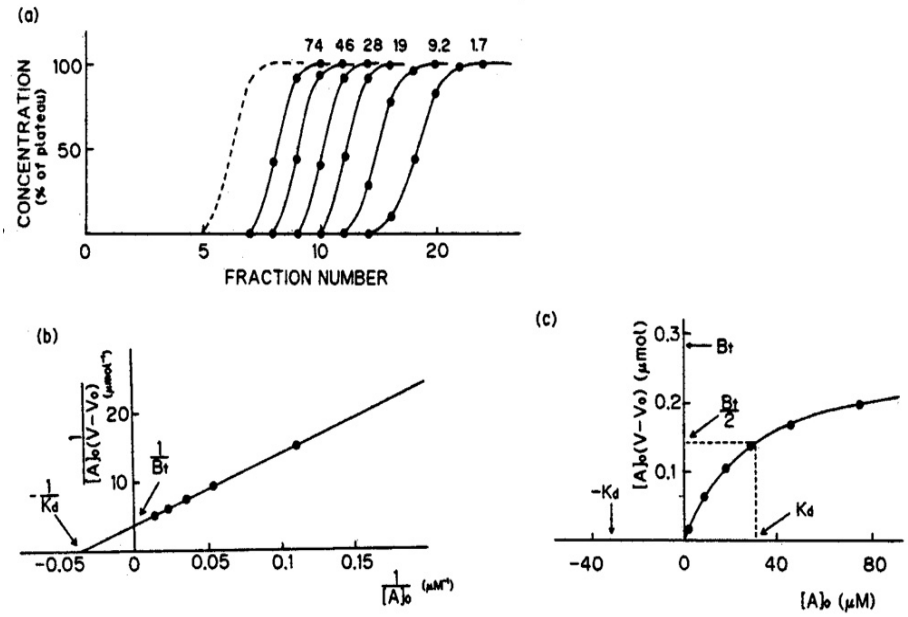

Figure 3. Frontal affinity chromatography according to Kasai *et al.* (1986).
a. Elution profiles for increasing analyte concentration (from right to left). The broken line (- - -) represents V_0 as determined with a non-reacting molecule.
b. Analysis of the data from a. See text for details.
c. Binding isotherm of bound versus free analyte concentration. See also figure 1.
Figure adapted from Kasai *et al.* (1986).

The kinetics in WAC, as well as other chromatographic parameters that govern the affinity separation, can be estimated by using simulation software (Wikström and Ohlson, 1992; Leickt, 2000). The program allows you to fit your experimental WAC-data and generates information on the kinetics of the studied interaction. Mathematical models for designing and predicting chromatography of proteins in general have been reviewed elsewhere (Jungbauer, 1996).

4.2. Optical real-time biosensors based on surface plasmon resonance

The term biosensor is used to describe equipment that measures the concentration of, or the binding to, biomolecules. The equipment can be based on several physical principles, such as changes in optical, electrochemical or thermoelectric properties (Turner, 1994). Optical biosensors can be further divided into surface plasmon resonance (SPR), grating couplers, differential interferometry, reflectometric interference spectroscopy, double beam waveguide interferometry and resonant mirror sensors.

The most widely used sensor for the study of antibody–antigen interactions is an optical biosensor based on SPR. The first commercially available instrument was BIAcore™ from Biacore AB (Uppsala, Sweden, website: www.biacore.com), which was released in 1990 (Malmqvist, 1993). The vast majority (~90 %) of biosensor publications cites the use of BIAcore instruments (Rich and Myszka, 2000). At least five other companies manufacture optical biosensor instruments including Affinity Sensors (Franklin, MA, USA, website: www.affinity-sensors.com) who offers the Iasys™ instruments (Davies and Pollard-Knight, 1993). The commercially available instruments vary in the biosensor design and they are hence suited for different applications (Ward and Winzor, 2000).

4.2.1. Detector basics

The principles of SPR in biosensing are explained in detail elsewhere (Liedberg et al., 1993). In brief, a semi-circular glass prism in the sensor is covered with a thin metal film (most sensors use gold or silver) on the flat side (fig. 4). When a polarized light beam shines through the prism onto the flat surface at an incident angle large enough (θ in fig. 4), no refracted light will pass through, i.e. the light is completely internally reflected. However, the electric field of the photons extends a short distance (approximately a quarter of a wavelength) beyond the reflecting surface, into the metal film. Here it interacts with free electrons and if the energy (proportional to θ) is accurate, the incident photons are absorbed and converted to surface plasmons. This resonance conversion is called SPR. Since the absorbed photons are not reflected (the plasmon energy is dissipated as heat), this results in a drop in the intensity at the corresponding angle that can be detected with a diode array detector (fig. 4). The energy for photon absorption is governed by the refractive index (*RI*) of the media in close proximity (< 1 μm) to the metal film, i.e. the sensor surface. A *RI*-change, for instance due to a change in concentration of molecules at the sensor surface, will alter the angle for the drop in reflected light intensity, and can thus be monitored in real-time.

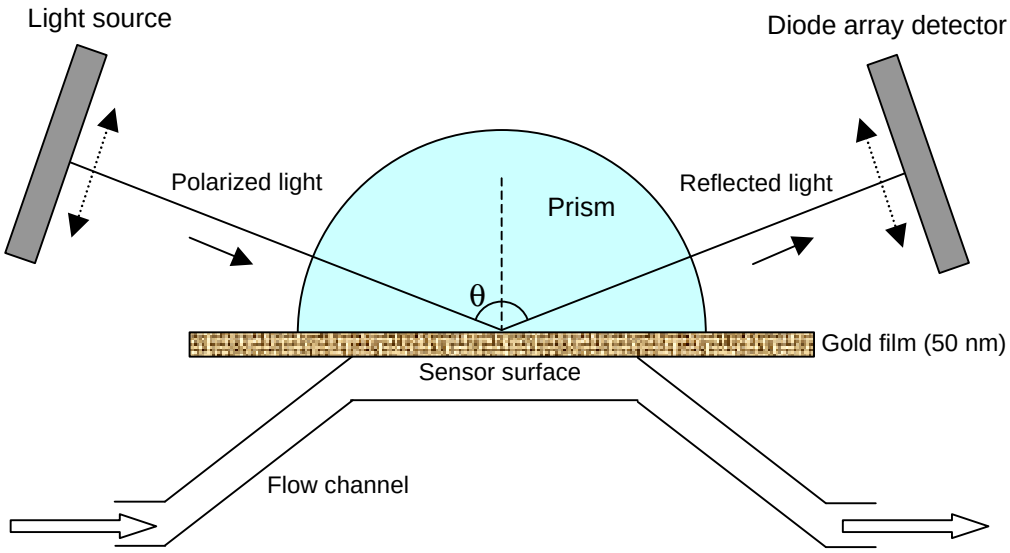

Figure 4. A schematic overview of the SPR detector (not to scale).

The angle for intensity minimum equals binding response and is measured with arbitrary units; response units (RU) for BIAcore or *arc* seconds for IAsys. For proteins, 1 pg/mm^2 corresponds to 1 RU. The optical biosensor displays the binding response in real-time in a sensorgram, which describes association and dissociation of molecular complexes in the vicinity of the metal film. One reactant of the molecular complex is immobilized on the sensor chip (the ligand) and the other is in solution (the ligate or analyte). Many different sensor surfaces are available, but the most widely used sensor chips have a hydrophilic matrix consisting of carboxymethylated dextran chains coupled to the metal film. The hydrated matrix extends typically about 100 nm from the metal surface and makes efficient immobilization of ligands (e.g. proteins) possible (Löfås and Johnsson, 1990). Several methods for immobilization of proteins to dextran matrices are available (Johnsson *et al.*, 1995). The "default" immobilization protocol is the amine coupling where primary amines in the protein react with *N*-hydroxysuccinimide esters on the dextran chains (Johnsson *et al.*, 1991). The analyte solution is exposed to the sensor surface, either in a flow (BIAcore) or in a stirred cuvette (IAsys).

4.2.2. Evaluation of SPR biosensor data

The data extracted from the sensorgrams can be fitted to a number of mathematical models that describe the kinetics and binding of interactions at the sensor surface (Roden and Myszka, 1996; Edwards and Leatherbarrow, 1997; Karlsson and Fält, 1997; Schuck, 1997a), even if the interaction deviates from "normal" behavior (Schuck and Minton, 1996a; Bowles *et al.*, 1997; Müller *et al.*, 1998). The evaluation of biosensor data has

improved significantly during the past years (O'Shannessy, 1995). It is now recognized that experimental design and careful choice of mathematical binding model are crucial for the quality of the results. To avoid the common pitfalls in kinetic analysis, one should subtract data from a reference surface and blank injections to correct for systematic artifacts. It is also important to use low ligand density and high flow rate to minimize mass transfer limitations in kinetic analyses, and to use pure samples free from multivalent or aggregated molecules to avoid multiphasic binding profiles (Karlsson and Fält, 1997). The consistency of kinetic data can be tested by simple calculations (Schuck and Minton, 1996b).

Many studies have compared results from biosensor experiments with binding data obtained with other methods, such as calorimetry and ELISA (Schuck, 1997b). Agreements between data could be found in many cases, but for some interactions there were significant differences. Many discrepancies could be attributed to poorly designed control experiments or to detrimental effects of the immobilization. Inconsistencies with results from ELISA experiments could be further resolved by determine the binding kinetics in a competition BIAcore experiment (Nieba *et al.*, 1996).

4.2.3. SPR biosensor applications

There are several advantages with an optical biosensor compared to other methods for interaction analysis: The interaction is monitored in real-time, no labeling is required, kinetic data can be determined, the consumption of reactants is low and it is easy to operate. The method is applicable to many different types of molecules (proteins, oligonucleotides, carbohydrates, lipids, small molecules, viral particles and even cells, see Table 1) and the field is expanding as new sensor surfaces become available. Today it is used to quantify active concentration, kinetics, affinity, thermodynamics (Zeder-Lutz *et al.*, 1997), stoichiometry and binding mechanisms. Qualitative applications include epitope mapping (Malmqvist, 1996), screening for binders and determination of selectivity. The drawbacks include limited sensitivity (depends on the molecular weight of the analyte), the fact that one reactant must be immobilized, and that the instrument and consumables are expensive, even though methods for reusing the sensor chips are available (Chatelier *et al.*, 1995). The most commonly employed methodical strategies are reviewed by Schuck (1997b) and several other reviews give an updated survey of the advances in SPR biosensors (e.g. Fivash *et al.*, 1998; Leatherbarrow and Edwards, 1999; Malmqvist, 1999; Rich and Myszka, 2000). Molecules bound to the sensor chip can be analyzed and identified in detail by combining the SPR biosensor with mass spectrometry (Krone *et al.*, 1997; Sönksen *et al.*, 1998).

The pioneer experiments analyzed the interaction between immobilized monoclonal antibodies and their antigens (Malmqvist, 1993) and the BIAcore has become an important tool in the screening for and characterization of antibodies and antibody fragments obtained from immunizations and recombinant protocols (Malmborg and Borrebaeck, 1995).

The SPR biosensor has been particularly successful in the analysis of weak affinity biomolecular interactions, a field previously hampered with a lack of proper analytical tools (van der Merwe and Barclay, 1996). It is possible to directly monitor interactions with low molecular weight analytes and low affinities if proper precautions are applied (chapter 5.2.; Karlsson and Ståhlberg, 1995; Malmqvist, 1999). The low sample consumption, label-free real-time detection and ability to analyze interactions in presence of dimethylsulfoxide are significant advantages in for example drug screening. Competition experiments are recommended if the response for direct binding of small molecules to immobilized ligand is too low or one wants to measure the interaction between molecules in solution (Karlsson, 1994; Nieba *et al.*, 1996). Studies of weak virus-receptor interactions have also been performed using BIAcore. Casasnovas *et al.* (1994) managed to immobilize intact human rhinovirus (300 Å in diameter) in the dextran matrix on the chip and measure its interaction to soluble ICAM-1. The interaction was relatively weak, with K_D in the range of 2-8 μM (depending on the exact method), but contrary to the "weak" examples in chapter 5.2., this was governed by slow kinetics and did consequently not exhibit the typical square-pulse sensorgrams seen in Figure 6. SPR biosensors have also been used for epitope mapping of viruses and for characterization of the interaction of viruses with neutralizing antibodies (van Regenmortel *et al.*, 1997).

Each method for measuring and detecting weak biomolecular interactions has its merits. One thing they have in common is that they all require a high concentration of the reactants in order for the weak affinity to be quantifiable and that control experiments should be included to ensure specificity. The optical SPR biosensor technology is so far outstanding because of the kinetic data that can be retrieved and the instant feedback on what happens on the sensor surface.

Table 1. A selection of the different type of interactions studied with SPR biosensors using the standard carboxymethylated sensor chip.

Reactants (ligand – analyte)	Literature reference (example)
antibody – antigen or *vice versa*	Malmborg and Borrebeack, 1995; Hock, 1997
antibody – DNA	LeBlanc *et al.*, 1998
receptor – ligand	Wu *et al.*, 1995
enzyme – lmw inhibitor	Markgren *et al.*, 2000
lectin – oligosaccharide/glycopeptide	Blikstad *et al.*, 1996; Haseley *et al.*, 1999
lectin – glycoprotein	Haseley *et al.*, 1999
oligosaccharide – lectin	Shinohara *et al.*, 1995
glycolipid – lectin	Mann *et al.*, 1998
glycopeptide – lectin	Shinohara *et al.*, 1994
glycoprotein – lectin	Hutchinson, 1994; Okazaki *et al.*, 1995
peptide – viral glycoprotein	Cormier *et al.*, 2000
lipopolysaccharide – peptide/protein	MacKenzie *et al.*, 1996; de Haas *et al.*, 1998
nucleotide – nucleotide	Bates *et al.*, 1995; Persson *et al.*, 1997
nucleotide – protein	Jost *et al.*, 1991; Bondeson *et al.*, 1993
various weak affinity biomolecules	chapters 4. and 5.2.
virus particles – receptor/mAb	Casasnovas *et al.*, 1994; van Regenmortel *et al.*, 1997
antibody – erythrocyte	Quinn et al., 1997
lmw analytes	Karlsson, 1994; chapter 5.2.

4.3. Miscellaneous biomolecular interaction assays

Perhaps the most classical method to determine the affinity for a biomolecular interaction is **equilibrium dialysis**. The set-up consists of two chambers separated by a semipermeable membrane through which only the smaller reactant (e.g. antigen) can move. The larger reactant (e.g. antibody) is placed in one chamber and after sufficient time to reach equilibrium, the concentrations can be measured. The concentration of free antigen will be identical in both chambers and the difference in total antigen concentration between the chambers will be the complex concentration. Affinity is then determined by calculating the slope of a Scatchard analysis or by any other graphical procedure (chapter 2.1.1.). No kinetic data can be obtained and reactants must differ in size (>3 fold). To improve the quality of the data for weak interactions, large amounts of purified material are needed in order to get a significant fraction of complexes. A closely related method, namely **saturation binding study**, has been used to determine affinities in the 40–60 μM range for a weak agonist that competed for binding to a cellular receptor with labeled high affinity monoclonal antibodies (Matsui *et al.*, 1991).

Traditional **enzyme-linked immunosorbent assay** (ELISA), where one of the reactants is adsorbed to e.g. a polystyrene surface, is used to detect different compounds or to estimate selectivity. True affinity constants of biomolecular interactions in solution can be measured using a modified ELISA procedure (Friguet *et al.*, 1985). Even binding rate constants can be estimated with further modified protocols (Li, 1985; Hardy *et al.*, 1997). In order to *detect* weak interactions the apparent affinity must be increased by avidity effects or by for instance lowering the incubation temperature. These procedures make however the *quantification* of weak binding characteristics well-nigh impossible.

Optical SPR biosensors dominate the real-time kinetic analyses of biomolecular interactions. However, for measurements of membrane-bound proteins binding to proteins in solution, subsecond **flow cytometry technology** have been reported to be superior to SPR biosensors regarding kinetic data assessment (Nolan and Sklar, 1998; Boulla *et al.*, 2000). The nonspecific binding of the fluorescent ligand must be subtracted from the specific interaction and as the study of weak interactions requires high ligand concentrations and thus exhibits large nonspecific binding, this can be cumbersome in the available experimental set-ups. Competition assays and new instruments could present a way to measure weak biomolecular interactions (Boulla *et al.*, 2000).

The absorption spectrum for a molecule in solution changes upon interaction. In **spectral titration**, the spectral shift is monitored at increasing concentration of one reactant and an adsorption isotherm is obtained and thus the affinity for the interaction. Spectral titration was used to analyze the binding of different dye conjugates to a protein and K_D values up to 37.0 μM were obtained (Mayes *et al.*, 1992). The complex formation could also be monitored in real-time to calculate the kinetic data. A possible error in this method is that the concentration of free proteins is overestimated if non-specific binding occurs, hence an erroneous assessment of K_D. The method is limited to interactions where a significant spectral shift occurs, either in UV or fluorescence spectra (fluorescence titration).

In **affinity capillary electrophoresis** (ACE), the migration shift of interacting molecules can be assessed for affinity determination (Busch *et al.*, 1997; Heegaard *et al.*, 1998; Kajiwara, 1999). For weak affinity interactions, one component is present in the capillary throughout the separation whereas the other is injected as a zone. The multiple association- and dissociation reactions during the electrophoresis present a quantifiable mobility change for the applied analyte. For the determination of K_D with this approach, the electrophoresis is performed in gel-filled capillaries. K_D values up to 3.6 mM have been reported for a pea lectin – sugar interaction (Shimura and Kasai, 1997). Weak anti-bodies can be utilized to separate antigens in gel-filled ACE comparable with a

miniaturized WAC system (Ljungberg *et al.*, 1998). Polyacrylamide gel **affinity electrophoresis** has also been used to study the interaction of carbohydrate binding proteins, for example lectins, with polysaccharides. The range of binding constants that can be determined with this method is 10 mM to 10 μM (K_D) (Tomme *et al.*, 2000).

With **isothermal titration calorimetry** (ITC), the heat change that accompanies the formation of biomolecular complexes in solution can be measured. The thermodynamic parameters and affinity data (K_D) can be obtained from a small series of experiments using only nanomoles of protein (Jelesarov *et al.*, 1996). The dynamic range of the latest, highly sensitive microcalorimeters is governed by the *c* value that is calculated by multiplying the affinity constant (K_A) with the total binding site concentration. As a rule of thumb, *c* should be in the range of 2 – 100 to get a good estimation of K_D. ITC-studies of interactions with 0.1 – 0.4 mM affinities have been published (Tomme *et al.*, 1996).

Equipment based on **electrochemiluminiscence** for interaction analysis has been commercially available for some years (Yang *et al.*, 1994). The method requires a luminescent label on one of the interacting molecules and is able to detect low affinity interactions, because of a high degree of cross-linking in the system (Ohlin and Borrebaeck, 1996). The technique has not been used to estimate affinity constants, except occasionally (Abraham *et al.*, 1996). Its applicability to measure low affinity interactions has not been explored yet.

Atomic force microscopy (AFM, also known as scanning force microscopy) is used to reproduce high-resolution topographic images of sample surfaces and to measure forces, e.g. adhesion, close to a surface (Moy *et al.*, 1994; Takano *et al.*, 1999). With AFM, the unbinding force for a *single* antibody–antigen interaction has been determined to be approximately 40-60 pN (Dammer *et al.*, 1996; Ros *et al.*, 1998; Harada *et al.*, 2000). The reported antibody – antigen interaction in Harada *et al.* (2000) had an affinity of 0.25 μM, as determined by ITC, which was close to the K_D-values predicted from AFM-data (unbinding force = 60 pN) using a simple equation. Another recent report demonstrates a clear logarithmic correlation between unbinding forces and k_{diss}-values (and also K_D since all k_{ass}-values in this study were in the same order of magnitude) for nine different single-chain antibody fragments (Schwesinger *et al.*, 2000). The AFM instruments are capable of measuring intermolecular forces down to 10 pN, which may promise studies of weak affinity single macromolecular interactions.

5. HOW TO UTILIZE WEAK BIOMOLECULAR INTERACTIONS - THE PRESENT INVESTIGATION

Weak interactions play an important role *in vivo*, as seen in the examples above. The fundamental understanding of these interactions requires analytical tools and methods capable of measuring them. Below are some examples of investigations where biomolecular weak interactions based on the antibody-antigen pair successfully have been studied with weak affinity chromatography (WAC) and optical biosensor.

5.1. Weak affinity chromatography

In qualitative chromatography, the goal is to separate or isolate compounds. The separation process is based on differences in the intrinsic qualities of the molecules in the mixture, such as size (in size exclusion chromatography), charge (ion exchange chromatography), hydrophobicity (hydrophobic interaction chromatography), chelating capability (immobilized metal affinity chromatography) or affinity for the immobilized ligand (affinity chromatography). The latter is a preferred method for isolating proteins from biological samples with a good purification factor and often with excellent yield in a single step. This high affinity procedure is in fact an adsorption/desorption routine with little resemblance to a true chromatographic separation. The concept of utilizing low affinity interactions in chromatography to obtain a dynamic and versatile analytical approach has been "in the minds" of researchers for quite some time, but was first implemented by Ohlson and co-workers with the advent of WAC (Ohlson *et al.*, 1988; Zopf and Ohlson, 1990). WAC allows numerous successive interactions to occur whose effects accumulate to produce the observed retardation and thus separate the injected components. It is especially suitable to solve separation problems with structurally similar analytes, see examples below.

Paper I describes a WAC system based upon a monoclonal antibody that exhibits different fine specificities for digoxin derivatives (Danielsson *et al.*, 1991). Unlike other WAC experiments where IgG has been the antibody class of choice (see below), we used a human monoclonal IgM antibody that had not been subjected to any affinity maturation. The immune defense uses IgM as an early, polyreactive, low affinity ($K_D > 10^{-5}$ M) but polyvalent neutralizer to suppress the intruder in infections until higher

affinity (IgG) antibodies have been produced (Kalinke *et al.*, 2000). These characteristics usually disqualify IgM antibodies as suitable reagents in immunoassays, but we could show that they perform excellent in WAC in regards to both activity and stability. A large amount of monoclonal anti-digoxin antibodies was covalently immobilized onto silica that was packed in an HPLC column. Digoxin and ouabain were differently retarded under isocratic conditions; both steroids eluted separated from the void volume (paper I). Eluted material was monitored using a UV-detector at the wavelength 230 nm, thus avoiding the need for labeled steroids. Presence of serum in the samples did not influence the retention of steroids, proving that the activity of the IgM matrix was retained even in the presence of non-specific binding proteins. The column was evaluated further with frontal chromatography (Kasai *et al.*, 1986; chapter 4.1.) to determine Q_{max} and K_D for each steroid and a number of control molecules. In order to establish the specificity of the interactions, all values were compared to data from a reference column with polyclonal irrelevant IgM immobilized onto the silica support, which showed virtually no retention for either steroids or control substances. The estimated K_D values from frontal chromatography were 25 μM for digoxin and approximately half the affinity for ouabain (60 μM), which is in fair correlation with previous studies using inhibition ELISA where the values were 14 and 28 μM for digoxin and ouabain respectively (Danielsson *et al.*, 1991). The selectivity, i.e. the quotient between the retention volumes of the steroids, of the column may not be so impressive and could be significantly improved by increasing Q_{max}. This can be achieved either by using a larger column, improving the coupling chemistry (only 20 % of the sites remained active after the immobilization in paper I) or by using antibody fragments, as Fab, Fv or scFv, which allow a higher density of active sites (Berry *et al.*, 1991; Berry and Davies, 1992; Berry and Pierce, 1993). Even short peptides (ten amino acids) derived from one hypervariable loop of a monoclonal antibody can be used (Welling *et al.*, 1991). Furthermore, the use of support materials with smaller pore sizes and thus an increase in available surface area can also be beneficial (Clarke *et al.*, 2000).

One of the problems with applications using weak-binding antibodies is that they are not (commercially) available. High affinity antibodies can be altered to exhibit lower affinity and enhanced polyreactivity, by exposure to high temperatures or extreme pH (McMahon and O´Kennedy, 2000). A way of screening for any antibody with low affinity or avidity using chromatography with immobilized antigens has been described (Leickt *et al.*, 1998). Recombinant antibody engineering with high diversity libraries could prove to be a valuable source for screening for weak reagents (Winter *et al.*, 1994; Söderlind *et al.*, 2000). Enzymatically or recombinant produced antibody fragments, e.g. Fab and scFv, have an apparent affinity (avidity) that is typically 10-1000-fold lower than the wild type complete antibody. Even the monovalent interaction is often

weakened when the antibody is cropped and fragmentation is hence a straightforward way of increasing the interaction reversibility and, as mentioned earlier, the amount of active material in the separation system (Roggenbuck *et al.*, 1994).

Much of the early WAC separations employed monoclonal antibodies from the same group as the antibodies used in the weak optical biosensor experiments (**papers II-V**). This group consists of mouse monoclonal IgG antibodies, all directed against the oligosaccharide tetraglucose (Glcα1-6Glcα1-4Glcα1-4Glc) (Lundblad *et al.*, 1984b). The antibodies have affinities in the 0.01–4 mM range of K_D (at 4–40 °C) for various oligosaccharides. The antigenic determinant is the Glcα1-4Glc carbohydrate sequence, present in for instance maltose (Glcα1-4Glc, Mw 342 Da) and panose (Glcα1-6Glcα1-4Glc). In a work by Ohlson *et al.* (1997), two of the antibodies, designated 39.5 and 61.1, were thoroughly studied as ligands in a WAC-HPLC system and the dependency of chemical and physical parameters on the isocratic separations were determined. It was concluded that the retardation, and thus the antibody-antigen affinity, showed a strong correlation to temperature, which could be controlled to fine-tune the separation. A span of 4-40 °C could be used without decreasing the lifetime of the column. Altering the pH value also affected the affinity, although non-physiological pH should be avoided since it decreases the stability of the antibodies. The typical mild conditions for WAC rendered a great long-term stability. Hundreds of separations could be performed during a couple of months with only minor loss in activity of the column.

An attractive alternative to anti-carbohydrate antibodies in WAC is to use a lectin as the immobilized ligand. Immobilized wheat germ agglutinin (WGA) was used in a WAC experiment for the separation of different carbohydrates (Leickt *et al.*, 1997). An isocratic chromatographic separation of five different saccharides within 25 minutes at 18 °C, corresponding to K_D values of 1 mM (for α-*N*-acetylglucosamine) up to 10 mM (for β-*N*-acetylgalactosamine), was presented. The 10 mM-affinity is the weakest binding reported so far for successful use of WAC.

Another example, using interactions with K_D values of 3-35 μM, is affinity retardation chromatography (ARC) for characterization of the laminin self-polymerization (Schittny, 1994). Analogously with the WAC experiments, a reference column was carefully designed to quantify the non-specific part of the interaction, such as binding to column material. The interaction between an enzyme and its substrate is often characterized by fast on and off rates, and enzymes have been used in WAC separation of substrate and substrate analogues (Ohlson and Zopf, 1993). The methodology for WAC using monoclonal antibodies are described in detail by Strandh *et al.*, (2000), who also compared WAC to alternative techniques such as capillary weak affinity gel electro-

phoresis (Ljungberg *et al.*, 1998), miniaturized chromatographic systems and weak affinity immunosensor (papers II and III).

To summarize, it was shown that WAC could be applied for different interactions. WAC allows molecules to be separated according to their slight differences in weak affinity to the immobilized ligand. Separation capacity is rather good and the retention volumes are small. Since no harsh elution step has to be implemented, the stability for both ligand and analyte is unaffected. WAC can be performed in any chromatographic system, but the combination with HPLC offers stability, reproducibility and possibilities for automation. The drawbacks with WAC include the scarcity of suitable ligands (or actually a lack of appropriate ligand screening procedures) and that a high ligand density in the column is needed.

5.2. Weak affinity surface plasmon resonance biosensor

Optical biosensors based on surface plasmon resonance has been a valuable complement to other methods, such as ELISA, microcalorimetry and two-dimensional immunodiffusion, in characterizing low affinity interactions, both in biological and in artificial or recombinant systems. The biosensor, described in detail in chapter 4.2., is used extensively to study specificity, thermodynamics and kinetics for protein-protein interactions, but can be used for almost any type of biomolecular interaction provided that one part can be immobilized (Table 1.).

5.2.1. Experimental design of weak affinity SPR biosensor experiments

There are many examples from the literature on protein–carbohydrate interactions that have K_D values typically in the micromolar range. They have been studied both to improve the understanding of the binding mechanisms and structure-function relationship, and to find suitable tools in the field of glycoscience. Lectins from plants and animals, as well as anti-carbohydrate antibodies, have been important model systems in the elucidation of the energetics and kinetics of carbohydrate-mediated events.

In general, study of weak affinity biomolecular interactions places special demands on the method, such as adequate signal-to-noise ratio, careful choice of binding model to detect possible multiphasic association and dissociation and design of reference and control experiments to exclude irrelevant binding. These points are of course valid in biosensor experiments as well, since it is easy to be deceived by the direct and

apparently easy collection of data. The feasibility of BIAcore to study weak interactions has been reported for numerous different interactions (van der Merwe *et al.*, 1993b; Karlsson and Ståhlberg, 1995; van der Merwe and Barclay, 1996; MacKenzie *et al.*, 1996; Margulies *et al.*, 1996).

In **papers II** and **III**, the BIAcore was used to study the interaction between an immobilized antibody and its carbohydrate antigens, exhibiting a tenfold weaker interaction compared other reported interactions. The selected antibody-antigen pair was discussed in detail in 5.1. This generic antibody-antigen interaction really pushes the limits of the BIAcore instrument in different aspects: (i) very low affinities (K_D>10^{-5}M); (ii) fast kinetics (both k_{ass} and k_{diss}); (iii) low molecular weight analytes (all antigens tested had a molecular weight of less than 700 Da); and (iv) high analyte concentrations that have to be used to compensate for low sensitivity resulting in a high ”bulk” response. Experiences from our experiments can be applied as guidelines for investigation of weak affinity, low molecular weight biomolecular interactions.

The murine monoclonal IgG antibody 39.5 was immobilized on the carboxymethylated dextran matrix, in close agreement with the manufacturer’s recommendation for covalent amine coupling. Reproducible results were only obtained at high antibody density (6,000 – 20,000 RU, corresponding to 6 – 20 ng/mm^2 of immobilized antibody). Lower immobilization levels produced detector responses too close to the background noise, due to Q_{max} values (R_{max} in Biacore terminology) of less than 10 RU. Carbohydrate antigens and control saccharides of different concentrations in running buffer were injected over the sensor surface. To exclude any nonspecific binding to the dextran surface of the biosensor chip and contributions from bulk refractive index (*RI*), data from a reference sensor surface with immobilized irrelevant proteins was subtracted in every point of the sensorgram. The apparent, uncorrected data was in the magnitude of 500 RU at equilibrium, of which specific binding contributed with less than 10 % as evident from the control experiments. Different proteins such as serum proteins, antibodies and enzymes were evaluated as reference surfaces and similar assessment of the non-specificity was obtained with all of them. It was important in the choice of reference ligand to immobilize it at the same density as the specific antibody. In most experiments, we selected mouse polyclonal IgG antibodies that provided a stable reference surface, even at immobilization levels of 20,000 RU or more. The importance of a reference surface that mimics the environment of your interaction of interest is further stressed by an artifact called the hook effect (fig. 5). At very high analyte concentrations, it is imperative that the relative contributions to the total refractive index at the sensor surface from the sample and the immobilized ligand are nearly identical in both flow cells. If no ligand, e.g. protein, is immobilized on the reference surface, then the response will be

based on the refractive index of the highly concentrated sample injections (RI_{sample}). If that index differs from the ligand refractive index (RI_{immob}) on the sensor surface, the correction will be erroneous and could cause an apparent loss ($RI_{sample} > RI_{immob}$, fig. 5B) or gain ($RI_{sample} < RI_{immob}$) in specific binding (Karlsson and Ståhlberg, 1995).

One can of course argue what the "perfect" reference system would be. Depending on the purpose, the reference has to be more or less carefully designed. Most scientists would agree that a molecule, identical to the active target in every aspect except a lack of the part responsible for the activity or binding, would be ideal. For example, an antibody mutated in the CDRs could be a perfect reference for the corresponding wild-type antibody. Alternatively, an antigen analogue that irreversibly blocked the antigen-binding site of the antibody, without interacting with the true analyte, could be considered.

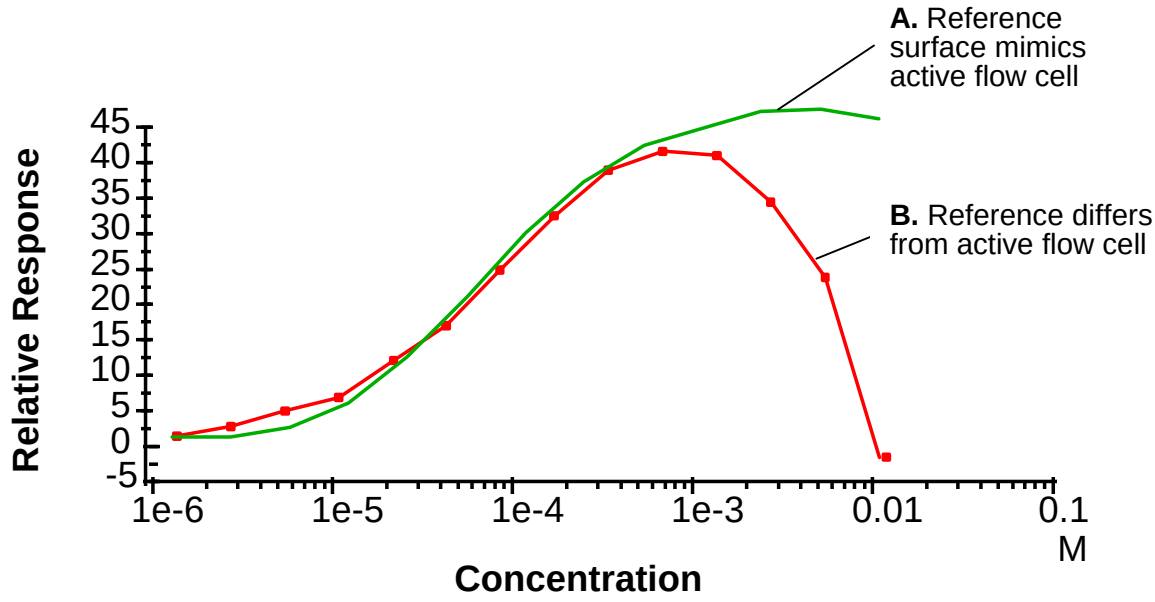

Figure 5. Two different adsorption isotherms obtained when injecting increasing concentrations of maltose on immobilized 39.5. Difference in response between 39.5-surface and reference surface is plotted *versus* concentration. **A**. Reference surface (high density of irrelevant antibody) mimics the active surface. **B**. Reference surface with nothing immobilized. *Figure adapted from paper III.*

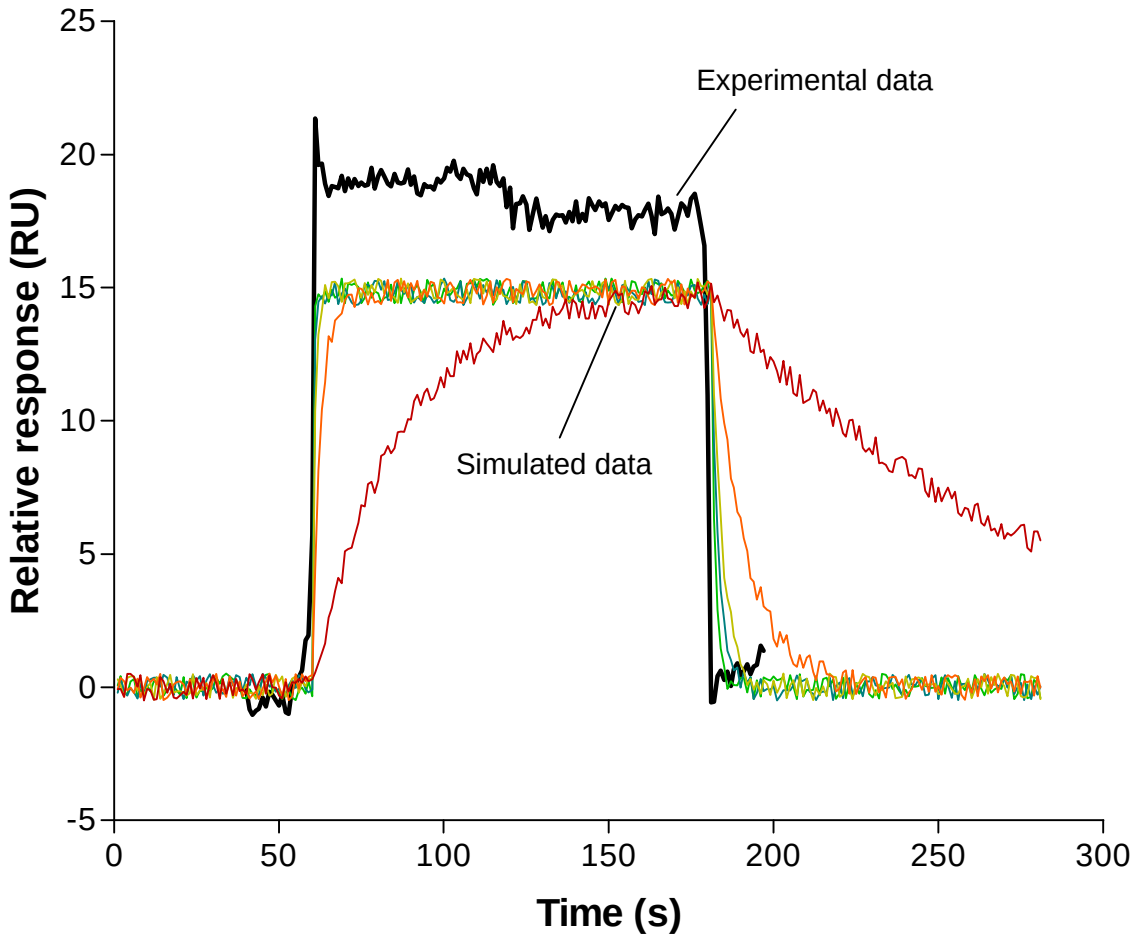

Figure 6. Overlaid square-pulse shaped sensorgrams obtained for weak affinity interactions (K_D = 0.19 mM). The k_{diss} for simulated data were 0.01–0.8 s^{-1} and the top sensor-gram was obtained from injection of 0.3 mM maltose over a 39.4-coated surface.

A series of representative reference-subtracted sensorgrams are illustrated in Figure 6. The typical square pulse appearance is due to the fast kinetics of the interaction; equilibrium is set within one or a few seconds, both for binding and for dissociation. The BIAcore instrument has a resolution of 0.2 s (the maximum sampling frequency is 5 Hz), so the binding rate constants could not be measured directly. However, an estimation of the kinetics can be done by comparing simulated square pulses similar to the experimental data, as shown in Figure 6. A simulated curve with k_{diss} = 0.8 s^{-1} has a good fit to the experimental data and corresponds to a half-life ($\ln 2/k_{diss}$) of the complex of less than one second (0.87 s). A special benefit with rapid dissociation rates is that the need for regeneration procedures of the surface is avoided and thus the stability and activity of the immobilized molecules are enhanced, similar to the long lifetime of the WAC columns (chapter 5.1.).

Due to large margins of error in kinetic data, it was impossible to calculate the affinity constant K_D by simple division of the rate constants [eq. 4]. Instead, the corrected equilibrium response levels were plotted vs. analyte concentration to make an adsorption isotherm (see for example Figures 1. and 5A.) that could be fitted to a 1:1 binding model to obtain K_D and maximum binding level (Q_{max}). The results showed good correlation data obtained with other methods; the affinity ranged from 0.06 mM (maltose, 15 °C) to 1.0 mM (tetraglucose, 40°C), which is comparable to 0.07 mM to 1.0 mM for β-maltose and maltotetraose at 30 °C, respectively, as determined with WAC (Ohlson *et al.*, 1997).

What are the limits for measuring rapid kinetics in the BIAcore instrument? The manufacturer (Biacore AB) states a working range of k_{ass}: 10^3–5 x 10^6 $M^{-1}s^{-1}$ and k_{diss}: 5 x 10^{-6}–10^{-1} s^{-1} for the instrument. It has been suggested that association rates faster than 10^5–10^6 $M^{-1}s^{-1}$ cannot be studied with the BIAcore since the assumption that the concentration of unbound analyte remains constant during the experiment then loses validity (Hall *et al.*, 1996; Bourdillon *et al.*, 1999). A borderline case, with respect to fast k_{diss}, is a study of an IgG monoclonal antibody specific to abequose (3,6-dideoxyhexose) subunits, present in the lipopolysaccharide of *Salmonella* bacteria (MacKenzie *et al.*, 1996). Binding of both monomeric and dimeric forms of single chain antibody fragments (scFv) to immobilized antigen was studied. It was evident from the sensorgrams that the dimers had biphasic dissociation ($k_{diss,fast}$ and $k_{diss,slow}$) and faster association rate (five-fold) compared to the monomers, due to presence of both monovalent and bivalent binding. The faster dissociation rate represented monovalent binding and was similar to the rates for pure monomers, whereas bivalency decreased the off-rate approximately 20-fold. The sensorgrams for the monovalent binding were similar to the square pulses in Figure 6, presenting very narrow analysis windows for the association and dissociation phases. Association rate constants for the scFv monomers were 2.3 – 6.3 x 10^4 $M^{-1}s^{-1}$ and the k_{diss} values were approximately 0.25 s^{-1}, to be compared with the stated BIAcore range (0.1 s^{-1}) and the estimations of the rate constants for the 39.5 system: k_{diss} ~ 1 s^{-1} and k_{ass} = 10^3 – 10^4 $M^{-1}s^{-1}$ (paper II). The difference in binding kinetics increased the affinity abequose 100-fold for scFv-dimers compared to monomeric fragments.

The excellent performance for SPR biosensors (e.g. BIAcore) has significantly facilitated the study of low affinity biologically relevant interactions. The majority of the data and suggested mechanisms for the biological events presented in chapter 3. originate from BIAcore interaction analyses, using the experimental design and precautions suggested above. The binding data for the aforementioned weak affinity biological system, i.e. different types of cell-adhesion interactions, are comparable to those obtained in paper II and III. The majority of the reports failed to quantify the

kinetics directly, owing to the limited resolution of the instrument. Different approaches to indirectly determine k_{ass} and k_{diss} have been described. The k_{diss} values for the monomeric CAM and CD62L-GlyCAM-1 interactions were determined by extrapolation to be at least 5 s^{-1} and 10 s^{-1}, respectively (van der Merwe *et al.*, 1993a; van der Merwe *et al.*, 1994; Nicholson *et al.*, 1998). The K_D values (50–100 μM) for these interactions were determined by plotting equilibrium data for different analyte concentration, according to Scatchard or any other graphical model, as in the study of the 39.5-maltose interaction (paper II). Also the study of the TCR–MHC/peptide recognition system has benefited from real-time binding experiments (chapter 3.2.), although these usually exhibit a somewhat stronger binding (K_D ~0.1 μM) (Corr *et al.*, 1994). In addition, the recently suggested immunological synapse for cluster formation in the T-cell has been mapped using SPR biosensor.

5.2.2. Continuous immunosensing

The perhaps most intriguing feature with weak interactions is that the equilibrium between bound and free molecules reflects the concentration of reactants in every moment. The dynamic nature of these interactions is important in living cells where the interacting molecules have to be reused. The same feature is also necessary when we want to monitor a process of any kind, with real-time data reflecting the condition of interest. In **papers IV** and **V** we combined a highly dynamic antibody–antigen reaction with a real-time SPR biosensor to introduce a novel, continuous immunosensor application (Ohlson, 1990). A number of conditions must be considered when designing a specific on-line sensor: (i) the sensitivity and specificity must be sufficient; (ii) regeneration of the sensor surface must be avoided; (iii) the system should be compatible and stable (iv); it should handle complex samples without pretreatment; and (v) the output should be reproducible and give real-time data. All these conditions can be met when combining the surface plasmon resonance biosensor with a weak, dynamic biomolecular interaction.

In **paper IV**, we used a model system consisting of immobilized monoclonal IgG antibodies (chapter 5.1.) that bound to different saccharides of varying concentrations in a continuous flow. Maltose and maltohexaose (Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc) were used as analytes and cellobiose (Glcβ1-4Glc) as control. Two antibodies with similar antigen specificities were used (Lundblad *et al.*, 1984a; Lundblad *et al.*, 1984b). They were murine monoclonal IgG, designated 39.4 and 39.5, showing an affinity for maltose of 0.19 mM and 0.087 mM at 25°C respectively. The discrepancy in the affinity

data for the 39.5–maltose interaction between paper III and IV (0.07 and 0.087 mM, respectively is probably due to inter-experimental variations, but has not been investigated further. The fast kinetics (k_{diss} = 1 s^{-1}, see Figure 6) implies that the interaction equilibrium is almost immediately set (within a second). The experimental setup was straightforward; an external peristaltic pump withdrew an analyte flow from a gradient chamber and presented it to the sensor surface where the specific interaction took place. The detector signal was processed and subsequently displayed.

In general, the biosensor response depends on analyte concentration, the molecular weight of the analyte, the immobilized ligand density and the affinity for the ligand-analyte interaction. In order to obtain adequate response signals in the system described, the low molecular weight of the analytes (<1000 Da) was compensated by high ligand densities and high analyte concentrations. In Figure 7, a sensorgram from a continuous immunosensing experiment is shown. The analyte concentration is altered stepwise and every plateau in the sensorgram reflects the factual analyte concentration. The steep "curves" separating them correspond to the time for the new equilibrium to set. The curves are not vertical since dispersion effects, mainly in the peristaltic pump, slow down the exchange from one analyte concentration in the system to the next. The peristaltic pump is also the major cause of the periodic noise in the sensorgram. As in every weak interaction experiment, the design of reference system is crucial. Analogously to the experiments in paper II and III, the response from a reference sensor surface (placed in series with the specific surface) with immobilized irrelevant mouse IgG, was subtracted to obtain specific binding data. When a crude sample containing serum was injected, a nonspecific signal was present. The amplitude was proportional to the contamination concentration and depended on the properties of the reference surface. Ideally, the reference should be able to compensate for any nonspecific binding. At 1% serum present in the samples, a net signal of ~1 RU was added to the specific response. Unsubtracted data was many orders of magnitude larger than the specific signal.

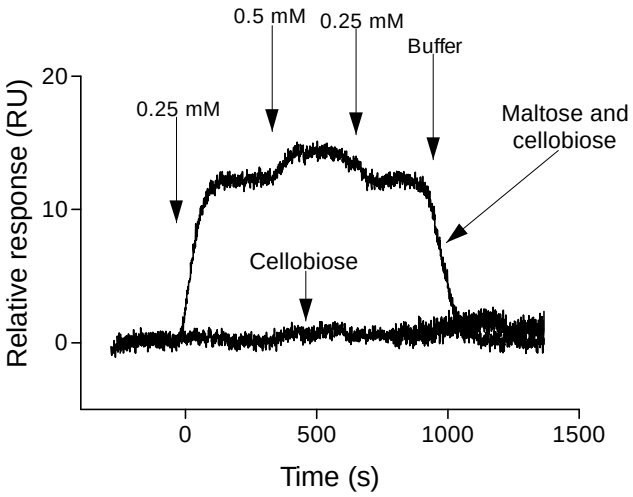

Figure 7. Continuous immunosensing with the SPR biosensor. Flow containing either a mixture of maltose and cellobiose (top sensorgram) or pure cellobiose (lower sensorgram) at different concentrations was continuously applied over the sensor surface with immobilized antibody 39.4. *Figure adapted from paper IV.*

What would happen if the affinity for the interaction was higher? If the dissociation rate is too slow, molecules from the previously applied concentration will still reside at the surface. The total amount of complexes, and thus the detector response, would not reflect the actual amount of free analyte present at that time. At increasing concentrations, the sensorgram profile would probably look the same as in Figure 7, but when the analyte concentration is lowered, a longer period would be needed to achieve a plateau (as governed by the $t_{½}$ for the complex). This is demonstrated by the results from high affinity equilibrium titration experiments with rising sensorgram profiles for increasing concentration (Myszka *et al.*, 1998; Schuck *et al.*, 1998).

In **paper V,** we applied the findings from the initial continuous immunosensing experiments and combined HPLAC with a detector based on SPR biosensor (LC-SPR). The readily reversible biosensor surface, specific for Glcα1-4Glc–containing carbohydrates, was used to analyze the effluent from two different chromatographic columns. The experimental set-up was a HPLC system where the column effluent was passed through a UV-VIS detector and was then directed to the microfluidic system of the BIAcore. In this way, the BIAcore functioned as a specific on-line detector, presenting real-time chromatograms (RU/s) based on the amount of antibody-antigen complexes formed in every moment. The chromatographic columns created a baseline separation of the injected saccharide/tagged saccharide mixture (as displayed by the UV-Vis detector) and presented a delicate task for the immunosensor; could the narrow peaks of the separation be correctly displayed as a sensor-chromatogram? A mixture of maltose, pnp-α-maltopentaoside, pnp-α-maltoside (all interacting with the 39.4 immobilized on the sensor surface) and pnp-α-glucopyranoside (control substance, not reacting with 39.4) was separated on a dextran-agarose column and presented as a correct sensorgram (fig. 8). All pnp-tagged saccharides were identified by the UV-Vis detector (response

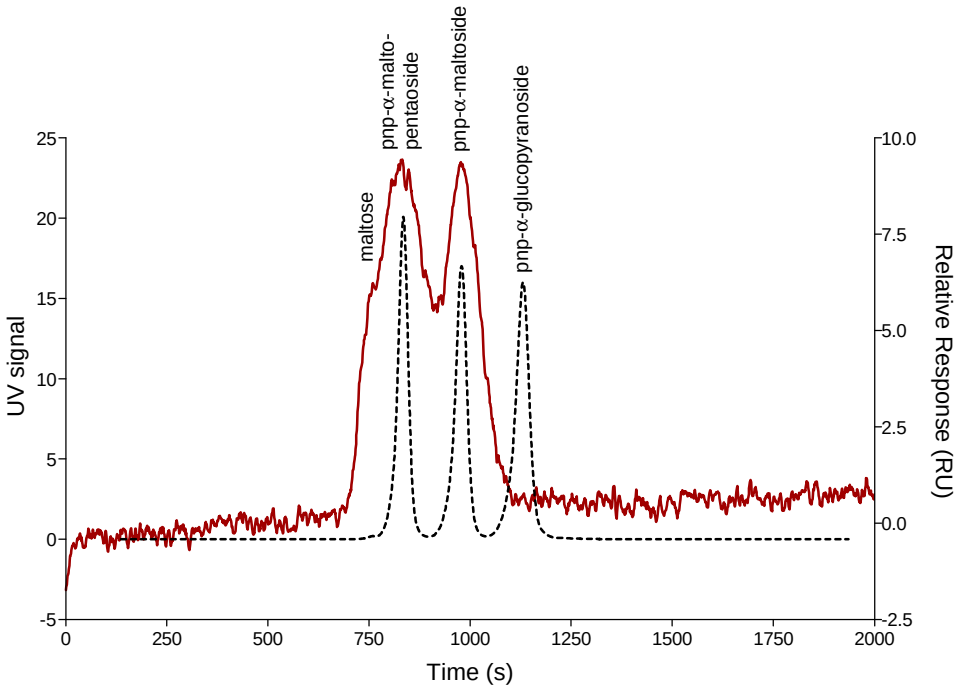

Figure 8. An example of LC-SPR. The eluate from a chromatographic separation of maltose, pnp-α-maltopentaoside, pnp-α-maltoside and pnp-α-glucopyranoside was monitored with UV-Vis detector (- - -) and SPR biosensor (——). *Figure adapted from paper V.*

depended on the analyte concentration and the absorption coefficient). Saccharides reactive with 39.4 were detected by the weak immunosensor and the response was proportional to the analyte concentration and the antibody–analyte interaction affinity. Together these two detectors presented a more complete picture of the separation; the UV-Vis detector saw compounds with absorbance at the selected wavelength (here 280 nm) and the SPR immunosensor detected molecules with affinity for the immobilized ligand. Some lagging was evident in the SPR-chromatogram, mainly due to dispersion effects in the fluid delivery system. In a similar approach, a longer $t_{½}$ produced a more pronounced lagging and subsequently an inferior peak resolution in the SPR-chromatogram (Blikstad *et al.*, 1996). We used PEEK tubing and connections, trying to minimize the tube lengths between column, detector and BIAcore, and we believe that a fully integrated apparatus would benefit from minimized dispersion and other systemic artifacts.

The system was quite robust for crude samples since the chromatographic step functions as a pretreatment step and eluting “impurities” in the void, and thereby avoiding disturbances in the specific immunosensing interaction. A drawback with the reported system was the poor sensitivity; saccharide concentrations down to only 0.1 mM could be detected. This limitation was attributed to dilution due to the dispersion in the system and the low molecular weight of the analytes. The amplitude of the relative

response in the SPR-chromatograms was less than 10 RU, which reduced the dynamic range of the SPR detector considerably.

The experiences and results presented in **paper IV** are applicable to virtually any area where there is need for a dynamic immunoassay, since the number of possible weak biomolecular interactions is virtually unlimited. Instead of taking a number of samples at different points of time and analyzing them individually, it is, using the continuous immunosensor, possible to have the answers when the bioprocesses occur. In our work, we used a manual biosensor, which only has two flow cells, one specific and one reference cell. In future applications with a different biosensor design, a number of surfaces, each with a specific immobilized ligand, could be utilized to get a more versatile and informative analytical device. The continuous immunosensor was applied to LC-SPR in **paper V**. Other SPR combinations have been reported (SPR-MS) (Krone *et al.*, 1997; Natsume *et al.*, 2000) and we strongly believe that LC-SPR will be a fruitful addition to the analytical toolbox (*Anal. Chem.* (2000), **72**:513).

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6. CONCLUDING REMARKS

This thesis discusses the underlying mechanisms and importance of weak affinity interactions in biological systems. It presents also an overview of the methods that are available for measuring these interactions and how they can be utilized in analytical situations.

Model systems comprising of low-affinity, antibody-antigen interactions have been used to demonstrate the benefits of dynamic equilibriums. In **paper I**, a monoclonal IgM antibody was used for the chromatographic separation of structurally related steroids. The results demonstrate the advantages with weak affinity chromatography; (i) small differences in specificity are amplified by a multitude of serial interactions, resulting in high performance separations based on affinity; and consequently, (ii) procedures for harsh elution of bound material and regeneration of column matrix are avoided. Furthermore, the weak affinity chromatographic systems were used in analytical affinity chromatography for the estimation of affinity constants. **Papers II and III** gave insights into the use of SPR biosensor for analysis of weak affinity interactions. A rationale for the design of such experiments to avoid inaccurate results has been suggested. The future possibilities for applications of dynamic biomolecular interactions were indicated in **papers IV and V**. Continuous immunosensing might have the capacity to be used in any area where there is a need for online monitoring of a biomolecule.

There should be plentiful opportunities to develop analytical procedures based on weak biological interactions not only for 'difficult' separations of e.g. structurally-similar substances in crude extracts but also for diagnostic challenges to analyze markers with subtle structural differences. Of special interest is the introduction of high through-put screening methods using weak affinity separations. By using miniaturization and parallel operations, cost and time for every analysis will be reduced dramatically. A bottleneck has been the access to libraries of suitable weak affinity ligands. However, the recent introduction of various combinatorial techniques should widen the availability of suitable weak affinity ligands. Drug design will also benefit from weak affinity techniques as multivalent pharmaceuticals can offer desired selectivity. Finally, yet importantly, the techniques described in this thesis might make it possible to characterize almost any weak biological interaction.

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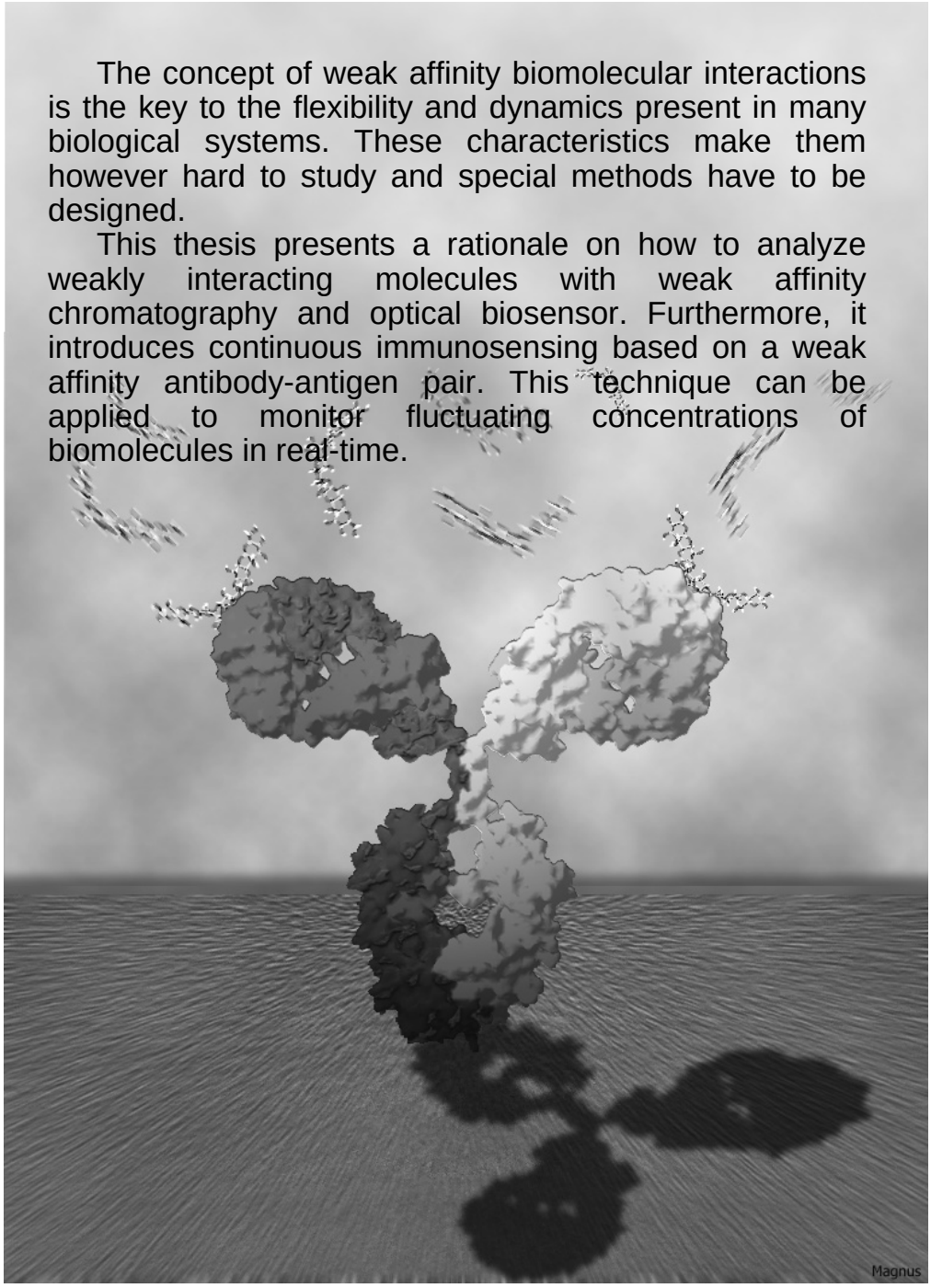
INSIGHTS INTO WEAK AFFINITY ANTIBODY-ANTIGEN INTERACTIONS

Studies using affinity chromatography and optical biosensor

The concept of weak affinity biomolecular interactions is the key to the flexibility and dynamics present in many biological systems. These characteristics make them however hard to study and special methods have to be designed.

This thesis presents a rationale on how to analyze weakly interacting molecules with weak affinity chromatography and optical biosensor. Furthermore, it introduces continuous immunosensing based on a weak affinity antibody-antigen pair. This technique can be applied to monitor fluctuating concentrations of biomolecules in real-time.



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