Fluorescence-based ligand assays for protein detection using affibody affinity proteins

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“We’re all one thing, Lieutenant. That’s what I’ve come to realize. Like cells in a body. ‘Cept we can’t see the body. The way fish can’t see the ocean. And so we envy each other. Hurt each other. Hate each other. How silly is that? A heart cell hating a lung cell.”

– Cassie from “The Three”, screenplay by Donald Kaufman
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**Abstract**

The detection and quantification of biomolecules, and proteins in particular, are of great interest since these molecules are of fundamental importance to our well-being. Body fluids, as for instance human blood, are well suited for sampling of protein levels. However, the complexity of the fluids and the low abundance of many of the interesting biomolecules makes detection and quantification difficult. This has spurred an interest into the development of many protein detection methods, and of these, ligand assays have proven particularly suitable. In this thesis, different types of ligand assays for protein detection have been developed using affibody molecules as ligands.

In a first study, a homogeneous competitive detection assay was investigated, based on anti-idiotypic affibody molecule pairs and fluorescence resonance energy transfer (FRET) as reporting system. The individual members of two anti-idiotypic affibody pairs, each consisting of a target binding (idiotypic) and an anti-idiotypic affibody ligand, were labeled with a donor fluorophore and an acceptor fluorophore, respectively. Incubation with the two target proteins IgA and Taq DNA polymerase resulted in a concentration dependent decrease in the FRET signal, allowing for target protein detection and quantification. For Taq DNA polymerase, detection in 25% human plasma was also possible in the same concentration span as in buffer.

In a second study, a homogeneous, non-competitive detection system was described. Affibody molecules of 58 amino acids directed against IgA and IgG were produced with chemical synthesis, and two fluorophores capable of FRET were site-specifically introduced. Binding of target protein induced a concentration-dependent change in the relative emission of the two fluorophores, which formed the basis for the detection system.

In two studies, affibody molecules were evaluated and shown to function well as capture ligands on microarrays. Synthetic affibody molecules directed against Taq DNA polymerase and IgA were produced with chemical synthesis, and two fluorophores capable of FRET were site-specifically introduced. Specific immobilization via a C-terminal cysteine or a biotin moiety, or random immobilization via amino groups, were studied in protein microarray experiments and SPR-based biosensor studies. The experiments showed that all immobilization chemistries resulted in functional capture molecules. A short spacer was also introduced, situated between the affibody and the cysteine and biotin moieties, which was shown to improve binding for all constructs. Multidomain affibody constructs of up to four N- to C-terminally linked domains were shown to increase the amount of bound target, compared to monomeric affibody ligands. Six dimeric affibody constructs directed against IgA, IgG, IgE, Taq DNA polymerase, TNF-α and insulin, respectively, showed low limits of detections for their targets and little or no cross-reactivity with the other target proteins. Dimeric affibody molecules directed against IgA and TNF-α were also shown to function in a sandwich format with antibodies for detection of targets in buffer and in human serum and plasma. Successful discrimination between normal and IgA-deficient sera showed that affibody molecules could be used for specific detection of protein in highly complex backgrounds on microarrays.

**Keywords:** Affibody molecules, biosensors, FRET, immobilisation, solid-phase synthesis, protein microarrays.
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List of publications

This thesis is based on the publications listed below, which will be referred to by their roman numerals.


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Analysis of biomolecules

Studies of biomolecules are of immense importance for our understanding of life processes. In particular, proteins are of great interest since this single class of biomolecules can have vastly different functional roles, including signaling, catalysis, protection, regulation, and mechanical support in our cells and bodies (Lodish et al., 2004). In bodily disorders and for the treatment of disease, proteins are frequently either supplemented or targeted. In fact, in modern medicine, almost all drug targets are proteins (Drews, 2000).

In diseases, such as cancers, bacterial or viral infections and genetic disorders, protein levels are changed, either through over- or underexpression of endogenous proteins in the body, or with the introduction of exogenous proteins from the virus or bacteria. As the understanding of the different proteins in our bodies grows it will become possible to know what normal levels of certain proteins are expected and what diseases and misconditions unbalances in these levels can result in or be the result of. One can envision a not too distant future, when a blood sample is taken and scanned for deviations from normal protein blood levels, to establish risk profiles for diseases that can subsequently be addressed with proper treatment, long before symptoms of the disease are detected by the patient.

Being able to efficiently and simultaneously detect and quantify all proteins found in human fluids would be a revolution in medicine. In human plasma and serum, for instance, virtually all proteins produced by the different cell types are likely to exist, either as secretions from cells,
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or as by-products as cells are degraded (Anderson et al., 2004). However, many of the proteins of interest are present only at very low concentrations, and hence not easily detected. This is further complicated by the great span in concentrations of plasma proteins; it is estimated that the difference is about ten orders of magnitude, from the most abundant protein, serum albumin (40 mg/ml) down to the rarest proteins (Anderson et al., 2002). The method which one uses to detect these proteins of interest in such a background must be precise, ideally so that all the other proteins do not interfere with the assay results.

As the result of many of years of multidisciplinary research, there are today many ways to detect and measure the concentration of proteins. Biophysical properties of proteins such as size, charge, mass, hydrophobicity and composition can be used to determine the presence and sometimes also the concentration of a certain protein. Some examples that can be used are electrophoresis (size), isoelectric focusing (charge), mass spectrometry (mass), reversed phase chromatography (hydrophobicity) and UV absorbance (composition). Mass spectrometry is often used together with 2-D electrophoresis, isoelectric focusing and gel electrophoresis, or liquid chromatography for detection of specific proteins in complex samples (Rodland, 2004). However, a great advantage for the analysis of complex mixtures is the availability of a protein-specific affinity ligand, which allows the specific detection of the target protein.

Interactions between proteins themselves or other biomolecules are vital in our bodies, without such, nothing would function. A fair degree of selectivity of proteins for their interaction partners is required, so that for example protein signaling cascades, and our immune system, can function properly. This ability of proteins to discriminate between biomolecules; for a certain ligand to bind only its designated target, even when other biomolecules are present, has been used in detection assays. In principle, such assays rely on the availability of a binding reagent (affinity ligand) directed against the protein/biomolecule (target analyte) of interest, and a reporter system that is capable of converting a binding event into a detectable signal (see figure 1). This simple concept is the basis of ligand assays.
Figure 1. The basic principle of ligand assays in which a specific analyte is recognized by a ligand, and the binding event is reported.
Affinity ligands

Some general features of an optimal ligand for use in ligand assay development can be described: (i) it should be specific (Vessman, 1996); it should only bind the target of interest, regardless of other molecules present, (ii) the affinity should be high so that low concentrations of molecules could be detected, (iii) it should be stable to the biological (e.g. proteases), chemical (e.g. pH) and physical (e.g. temperature) conditions associated with the application in mind, (iv) it should allow specific incorporation of immobilization tags or reporter labels without significantly affecting the function of the ligand.

In principle, three main routes can be depicted for the development of an affinity ligand towards a given analyte of interest. Firstly, existing biomolecules can be investigated for their ability to bind a given target analyte. For example, the ability of avidin/streptavidin from hen eggs or bacteria, respectively, to bind biotin has been used extensively in a vast number of assay formats. Secondly, laboratory animals can be immunized for production of polyclonal or monoclonal antibodies. Thirdly, novel affinity ligands can be developed by chemical synthesis or gene technology, bypassing the need for laboratory animals. However, these routes are not entirely strict, in that combinations of different routes are used.

The difficulties in finding ligands fulfilling all of the desired criteria has spurred an interest into different types of binding molecules, all with potential advantages, but none perfect.
Antibodies

The most commonly used affinity ligands for specific protein detection are, by far, antibodies. These antigen-binding molecules are secreted by B-cells of the immune system of vertebrates for a number of functions. They circulate the body and are found in mucous membranes and cell walls. It is up to antibodies to bind to, coat, and inactivate intruders such as toxins, viruses and bacteria that enter the body, and to convey signals to other parts of the immune system, in order to activate them against the newly detected threat (Kuby, 1997).

In the 50s and 60s, Rodney Porter and Gerald Edelman elucidated the structure of antibodies through the fragments created when they were subjected to enzymatic cleavage and breaking of their disulfide bonds (Edelman, 1959; Porter, 1959). Porter and Edelman were awarded the Nobel prize in Physiology or Medicine in 1972.

Antibodies, most often consisting of two pairs of identical heavy and light chains, can be divided into two parts based on functional differences (see figure 2): (i) the Fc-region, consisting of two heavy chain-parts held together with disulfide bonds; a constant region with effector binding sites for complementation activation and attachment points for receptor proteins (Woof et al., 2004). Depending on the isotype or subclass, the Fc portion has different structures and confers different functions. In most species, five main isotypes are present, namely IgG, IgM, IgA, IgE and IgD, differing for example in the number of constant domains (C\text{H}-domains), glycosylation patterns, and properties related to formation of higher valency forms (dimers or pentamers), (ii) the Fab-regions, variable regions consisting of a heavy chain-part and a light chain that make up each “arm” of the Y-shaped antibody. The Fab contains the Fv-region, the variable region, of which 15-20% is made up by complementarity determining regions, CDRs, which determine the specificity of the antibody. The CDRs consist of six loops, three loops contributed by the heavy and light chains each, with variable lengths and amino acid sequences. It is these differences that make up the different specificities of antibodies (Kuby, 1997).
Figure 2. An antibody typically consists of two light and two heavy chains and can be divided into a constant region and a variable region. The six CDR-loops in the variable region make contact with the antigen and define the antigen-specificity. The Fc-region can be of five different main subclasses, or isotypes; IgG, IgA, IgE, IgM, IgD, which make the antibodies suitable for diverse biological functions.

The genes encoding the variable regions of antibodies are created from germline segments of DNA which are shuffled differently in individual maturing B-cells to create a high genetic diversity from a low number of genes. A clonal selection of B-cells occurs via antigen binding to cell surface-anchored antibodies and further mutations are subsequently introduced into these antibodies via somatic hypermutation, resulting in point mutations mostly in the CDRs. B-cells producing higher affinity antibodies become stimulated and proliferate and can be subjected to further hypermutation. In this intricate way, antibodies are matured to increased affinity for their antigens (Kuby, 1997). During the lifetime of a B-cell clone its rearranged genes conferring the antigen binding properties become linked to different Fc-encoding genes, a result of so-called class switching.

**Polyclonal antibodies**

It was realized early that preparations of antibodies from immunized animals could be used for applications based on molecular recognition (Berson et al., 1956; Laurell, 1990; Opitz, 1990; Yalow et al., 1959). Immunization of laboratory animals with an antigen results in the generation
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of a pool of antibodies, with each antibody species produced by a specific B-cell clone and recognizing a specific epitope of the antigen. These polyclonal antibodies are used extensively as affinity reagents for in vitro diagnostics (see Ligand assays and Protein microarrays). Mice, rats, rabbits or goats are commonly used animals for polyclonal antibody production. There are both advantages and disadvantages of using polyclonal antibodies, compared to alternative reagents. One advantage is the relative ease of which the antibodies can be produced. Antibodies can often be collected already after a couple of months after immunization of an animal. Another advantage is that antibodies from different B-cells can be directed against different epitopes on the same target, which can result in cooperative binding. If immobilized onto a solid support, these could provide synergy effects (avidity) that enhance the detection limit through a higher apparent affinity (Wingren et al., 2005). These large populations of antibody types are, as a pool, also less sensitive to changes in the assay conditions; an increase in pH, temperature, salt concentration or denaturant may affect the antigen so that a 3-D epitope used by a certain antibody is disrupted or that binding to a linear sequence becomes impossible. The use of a population of different antibody types increases the chance that at least one type of antibody can still bind the target and therefore, they can be used in a variety of platforms (Angenendt et al., 2003; Engvall et al., 1971; Uhlén et al., 2005; Wide et al., 1967; Yalow et al., 1959). However, one major disadvantage is that polyclonal antibody production is not entirely reproducible; even if the same type of animal is immunized with an identical antigen, this could result in a different polyclonal serum. This is due to the way in which B-cells are maturing; shuffling and somatic hypermutation do not necessarily yield the same sequence twice, especially if different germline segments are used as starting material. Purification of polyclonal sera by protein A or protein G columns could be used for enrichment of specific isotypes, depending on the animal species used. In addition, strict purification of polyclonal sera on antigen columns can be applied to refine only the antigen-specific antibodies, which constitute a relatively small fraction of all serum antibodies (Lipman et al., 2005). This strategy has been implemented in a high-throughput fashion within the Swedish Human Proteome Resource Project (Uhlén et al., 2005, www.proteinatlas.org).

Monoclonal antibodies from hybridoma

In 1975, many of the problems associated with polyclonal antibodies were addressed when
Georges Köhler and Cesar Milstein published a paper describing the fusion of an activated, antibody-producing B cell with an immortal cancer cell (Kohler et al., 1975). The product, called hybridoma, was an immortal, dividing cell line producing identical antibodies; monoclonal antibodies. For this accomplishment, Köhler and Milstein were awarded the Nobel Prize in Physiology or Medicine in 1984. Individual hybridomas are screened for antibody production and for target specificity, because only a fraction of the B-cells produce antibodies against the antigen of interest. Since identical antibodies binding the same epitope are expressed from a hybridoma, monoclonal antibody reagents can be reproducibly generated, have a definable composition and the affinity for the antigen can be determined and only antibodies suitable for the application can be selected. In some cases, the monospecificity may be less ideal, for instance in changing environments, as discussed for polyclonal antibodies. Also, monoclonal antibodies are, compared to polyclonal antibodies, labor-intensive in production and it may take well over a year to produce a good antibody, for a review on distinguishing features of monoclonal and polyclonal antibodies, see Lipman et al (Lipman et al., 2005). When produced, hybridoma cells are very practical; they can be frozen, thawed and still produce antibodies.

**Recombinant antibodies**

The isolation of cDNA from hybridoma or other sources allows for the transfer and engineering of the antibody genes into alternative expression hosts. Many of the therapeutic antibodies used in the clinic today are produced in CHO cells after some engineering (such as humanization). However, the use of other cells than mammalian cells is also possible. Inspired by two reports published in Science 1988, which described the production of functional antibody fragments in *E. coli* (Better et al., 1988; Skerra et al., 1988), technology for the production of various antibody constructs in *E. coli* has now become significantly developed and widespread (Fernandez, 2004).

This route for recombinant production of monoclonal antibodies allows for various types of genetic engineering of antibody genes, including the subcloning and expression of fragments (described below) or genetic fusion to other protein domains. In present day, full-length hybridoma-derived antibodies are used side by side with recombinant antibodies or fragments of antibodies. Advantages of using antibody fragments include the removal of the Fc region,
when effector functions are not needed, and that fragments are better suited for expression and selection. Some of these fragments will be presented briefly below (see figure 3).

![Figure 3](image)

**Figure 3.** Schematic illustration of antibody fragments that can be created with recombinant methods. The black lines illustrate the fragments, and the grey lines outline the antibody from which the fragments are derived.

**Antibody fragments**

Originally, **Fab fragments** were found to be a product after enzymatic cleavage of a whole antibody with papain and pepsin, and were named Fabs simply because they were the antigen binding fragments resulting (Porter, 1959). The smaller single-chain Fv, **scFv**, (~28 kDa) consists of the variable region’s light and heavy chains that are genetically joined with a flexible linker (typically 15 aa) so that the light and heavy chain can be functionally produced in one polypeptide chain, hence the name single chain. **V<sub>H</sub> and V<sub>L</sub> domains** contain either the light or the heavy chain variable region and are even smaller than the scFvs (Holliger et al., 2005). Camelids (camels and llamas) and cartilaginous fish (wobbegong and nurse sharks) naturally produce antibodies that only consist of heavy chains, where one of the CDRs in the V<sub>H</sub> region is a long loop, stabilized with disulfide bridges. **V<sub>H</sub> fragments** of this class of antibodies, denoted
A multitude of engineered fragments of antibodies has been produced by recombinant means. For example, dimers and trimers of identical Fab fragments have been produced so that binding benefits from the avidity effects of many proximal binding sites. ScFvs have been expressed as non-covalently linked dimers (diabodies), trimers (tribodies) and tetramers (tetrabodies) (Holliger et al., 2005). These types of multimeric antibody fragments have also been produced as multispecific ligands with different specificities for each domain. In this way, one domain of the multimeric constructs can target an epitope, on for instance a cancer cell, while the other acts as a binder that attracts immune cells, thereby activating the endogenous immune system against the cancer cell (Kipriyanov et al., 2004). Further, direct fusions of antibody fragments to reporter functions have been described, as a means to obtain easily produced conjugates for diagnostic ligand assays (Ducancel et al., 1993).

**Antibody phage display**

In 1990, five years after the basic principles had been described (Smith, 1985), phage display was utilized for display and selection of antibody fragments expressed on the surface of a phage (a bacterial virus) (McCafferty et al., 1990). This significant step in affinity ligand development history had been preceded by several years of work involving peptide phage display, where short randomized peptide sequences were subjected to affinity selections (Parmley et al., 1988; Parmley et al., 1989). The phage display technology, capable of creating and maintaining a link between phenotype and genotype during a selection process allowed for the construction of large libraries of antibody fragments from which rare clones capable of interacting with a desired target could be “fished out” for later identification and recombinant production.

Phage libraries of antibody fragments can be constructed using antibody genes from different sources, including B-cells from immunized or non-immunized (naive) laboratory animals or humans, or from *in vitro*-generated pools using oligonucleotide-based gene technology, (Hoogenboom, 2005).
**Alternative selection systems**

Since the introduction of the phage display system, other selection systems have been developed, for example yeast display, bacterial display, bead display, ribosome and mRNA display (Lin *et al.*, 2002). Common for all these is the capability of creating a link between genotype and phenotype, to allow the genotype to be elucidated for a binding phenotype. For all selection systems, more than one round of selection is used so that after each selection cycle, binding variants are retained, amplified, and subjected to subsequent rounds of selection. In this way, binders with specificity for the target will be enriched until a suitable number of binders remain.

**Alternative affinity ligands and their generation**

With the advent of recombinant methodologies for the creation and selection of novel specificities and a need for stable affinity ligands that are easily produced, an interest in alternative non-immunoglobulin-based binding elements was created. Libraries of random linear peptides have been used extensively as ligands, for example in epitope mapping work or affinity ligand development (Cortese *et al.*, 1994; Labrou, 2003). To increase binding affinities, various approaches have been taken to constrain peptides, for example via the introduction of cysteine pairs forming disulfide bridges (Szardenings, 2003). An alternative to this route is to take advantage of already folded proteins for the development of novel affinity ligands. Briefly, combinatorial mutagenesis has been applied to surface-exposed positions of various proteins for the construction of large pools of variants, from which new variants can be selected (Binz *et al.*, 2005; Hey *et al.*, 2005; Hosse *et al.*, 2006; Nygren *et al.*, 2004).

Some general issues could be considered when choosing a particular protein scaffold and regions thereof for such projects. First of all, the scaffold candidate should ideally be a single subunit protein to facilitate library construction and expression and it should be tolerant to the introduction of multiple substitutions, without losing its overall three-dimensional structure. If the native protein is already involved in biomolecular interactions it might be suitable to randomize the amino acids involved in the binding. The randomization should cover a sufficiently large surface area, so that entirely new binding interfaces to many targets can be possible. Scaffold proteins should be chosen on the basis of structure, composition and
intended use. For instance, if novel binders are to be used in the reducing cytosol, a scaffold protein dependent on disulfide bridges is less suitable. For the development of binders to small haptens, it could be advantageous to choose a scaffold protein containing a cavity. It is obviously convenient if the developed affinity ligands can be produced in large quantities in a simple manner, in bacteria or by chemical synthesis. The candidate scaffold should also be compatible with prerequisites related to the particular selection system to be used. In the following, some affinity ligands based on alternative scaffolds are presented briefly:

**Affibody molecules**

The 58 amino acid three-helix bundle protein Z, once created by protein engineering of the B domain of the five-domain protein A from *Staphylococcus aureus* (Nilsson *et al.*, 1987) has been randomized in 13 of the surface-exposed amino acids on helices one and two to give libraries of binding molecules, named affibody molecules (see figure 4). Using phage display technology, affibody molecules have been selected against a variety of targets, for instance insulin (Nord *et al.*, 1997), IgA (Rönnmark *et al.*, 2002), Her2 (Wikman *et al.*, 2004) and other targets (Hansson *et al.*, 1999; Nord *et al.*, 1995; Sandström *et al.*, 2003), and they have been used in many biotechnological applications based on molecular recognition, such as affinity purification including harsh alkaline regeneration conditions (Nord *et al.*, 2000) and other applications (Andersson *et al.*, 2003; Gräslund *et al.*, 2002; Rönnmark *et al.*, 2002; Rönnmark *et al.*, 2003). Affibody molecules are most often produced in *E. coli*, but as will be described (present investigation, papers II and III), they have also been produced with chemical synthesis which allows specific incorporation of proteinaceous and non-proteinaceous chemical moieties. The first selections made from libraries of approximately $10^7$ members yielded binders with affinities ($K_d$ values) in the low µM-range (Nord *et al.*, 1997), (Rönnmark *et al.*, 2002). After affinity maturation, binders in the nanomolar affinity range have been obtained to *Taq* DNA polymerase (Gunneriusson *et al.*, 1999) and Factor VIII (Nord *et al.*, 2001). More recent selections from larger naive libraries have directly yielded binders with higher affinities, for instance against the human receptor Her2 ($K_d=50$ nM) (Wikman *et al.*, 2004), in accordance with the observation by Griffiths and co-workes (Griffiths *et al.*, 1994) and Ling, (Ling, 2003) that the use of larger libraries should give higher affinity binders since the chance of finding a suitable binder increases with library size. Affinity maturation of the 50 nM affinity Her2 binding affibody molecule was recently
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described to result in a further 2,200-fold improved binding affinity, reaching an impressive
dissociation constant of 22 pM (Orlova et al., Cancer research, In press). Affibody molecules
to different targets have been used as
ligands for all ligand assay work (I-IV)
in this thesis.

Figure 4. Schematic illustration of the
affibody three-helix bundle protein. The
positions of the randomized amino acids
in helices, one and two are marked in the
figure.

Ankyrin repeats

Ankyrin repeats are made up of repeating protein domains, which are flanked with N- and
C-terminal capping domains. Each repeat is made of 33 amino acids, and forms a secondary
structure of a β-turn followed by two antiparallel α-helices which in turn are followed by a loop
that ends in the next repeat domain. By comparing the sequences of many naturally occurring,
protein-interacting ankyrin repeats, it became clear that the surface-exposed residues were
varied, and that these variations gave rise to different specificities for different repeats (Binz et al.,
2003). Therefore, in order to construct libraries for selection towards any given target, Plückthun
and co-workers chose six amino acids in the β-turn and the first α-helix to be randomized, and a
varying number of repeat domains were used for selections against the maltose binding domain
and two map kinases. Low nM binders were found for all three proteins, and one of the ankyrin
repeat binders directed against the maltose binding protein was co-crystallized with the maltose
binding protein (Binz et al., 2004). The structure showed that the varied amino acids made target
contact, and thus confirmed that the randomization approach had been successful. Generally,
ankyrin repeats should be expected to be excellent binders for a variety of targets since their
sequential arrangement of domains allows them to adjust to different binding surfaces, while
retaining their rigid subdomain fold. Ankyrin repeat proteins also lack cysteine residues, which
makes them possible to use for intracellular applications.
**Anticalins**

Lipocalins are vitamin and steroid transporters in human plasma and blood. The lipocalin motif, a rigid B-barrel structure of 160 to 180 amino acids with four hypervariable loops that form the binding site, has been used as a scaffold for randomization. Residues in the four loops were variegated to form a new class of binders, anticalins, and ligands directed against fluorescein \((K_d=35\text{nM})\) (Beste et al., 1999) and the plant steroid digoxigenin \((K_d=30\text{nM})\) (Schlehuber et al., 2000) have been selected. Proving that anticalins are suitable also against macromolecular targets, ligands against CTLA-4 (cytotoxic T lymphocyte-associated antigen) have been selected (Schlehuber et al., 2005).

**Knoottins**

The cystine knot superfamily is based on a small and tightly packed protein motif that is stabilized by three disulfide bridges (Craik et al., 2001). The fold is a triple-stranded antiparallel \(\beta\)-sheet connected with three loops, made up of around 25-35 amino acids, which makes them a suitable scaffold for randomization. The cellulose-binding domain (CBD) of cellobiohydrolase I from the fungus *Trichoderma reesei*, has been used as scaffold for randomizations. Novel ligands to alkaline phosphatase (Smith et al., 1998), alpha amylase (Lehtio et al., 2000) and a metal/chelator complex (Wernerus et al., 2001) have been described.

**Aptamers**

Aptamers are particularly interesting since they differ from the previously described scaffolds in that they are non-proteinaceous. Consisting of synthetic single stranded DNA or RNA (5-25 kDa) which form defined structures, the variation in these binders is made up of only four bases; uracil/ thymine, cytosine, guanine, and adenine (Proske et al., 2005). This seeming limitation in diversification, compared to the twenty different amino acids available for variation for proteinaceous scaffolds, is compensated for by the large library size which can be achieved with aptamers, using the SELEX selection system (Tuerk et al., 1990). In this system, a random aptamer library of DNA or RNA is incubated with a target for which a binder is desired. After incubation, non-binding aptamers are washed away and the retained aptamers are amplified with RT-PCR. This allows for a stepwise enrichment of binding aptamers for typically 6 to 18
iterative cycles, until only a few high-affinity aptamers dominate the remaining library pool. Since 1990 (Ellington et al., 1990; Tuerk et al., 1990), aptamers have been created for a wide variety of targets such as small molecules, amino acids, peptides, and proteins, with affinities for their targets typically in the low nM to pM range. Though aptamers are naturally sensitive to DNA or RNA degrading enzymes, they can be stabilized with the use of 2’-fluoro- or 2’-amino-substituted pyrimidines that enhance their half-life. In 2004, the first aptamer drug, Macugen (Pegaptamib, Pfizer and Eyetech) was approved by the FDA for treatment of age-related macular degeneration, proving that aptamers have large potential also for clinical applications (Proske et al., 2005).

Photoaptamers, an aptamer variant, can be cross-linked with UV-light to their target proteins after capture, which allows harsh washing to decrease background signals (Smith et al., 2003). Interestingly, captured proteins can be detected with activated dyes, which couple to the free amino groups on the captured proteins, thereby allowing detection. For example, Bock et al have investigated the use of photoaptamers in capture microarrays (Bock et al., 2004) and were able to measure concentrations below 10 fM for several analytes including interleukin-16 and VEGF.
Solid phase peptide synthesis

Today, small proteins and peptides can be synthesized with peptide chemistry as an alternative route to recombinant protein production. This is possible due to the solid phase peptide chemistry, pioneered by Bruce Merrifield. In 1963, Merrifield published a paper describing the stepwise chemical synthesis of a tetrapeptide on a nitrated polystyrene resin using the benzyloxycarbonyl (Z) protecting group, which was removed with a strong acid (Merrifield, 1963). The solid phase allowed an excess of reagents to be used which could drive the coupling reactions to completion and that could be removed by filtration after coupling. Until then, peptide synthesis had only been performed in solution, and the new solid phase synthesis method was met by skepticism by the peptide synthesis community, on account of that the isolation and analysis of intermediates of reaction paths became unfeasible (Marshall, 2003). A year later, Merrifield introduced the t-butyloxycarbonyl (Boc) group as temporary amino protecting group instead of the Z group and the benzyl ester as a linker to the polystyrene resin. Milder reaction conditions could be used, and the synthesis of the nonapeptide bradykinin was possible (Merrifield, 1964). The successful synthesis of such a long peptide by solid phase peptide synthesis, made possible by the improvements of the chemistry, would change the peptide synthesis field dramatically, and in 1984, Merrifield was awarded the Nobel Prize in Chemistry. In 1972, Carpino introduced the base-labile fluorenylmethyloxycarbonyl (Fmoc) group (Carpino et al., 1972). In the late 1970’s, Sheppard and Atherton developed the Fmoc chemistry where the Fmoc group was used as temporary protecting group instead of the Boc group, and t-butyl derived protecting groups (Boc, for instance) were used as permanent side chain protecting groups (Atherton et al., 1978;
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Atherton et al., 1978). This allowed the synthesis to be performed under milder conditions, and though Boc chemistry is still in use, Fmoc chemistry has become the major route for solid phase peptide synthesis today (Sheppard, 2003).

**Principles of Fmoc solid phase peptide synthesis**

The first amino acid, corresponding to the C-terminal residue of the final protein, is coupled via a linker to a solid support, often a polystyrene resin (see Figure 5). The carboxyl group of the next amino acid is activated with a good leaving group and coupled to the amino group of the resin-bound first amino acid. Any reactive functional groups on the side chains, like amine and carboxyl groups, are protected with permanent protecting groups that are removed after completed synthesis. The N-terminus of the added amino acid is protected with a temporary protecting group, the Fmoc group, which is removed with base (typically 20% piperidine) before the coupling of the next amino acid. In this way, only one reactive group of the growing peptide chain, the N-terminus, is available for reaction with the carboxyl group of the next amino acid. The coupling cycles are repeated until the entire peptide sequence is complete. Following this procedure is the final deprotection of the permanent side-chain protecting groups by acid and cleavage of the linker to release the peptide from the resin (often with TFA). After work-up of the cleavage reaction, the peptide is typically further purified with RP-HPLC. It should be pointed out that the synthesis described above progresses from C to N-terminus of the peptide sequence, rather than from N to C-terminus which is the synthesis route for ribosomal translation.
Solid phase peptide synthesis is not limited to the naturally occurring amino acids; also unnatural amino acids, D-amino acids, polymers, fatty acids and other organic building blocks can be incorporated with great precision into the growing peptide chain. Synthesis yields are potentially high. The use of orthogonal protecting schemes, where certain side chain protecting groups can be removed specifically, gives great flexibility for site-specific incorporation of building blocks after completion of the peptide. An example of this is found in paper II in this thesis, where fluorescent reporter groups and an immobilization anchor are incorporated after the completed synthesis of an affibody molecule. Also, peptides that are difficult to produce by recombinant means, for instance amyloid fiber forming peptides, can be synthesized with solid
phase peptide synthesis (Tickler et al., 2004). On the other hand, solid phase peptide synthesis is not well suited for the synthesis of long peptide sequences or proteins. When the peptide sequence grows, side products accumulate and incomplete couplings decrease the final yield. Synthetic peptides of 20-30 amino acids can routinely be synthesized by solid phase methods, but whereas longer peptides of up to 100 amino acids often are possible to synthesize, more side-products and lower yields can generally be expected. For the synthesis of long peptides and proteins, fragment condensation techniques, where several short and separately synthesized peptides are ligated to a longer peptide, are often used as alternative methods. Another consideration is that reporter groups incorporated into the peptide must be able to withstand the conditions for the final deprotection of the permanent side-protecting groups and cleavage from the resin, which often involves 95% TFA; a very strong acid compared to ordinary biochemical environments.
Ligand assays

In their most fundamental form, ligand assays rely on a ligand, typically belonging to any of the classes previously described, for the recognition of an analyte, and a reporter system that relays the binding event. A comprehensive history of ligand assays is outside the scope of this thesis, however, some assays using new fundamental principles will be presented.

The 1950s work by Yalow and Berson on insulin, which led to the invention of radioimmunoassay (RIA) (Yalow et al., 1959; Yalow et al., 1960), was a major milestone in ligand assays. Using RIA, human insulin serum concentrations could be determined with the use of polyclonal antibodies from guinea pigs, raised against crystalline beef insulin. Unlabeled human insulin competed with iodine-131 radiolabeled beef insulin for the antibody binding sites. The separation and the subsequent detection of bound and unbound insulin were performed with paper-chromatography. The concentration of unlabeled insulin in the sample affected the amount of bound radiolabeled insulin so that the concentration of unlabeled insulin could be determined. The main principle of RIA, the competitive detection, would have fundamental implications. Now sensitive detection of all sorts of analytes was possible, if an antibody could be raised against them, and a radiolabeled competitor-analyte could be created (Ekins, 1960; Murphy, 1964). For the development of the radioimmunoassay, Rosalyn Yalow was awarded the Nobel Prize in Physiology or Medicine in 1977.
The next step in ligand assay development came with the first non-competitive assay, the radioallergo sorbent test (RAST), described by Wide et al in 1967 for the diagnosis of allergy (Wide et al., 1967). In this assay, antibodies directed against IgND (IgE) were attached to a solid phase, added to allergic sera, and after incubation, the analyte was detected with a second iodine-131 radiolabeled antibody also binding the analyte, but at a different epitope. The classification “non-competitive” relates to the way in which the analyte is detected; no competition for the capturing antibody takes place and the analyte is measured directly by the detecting antibody.

In 1971, Engvall and Perlmann (Engvall et al., 1971) published a paper describing an assay using an enzyme (alkaline phosphatase)-linked antibody, to quantitatively detect the IgG concentrations in rabbit serum. The assay was named ELISA; enzyme-linked immunosorbent assay. ELISA in this setup was competitive, and native IgG was allowed to compete with alkaline phosphatase-linked IgG for binding to anti-IgG antibody coated cellulose. After washing, enzyme activity was measured through changes in absorbance at 400 nm caused by the hydrolysis of p-nitrophenylphosphate (NPP) to the yellow product p-nitrophenol. This publication was shortly followed by the publication of Van Weeman and Schuurs (Van Weemen et al., 1971) where the enzyme horseradish peroxidase was used as a reporter for the detection of gonadotropin concentrations in urine. Safety and health issues, special laboratory facilities and expensive equipment regarding radiolabel-work had created a need for a simpler assay, something that ELISA would grow to be. In the 1980s, the sensitivity of RIA was matched by ELISA, and fully automated test systems were commercially available (from Boehringer-Mannheimer and Abott, for instance).

In 1974, Dr Soini, research manager of Wallac, a Finnish company selling mostly alfa- and gamma radiation measurement devices, in collaboration with Roger Ekins of Middlesex Medical School of Medicine, London, became convinced that the future of immunoassays was not in radiolabels, for the reasons mentioned above, but in fluorescence. The ideas of Ekins combined with the persistent work of Soini, eventually resulted in the first commercial immunoassay based on fluorescence, Delfia (Marshall NJ, 1981; Miettinen, 2000). Since then, the number of immunoassays utilizing fluorescence has increased dramatically, today a PubMed search limited
to papers published in the last five years on “fluorescence” and “assay” yields a total of roughly 16,000 hits, compared to 6,000 for “radioimmunoassay”.

**Ligand assay formats**

Numerous formats for ligand assays have been developed in solution or on solid phase, in competitive and non-competitive formats, relying on various reporter systems and labels for detection (see figure 6). Analytes can be labeled for direct detection, or a secondary detecting ligand binding the analyte can be labeled for sandwich detection. Alternatively, a third detecting ligand, binding the secondary, can be introduced to increase the number of labels for a particular binding event.

In solid phase formats, the ligand can be immobilized onto a solid support for detection of analytes in solution, or the analyte can be immobilized on a solid support for detection by a ligand in solution. In solution phase assays, both ligand and analyte are in solution. Solid supports are often used to allow separation or washing steps before the addition of a secondary binding agent to allow detection after analyte capture. These types of assays are called heterogeneous. Heterogeneous assays are usually slower, and often require trained personnel to carry out the subsequent steps after addition of the sample containing the analyte (Burtis, 1994). In contrast, homogeneous assays require no such steps prior to detection after analyte addition. The main benefits of homogeneous assays are the great simplicity which allows untrained personnel to run the assay, and often short assay times. Many assays using solid supports are non-competitive (and heterogeneous). These assays tend to be more sensitive for low-concentration analyte detection and determination since they rely on direct detection of analytes. In contrast, competitive assays detect a labeled competitor after competition with the analyte for ligand binding. The principles governing competitive and non-competitive assays will be described in conjunction with a description of “the law of mass action”, see below.
Figure 6. Examples of different detection formats used in ligand assays. A distinction has been made between competitive and non-competitive formats and of solid- and solution-phase assays. Schematic illustrations are used to demonstrate the underlying principles of the formats, which will be described in more detail in the text. CEDIA; Cloned enzyme donor immunoassay (Armbruster et al., 1995). FCS; Fluorescence correlation spectroscopy (Tetin et al., 2004).
The law of mass action

The previously described competitive (Engvall et al., 1971; Yalow et al., 1959) and non-competitive (Wide et al., 1967) assays follow some basic principles for protein complex formation (in solution), from which interesting observations can be made. For the simpler, non-competitive assay, the relation between the concentration of free ligand, \( L \), and its analyte, \( A \) (free concentration), can be described by the law of mass action in the formula:

\[
L \cdot A / (LA) = \frac{k_{\text{off}}}{k_{\text{on}}} = K_{d}^{A} = 1/K_{a}^{A}
\]

where \( LA \) is the concentration of complexes formed, \( k_{\text{on}} \) is the association rate constant in \( M^{-1} \text{ min}^{-1} \) (the number of association events per minute and molar), \( k_{\text{off}} \) is the dissociation rate constant in \( \text{min}^{-1} \) (the number of dissociation events per minute) and \( K_{d} \) is the dissociation constant in molar (M) (see figure 7).

The overall affinity denominator of the interaction, \( K_{a}^{A} \), is important since it can be used to calculate how much analyte is bound by the ligand at equilibrium. The lower the \( K_{a}^{A} \), the higher the fraction of ligand and analyte in complex. The amount of analyte in complex (bound by the capture ligand) is most likely deciding the limit of detection for the assay and therefore, ligands with lower dissociation constants give more sensitive assays. However, since the association rate is concentration-dependent, an increase in the concentration of capture ligands results in an increase of bound analytes similar to an increase in affinity. This leads to the conclusion that for non-competitive assays, the concentration of capture ligands should be maximized to decrease the limit of detection.
Fluorescence-based ligand assays for protein detection using affibody affinity proteins

The competitive assay format is based on competition between two molecules for one binding site. Often, a labeled analyte of known concentration is competing with an unknown concentration of unlabeled analyte for binding to a ligand. After competition, the amount of unlabeled analyte is determined from the amount of labeled analyte bound. The presence of these three components leads to a slightly more complex formula describing the formation of complexes for these three participants:

\[(L) = \frac{[(LC)K_{dC}]}{(C)} = \frac{[(LA)K_{dA}]}{A}\]

where \(C\), the labeled analyte competitor, and \(A\), the analyte, are competing for binding to \(L\), the ligand. \(K_{dC}\) is the dissociation constant for \(L\) binding to \(C\) and \(K_{dA}\) is the dissociation constant for \(L\) binding to \(A\). \(L\), \(C\) and \(A\) are the concentrations of unbound molecules and \(LC\) and \(LA\) are the concentrations of the complexes of \(L\) and \(C\), and \(L\) and \(A\), respectively (see figure 8).

Figure 8. In the competitive assay format, the unknown concentration of the analyte (\(A\)) is determined from the concentration of ligand-competitor complexes (\(LC\)). The labeled competitor (\(C\)) competes with the analyte (\(A\)) for binding to the ligand (\(L\)). The two dissociation constants \(K_{dA}\) and \(K_{dC}\), and the concentrations of analyte, competitor and ligand, determine the concentrations of the formed ligand-competitor and ligand-analyte complexes.

In a competitive format, the number of ligands, \(L\), and competing labeled ligands, \(C\), must be in the same concentration range as the competing analyte, \(A\), that one wishes to measure. If the concentration of the competing ligand, \(C\), is too high or too low, there will be no measurable competition between \(C\) and \(A\) and the assay will be insensitive. If the concentration of \(L\) is higher than that of \(C\) and \(A\), \(C\) and \(A\) will not compete efficiently since the number of free \(L\) allows binding of both \(C\) and \(A\) at the same time. From this follows that if one wishes to detect a low concentration of \(A\), both \(L\) and \(C\) must be of low concentration to allow efficient competition.
and thus a sensitive assay. Also the dissociation constants are important: if the concentration of A is lower than the dissociation constant $K_d^A$, only a small fraction of A is bound to L, and the sensitivity of the assay decreases. The competitive assay format also depends on the dissociation constant $K_d^C$ and the fractional coverage of C to L, since it is C that is labeled and thus it is the difference in bound or free C, as a result of the competition to A, which is detected. So, as for A, the sensitivity of the assay will decrease with the decreased fractional coverage of C. These are the reasons why the limit of detection for competitive assays is generally higher than for non-competitive assays. Thus, the $K_d$ values in competitive assays are more important, compared to the direct detection in non-competitive assays, where sensitive detection can be performed many logarithms under the dissociation constants of the capture ligands, by increasing the concentration of capture ligands and using signal amplification techniques (see signal amplification below).

**Detection labels**

There are many types of labels that can be used for detection in ligand assays. Radiolabels were used early, as in the described RIA (Yalow et al., 1959) and RAST (Wide et al., 1967) methods, followed by enzymes (Avrameas, 1970), as are used in ELISA (Engvall et al., 1971). Today, fluorophores are the most used labels, for their sensitivity and easy handling (see Fluorescence). For sensitive detection, signals often need to be increased, via the use of more labels per binding event, or by DNA-based amplification systems using for example PCR or rolling circle amplification (see DNA based detection). Some formats avoid labeling altogether and use inherent properties of the analyte for detection (see Label-free detection).

For successful detection of a binding event, the signal of the label must be sufficiently strong to be detected. In most systems used today, a single fluorophore (or radiolabel) cannot be detected, and multiple fluorophores and binding events are needed. Many signal amplification techniques have been developed that increase the signal intensity per binding event, to improve the limit of detection of the assays. For example, more than one labeled detecting ligand (antibody) can be used for the detection of a bound analyte in sandwich assays. An analyte-specific detecting antibody can be detected with several labeled secondary antibodies. For ELISA-type assays, the
enzyme itself also gives rise to signal amplification as a single enzyme can process many substrate molecules. A similar approach to reporter-labeled secondary antibodies is the use of biotinylated target-specific antibodies that are bound with several labeled streptavidin molecules. Although most assays using signal amplification are used in non-competitive sandwich formats, signal amplification can also be used in other non-competitive or competitive formats.

**Fluorescence**

As mentioned previously, a number of different labels such as radiolabels, enzymes and fluorophores, have been used as reporters in ligand assays until today. Since fluorophores are by far the most used they deserve special attention. Most assay formats can make use of fluorophores as labels, for instance, the previously described competitive and non-competitive ligand assays that originally were based on radiolabels can also be used with fluorophores. With fluorophores, sensitive detection is possible while avoiding safety issues and the use of special lab areas.

A fluorophore emits fluorescence when an electron has been excited and falls back to its ground state. In more detail, an electron is excited by a laser or a lamp. The excitation energy is of a wavelength that corresponds to the energy difference between its ground state and excited state. The excited electron will fall to the lowest possible vibrational band in the excited state, losing energy as heat, by a process called internal conversion. When the electron falls back from the excited state to one of the vibrational bands of the ground state, it can emit the energy as a photon, and when this happens the molecule will fluoresce. From this it can be understood that only wavelengths corresponding to the energy differences of the vibrational bands of the ground state and the excited state are accepted as excitation wavelengths, and that the fluorescence wavelength of the emitted photon (the fluorescence) is possible in only a certain number of set wavelengths. These wavelengths will also always be longer than the excitation wavelengths, since they contain less energy. The difference in excitation and emission wavelength is called the Stoke’s shift, and is important since it allows excitation at one wavelength and detection at another, which is the basis for the sensitivity of fluorescence-based assays.

Fluorescent proteins, semiconductor crystals (quantum dots), and organic dyes can all be used
as fluorescent labels in ligand assays. Proteinaceous fluorophores such as the green fluorescent protein (GFP) and variants thereof (Verkhusha et al., 2004), have become very popular since they allow genetically linked fusion proteins to be expressed in cells. Quantum dots are small nanoparticles of around 2-10 nm, for which the fluorescence properties varies with size and material, that have been used as reporter dyes quite recently for *in vitro* and *in vivo* applications (Jaiswal et al., 2004; Michalet et al., 2005; Ozkan, 2004). Their quantum yield and stability to photobleaching surpass that of organic dyes. Organic dyes are still the most popular fluorophores (Haugland, 2002). The organic Cy3 and Cy5 dyes (Amersham Biosciences) are used extensively in DNA microarray work, and are also commonly used in protein microarrays experiments (see Protein microarrays). In microarray experiments, they are typically used to compare mRNA levels: two different samples are labeled with Cy3 and Cy5, the samples are pooled and allowed to bind to the array, after which the relative fluorescence intensities are compared (Schena et al., 1995). Other examples of commonly used fluorophores include fluorescein, tetramethylrhodamine (see figure 9) and the Alexa dyes produced by Molecular Probes.

![Figure 9. Structures of the fluorophores (a) Cy3, (b) Cy5, (c) Fluorescein, and (d) Tetramethylrhodamine.](image-url)
Direct labeling of proteins or protein mixtures is possible with the use of reactive derivatives of fluorescent dyes (Haugland, 2002). Popular reactive groups are the N-hydroxysuccinimide (NHS) esters and isothiocyanates that are easy to couple to amino groups on lysine side chains or to N-terminal amines, and form covalent bonds after reaction. Using thiol-specific reagents such as haloacetyls or maleimides, cysteines can be specifically modified, although cross-reactivity with amines may occur.

**Fluorescence resonance energy transfer (FRET)**

In 1948, Förster published an article describing the radiation-free transfer of energy between two fluorophores, a donor and an acceptor (Förster, 1948). When the donor was excited, energy would be transferred to the acceptor through resonance, if the relative orientation of the dipoles of the donor and acceptor was correct, the donor emission spectrum overlapped the acceptor excitation spectrum, and the molecules were sufficiently close in space, typically under 10 nm (see figure 10). The phenomenon has been named fluorescence resonance energy transfer (FRET). The energy transfer is highly dependent on distance between the donor and acceptor and its efficiency decreases with the sixth power of the distance. This property of FRET has been used in ligand assays. For example, Lichlyter et al (Lichlyter et al., 2003) used an antibody conjugated to a donor fluorophore, Alexa Fluor 546. The antibody was bound by the Fc-binding protein A conjugated to an acceptor fluorophore, Alexa Fluor 594. Upon target-specific binding by the antibody, the conformation of the antibody changed, which affected the distance between the donor and acceptor fluorophores; altering the FRET, and thus detection was possible. Arai et al (Arai et al., 2000) used the variable light and heavy chain fragments of an anti-hen egg lysozyme antibody and fused each of the chains to EGFP and EBFP, two fluorescent proteins constituting a FRET pair. Addition of lysozyme caused reassociation of the two variable binding domains which was measurable as an increase in FRET. Both these examples used alterations in distances caused by either changes in the conformation or organization of molecules to monitor binding.
Figure 10. Important characteristics of FRET. In a), the donor is excited and emits fluorescence, since the donor and acceptor fluorophores (to the right) are separated in space. However, in b), the donor is excited, energy is transferred via FRET to the acceptor, and the acceptor emits fluorescence, since the two fluorophores are close in space and have overlapping emission and excitation spectra.

**Fluorescence quenching**

When a fluorophore is quenched, its fluorescence is decreased. This can happen through dynamic quenching, for example by collisional quenching or FRET, or by static transfer. In static transfer, the fluorophore and quencher bind each other, due to hydrophobic or electrostatic interactions, and form a ground state complex in which the fluorescence is decreased. In static transfer, if the two fluorophores are identical and would align perfectly, the fluorescence could theoretically be zero; however, in reality most quenchers cannot completely quench all fluorescence (Johansson et al., 2003). Quenching has been used as the basis of detection in many immunoassays. For example, Hamaguchi et al used an anti-thrombin aptamer that upon binding increased its
fluorescence (Hamaguchi et al., 2001). The 5’ and 3’ ends of the aptamer were extended with a complementary binding sequence so that the 5’ and 3’ ends were brought together when no thrombin was present. The 5’-end was further labeled with carboxyfluorescein and the 3’-end was labeled with DABCYL (a quencher), and in this way, the fluorescence was quenched when no thrombin was present. When thrombin was added, the protein-binding aptamer structure was formed instead of the complementary 5’ and 3’ base-pairing, which resulted in the separation of the fluorophore and quencher, and thus the emission of fluorescence. In general, quenchers are practical for systems that measure total fluorescence and lack the ability to distinguish between different emission wavelengths.

**Environment sensitive fluorophores**

Fluorophores whose fluorescent properties are changed by their proximal surroundings can be used in biosensors. Renard et al (Renard et al., 2002) described an assay based on environment-sensitive fluorophores, where the fluorescence was changed by the electronic environment conferred by a bound target proximal to the fluorophore. A cysteine was genetically introduced into the variable region of a scFv directed against lysozyme to facilitate a site-specific coupling of an environment-sensitive fluorophore (IANBD). The introduced fluorophore was demonstrated to have no influence on binding affinity. Concentration-dependent titration of lysozyme was detectable as a change in fluorescence in both buffer and in serum, showing that the assay could be used for detection of proteins in complex samples, such as body fluids. In a later publication based on the same concept (Renard et al., 2004), the dynamic range, that is, the range where concentration can be measured, was increased by using lysozyme-binding scFv variants with point mutations introduced in the binding region that decreased the affinity. Since the epitope was still the same for all variants, the different affinities created a longer span of detectable concentrations.

In another example, Usui et al (Usui et al., 2004) used libraries of peptide polymers constructed from either α-helical, loop, or β-sheet secondary structures fused to fluorophores for the profiling of proteins. Different peptides in the libraries bound proteins differently, which was reported through changes in fluorescence upon binding, and the sum of all members of the protein library gave a characteristic fingerprint of the particular protein.
DNA-based detection

An interesting approach to signal amplification is immuno rolling circle amplification published by Schweitzer et al in 2000 (Schweitzer et al., 2000), based on the previously described method immuno-PCR (Sano et al., 1992). In immuno-RCA, analytes are detected with an antibody that is coupled to a single stranded DNA-fragment. This fragment is then hybridised to a circular DNA which can serve as a template for Taq DNA polymerase. The circular DNA allows the fabrication of a long stretch of DNA after the addition of nucleotides and DNA polymerase. To this product, fluorescently labeled oligonucleotides are added that hybridize to the DNA-strand, and thus allow fluorescent detection of bound antibody-antigen complexes. In immuno-RCA, the detection limit for PSA, prostate-specific antigen, was reported to be three orders of magnitude lower than that for standard ELISA.

Another DNA-based detection technique is proximity ligation (Fredriksson et al., 2002; Gustafsdottir et al., 2005). Two antibodies binding separate epitopes on an analyte are labeled with different single strand DNA oligomers. Upon binding, the two oligonucleotides are brought in close proximity, a DNA-fragment complementary to both strands is added and hybridized, and the two fragments are ligated with DNA ligase. The ligated DNA is then amplified with real-time PCR or rolling circle amplification, and detected. The benefit of proximity ligation compared to immuno-RCA is that two recognition events are needed for signal generation and amplification. This decreases background, and thus decreases the limit of detection.

In the bio-bar code assay for protein detection (Nam et al., 2003), a target-specific antibody and DNA fragments are immobilized onto a gold particle. A magnetic particle coated with another target-specific antibody, a sandwich partner, is also used, so that if target is present, a complex is formed between the gold particle and the magnetic particle. These complexes can be separated from the solution using a magnet, and after brief washing, the DNA is removed from the gold particle, PCR-amplified, and detected, or the DNA on the gold particles is allowed to hybridize to immobilized microspots of complementary DNA, and the gold particles are precipitated with silver and are detected with light scattering.
Label-free detection

Labeling proteins and biomolecules is not problem-free. The incorporated label may impair protein function if a binding site is disturbed and could sterically hinder ligand binding to the protein if the attachment occurs near the binding epitope. Labeling protein mixtures of different compositions may result in an unreproducible labeling efficiency of the analyte proteins, if sample components prone to coupling to the reactive groups are present at different concentrations in different samples. Labeling of complex protein mixtures could also increase background since all non-specifically bound proteins will fluoresce, and thereby increase the background noise. On the other hand, if a sandwich approach is used instead of analyte labels, a problem can be that a high number of labeled secondary antibodies, for large-scale parallel analysis, will likely decrease sensitivity as the secondary antibodies start to cross-react. For instance, Schweitzer et al separated 70 antibody pairs into two pools to avoid cross-reactivity (Schweitzer et al., 2002). Also, for many analytes two ligands binding separate epitopes can be hard to raise.

These issues has spurred an interest into label-free detection methods, such as mass spectrometry (Zhu et al., 2005), surface plasmon resonance (Pattnaik, 2005), atomic force microscopy (AFM) (Kienberger et al., 2006), quartz micro-balance technology (Marx, 2003), and electrical detection (Zheng et al., 2005). Using physicochemical properties of the analytes for detection, such as mass and charge, the need for a label, and problems associated with labeling, can be avoided. Also, only one ligand is needed for the assay. Generally, label-free detection systems are not yet as sensitive as label-based, but are promising in theory. Some label-free detection systems will be presented briefly:

Surface plasmon resonance (SPR)

Many detection systems using surface plasmon resonance have been created, and also commercialized; Biacore, Genoptics and Reichert are some examples of companies active in this field. The Biacore platform (Jönsson, U. et al, 1991) has been used extensively for studies of ligand interactions to determine association and dissociation rate constants between analyte and ligand. In the standard Biacore setup, the capture ligands are attached to a dextran polymer
on a gold chip which is placed in a flow chamber that allows continuous flow over the surface. Light is reflected by total internal reflection on the dry side of the chip and the angle (resonance angle) for which an energy loss (detected as darkness) is occurring by virtue of the SPR phenomenon is detected with a photodiode array in real-time. When analytes in the flow buffer bind to the immobilized ligands, the mass accumulation near the gold surface causes a shift in the resonance angle conferred by a shift in the refractive index. Since the detection principle ultimately depends on the mass of the accumulated analytes, the amount of bound analytes can be monitored. Although mostly used for relatively small sets of samples, the SPR principle has also been used for large scale analysis. Säfsten et al (Säfsten et al., 2006) screened 386 crude hybridoma samples for monoclonal antibodies with desired kinetic profiles in 12 hours using the Biacore A100 (containing 16 spots for immobilization in total). Though slower than standard ELISA screening techniques, the information obtained was more detailed, and affinity ranking and determination of off-rates was possible. The newly introduced Biacore Flexchip with 400 surfaces for interaction studies will likely take these kinds of screening applications one step further.

**Mass spectrometry**

In mass spectrometers, the analyte is ionized and led into an analyzer, where the mass of the analyte is determined. There are different variants of analyzers, for example quadrupoles that act like filters and allow only analytes of a certain mass-to-charge ratio to pass to the detector, and time-of-flight (TOF) analyzers, where the time for the analyte to reach the detector is measured (Cole, 1997). A breakthrough in mass spectrometry came with two Nobel Prize awarding new ionization techniques, Matrix assisted laser desorption ionization (MALDI) (Karas et al., 1988; Tanaka et al., 1988) and electrospray ionization (Fenn et al., 1989). With these technologies came the possibility to build cheap instruments that could analyze large biomolecules. Mass spectrometry is used in a variety of fields related to ligand assays including biomarker discovery, diagnostics, and peptide synthesis. In a ligand assay variant, immobilized antibodies are used to capture target molecules from complex mixtures and detection is performed with the MALDI analogue SELDI (Zhu et al., 2005). A great advantage of mass spectrometry is that detection also conveys the mass of the analyte, and thereby verifies its identity.
Electrical detection

Electrical detection using the inherent charge state of the analyte molecules can be used for detection. For instance, nanoscale field effect transistors (FETs) constructed from semiconducting nanowires can be used as molecular detectors. When a capture ligand immobilized on the nanowire surface binds a charged analyte, this event results in the accumulation or depletion of carriers (electrons or holes) in the nanowire that can be monitored. For example, biotinylated nanowires have been used for streptavidin detection, and wires coated with calmodulin have been used for Ca\(^{2+}\) detection (Cui et al., 2001). Recently, prostate serum antigen, PSA, was detected at levels down to 0.9 pg/ ml in human serum, showing that nanowires indeed can be used for detection of low-abundant proteins in complex sample mixtures (Zheng et al., 2005).
Protein microarrays

The fruitful work in the DNA microarray field during the 1990s (Fodor et al., 1991; Schena et al., 1995) was an inspiration for the miniaturization and development of parallel protein assays in a microarray format. However, already in the end of the 1980s, Roger Ekins had described a miniaturized, parallel assay in which spatially separated microspots of antibodies with specificities against different proteins were to be used for protein detection, making possible the parallel analysis of many proteins at the same time (Ekins et al., 1990; Ekins et al., 1989; Ekins, 1989). The miniaturization allowed many spots to be fit in a small area and required new equipment capable of detecting not only total fluorescence, but also the spatial placement of the fluorescent spot. The small amount of analyte captured by the microspots was predicted to be negligible in comparison to the amount of analyte remaining in solution; thus the concentration would be unaffected, and an accurate estimate of the concentration in solution would be possible.

Simply described, a protein microarray is an array for protein analysis consisting of capture ligands or analytes, immobilized on a solid support in microspots, separated from each other in space (though bead-based arrays using color codes instead of the spatial separation have also been created (Gordon et al., 1997) (see figure 11).
Technological aspects of protein microarrays

DNA microarray technology became an established method for expression analysis already in the mid-1990s, when for instance Schena et al. used robotically printed complementary DNA on glass slides for the parallel quantification of gene expression of 45 Arabidopsis genes with two-color fluorescence (Schena et al., 1995), and earlier, expression profiling chips were produced with photolithographic synthesis methods (Fodor et al., 1991). In the field of protein microarray technology, important technological advancements have been reported in quite recent years. For instance, robotic printing of proteins on glass slides for protein and small molecule interactions and evaluation of kinase activity was reported in 2000 by Macbeath and Schreiber (MacBeath et al., 2000), the production of a protein array containing thousands of features for interaction studies was published in 2001 (Zhu et al., 2001), and the optimization of surfaces and immobilization techniques for optimal array performance were reported (Peluso et al., 2003) cahill, joos (Kusnezow et al., 2003). As a matter of fact, protein microarray technology is still developing, and for a good reason: though it may seem easy to just transfer DNA microarray technology to protein microarrays, two fundamental differences separate the two fields. In DNA microarrays, construction of stable, high-affinity binding elements is simple;
a complementary DNA oligonucleotide can be used that binds with high affinity to the analyte (single stranded DNA or RNA). This led to the possibility of producing expression profiling chips early with photolithographic synthesis methods (Fodor et al., 1991) or robotic printing of DNA (Schena et al., 1995). Secondly, DNA and RNA can be copied and amplified with PCR. This makes possible the detection of minute amounts of DNA, in principle a single copy.

On the contrary, for protein microarrays, a protein amplification method is currently not available, but detection of low analyte concentrations is still desirable. Therefore, specific high affinity ligands and signal enhancing technologies are needed. The great concentration spans of proteins in complex body fluids (Anderson et al., 2002) make ligand specificity important; cross-reactivity to high-abundant proteins must be minimized or the true signals will disappear in the background noise. Where unspecificity in DNA hybridization can be adjusted with increased temperature, similar handling of proteins will result in irreversible denaturing. Capture ligands or analytes coupled to the solid support in the arrays must use suitable chemistry that allows non-destructive immobilization and proper presentation of binding sites. The ligand itself must be stable on the solid support for long periods of times to allow storage without being denatured or in other ways destroyed. Optimal surface chemistry should minimize non-specific binding to increase the signal-to-noise, and the solid support must be compatible with the detection method, for example, a commercial scanner might require transparent slides of a certain size. These aspects are summarized in figure 12.

![Figure 12](image-url)  
**Figure 12.** Examples of potential problems in microarrays. These issues are discussed further in the text.
Fluorescence-based ligand assays for protein detection using affibody affinity proteins

For sensitive detection, signal intensities per binding event can be increased, or the background noise can be reduced, since it is always signal to noise that is measured. To increase signal intensities, Schweitzer et al. (Schweitzer et al., 2002) further developed the rolling circle amplification (RCA) for microarray applications, and immobilized 75 antibodies directed against cytokines. After incubation with analytes, detection was performed using a biotinylated antibody, which in turn was detected with an anti-biotin antibody with a fused DNA tag that was used for RCA. Sensitive detection of low-abundant cytokines in secretions of dendritic cells after treatment with lipopolysaccharides or tumor necrosis factor α was possible.

To decrease background noise, Zeptosens developed a fluorescence scanner using a planar waveguide (Pawlak et al., 2002): laser light was led into a material with high refractive index (Ta₂O₅) on a glass slide, which created an evanescent field that allowed excitation of fluorophores only up to 150 nm away from the surface. A camera under the slide was used for detection of the fluorescence. The sensitivity is increased since only fluorophores in the proximity of the surface, bound to the capture molecules, are excited, thus limiting background fluorescence.

Cross-reactivity is often detected in large-scale studies of protein interactions. The use of well characterized ligands becomes important so that false positives can be avoided. To estimate cross-reactivity, Lueking et al. (Lueking et al., 2003) characterized two monoclonal mouse antibodies, anti-GAPDH and anti-HSP90β, on a chip containing ~2,400 human fusion proteins. The anti-GAPDH antibody cross-reacted to one of the proteins with similar intensities to its cognate ligand, and weakly to 1% of the 2,400 proteins; with more than 10% of the intensity of the cognate ligand. The anti-HSP90β antibody cross-reacted to two proteins with similar intensities to that of the cognate ligand and weakly to seven other proteins. In another example, Schweitzer and co-workers (Michaud et al., 2003) used a yeast protein microarray containing ~5,000 yeast proteins to screen 11 antibodies for unspecific binding and found cross-reactivity in varying degrees. These studies indicate that finding ligands with absolute specificity only for their analyte can be very difficult. One way of limiting the problems can be to divide cross-reactive reagents into separate pools or arrays, for instance, in the previously mentioned RCA application for microarrays, Schweitzer et al. (Schweitzer et al., 2002) used two separate subarrays containing 37-38 antibodies each and two pools of secondary antibodies to avoid cross-reactivity and non-specific signals.
Less non-specific binding leads to lower background noise and the possibility of detecting lower concentrations of analyte. This has led to an interest in the creation of new surfaces, often hydrophilic, and the use of protein-repelling polymers, such as PEG (Ruiz-Taylor et al., 2001) or dextran (Löfås et al., 1990) Also, 3-D surfaces allowing immobilization of a higher density of ligands in a defined surface area (by using the z-dimension) will lead to more bound analytes and potentially higher better signal-to-noise. For two reviews on solid supports and immobilisation, see Kusnezow et al 2003 (Kusnezow et al., 2003) and Tomizaki et al 2005 (Tomizaki et al., 2005). Blocking the surface after spotting and/or using blocking molecules with the analyte can also decrease the background noise, since the blocking molecules coat the solid surface and prevent non-specific interactions. Surfactant molecules like Tween 20 (Bird et al., 1988) and Pluronic F108 (BASF Corporation, for instance used by Nord et al in (Nord et al., 2003)) can be used, or proteinaceous blocking agents like casein, Superblock (Pierce) and Topblock (Sigma-Aldrich).

**Protein immobilization**

Protein immobilization to solid supports is not problem-free. Immobilization can lead to the destruction of the binding epitope on the immobilized protein, and buried hydrophobic residues needed for the stability of the protein can make contact with the solid surface, leading to denaturation of the protein. For example, Haab et al printed 115 antigen/antibody pairs (either antibody or antigen) and probed them in a competitive fashion with a Cy3-labeled standard protein mixture and six Cy5-labeled protein mixtures, of antibodies or antigen, depending on which was spotted onto the microarray. Interestingly, only 20% and 50% of the spotted antibodies and antigen provided accurate antigen or antibody concentration determination, respectively (Haab et al., 2001).

Random and specific, covalent and non-covalent methods can be used for protein immobilization, as illustrated in figure 13. Though intuitively specific immobilization may seem optimal for all array performance, the many factors influencing proteins on solid supports can lead to similar results for random and specific immobilization (Kusnezow et al., 2003)).
Figure 13. Random immobilization can result in a heterogeneous surface where some ligands are unavailable for capture, in contrast to directed immobilization where ligands can be oriented for optimal binding. Ligands can be attached with chemical bonds (covalent immobilization), or non-covalently with affinity tags (affinity) or by adsorption.

**Covalent immobilization**

Proteins can be immobilized via their lysine side chains or N-terminal amino groups via NHS-esters, isothiocyanates, epoxy groups or other amine-reactive reagents. The result is a population of covalently and randomly immobilized proteins. The hinge region of antibodies contains disulfides, which can be reduced to thiols and used for surface immobilization (Vijayendran et al., 2001), for example by coupling to maleimides or haloacetyl groups. The carbohydrate groups on the Fc-region of antibodies can also be used for covalent, directed coupling. The
carbohydrates can be oxidized to aldehydes and be coupled to solid supports containing for example hydrazides (Hoffman et al., 1988) or amino groups (Weiping et al., 1999) For proteins naturally lacking cysteine residues, these amino acids can be introduced at a genetic level and be used for specific immobilization, if the ligand can be recombinantly produced. If chemical synthesis of the protein is possible, greater flexibility can be achieved as site-specifically incorporated cysteines and aldehydes (for coupling to, for instance, hydroxylamine), can be used, among other methods.

**Non-covalent immobilization**

Proteins can be adsorbed to surfaces via hydrophobic and electrostatic interactions, van der Waals and hydrogen bonds, that retain the protein on the solid support. Since hydrophobic interactions often involve exposing buried residues needed for stability, parts of or the entire protein might be damaged by this immobilization technique (Butler, 2000; Butler et al., 1993; Metzger et al., 2002). Further, the proteins are immobilized in a random fashion, creating a heterogeneous protein population; binding sites used for protein interactions can be inaccessible or destroyed, which results in less binding activity.

Proteins can also be immobilized using non-covalent, specific interactions. Often a protein is attached to the surface and is bound by the ligand (or analyte) resulting in non-covalent immobilization. Butler et al (Butler et al., 1993), compared adsorption, biotin-avidin immobilization, and anti-globulin immobilization of monoclonal and polyclonal antibodies and found a 5-fold increase in performance for monoclonal antibodies using biotin-streptavidin immobilization compared to adsorption. While the anti-globulin immobilization resulted in a high fraction of functional antibodies, the lower density of antibodies gave results similar to those for adsorption. Streptavidin-biotin attachment has also been utilized by Peluso et al for the random and oriented attachment of antibodies or Fabs (Peluso et al., 2003). Specific immobilization of Fabs compared to random immobilization resulted in up to 10 times better performance. Recombinantly produced proteins can be immobilized by virtue of genetically introduced tags; for instance, Zhu et al (Zhu et al., 2001) used His-tagged yeast proteins that were coupled to a nickel-coated surface.
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To avoid the time-consuming protein production and purification steps, Ramachandran et al. (Ramachandran et al., 2004) integrated protein production into the microarray by immobilizing biotinylated plasmid DNA together with anti-GST antibodies onto avidin slides. This allowed cell-free production from the plasmids of human proteins with GST fusions, which were subsequently captured by the anti-GST antibodies, producing an array of proteins that could be used for interaction studies.

**Kinetics in solid phase assays**

Reaction kinetics are different in solid/solution phase interfaces than in solution phase assays, and mass-transport issues can limit assay sensitivity. In assays relying on depletion of the analyte for detection, most analytes will have to cross large distances to reach the solid support (Butler, 2000), which will be time-consuming. Therefore, in theory, smaller microspots can be used for capture, to allow detection to be performed under ambient analyte conditions, where analytes in solution are insignificantly consumed (Ekins et al., 1989), and depletion effects are less pronounced. In reality, most solid-phase based assays will be diffusion-limited when analyte concentration becomes lower (Kusnezow et al., 2006), leading to longer incubation times required for enhanced array performance (Saviranta et al., 2004). Agitation or stirring the solution is often used to decrease these issues (Butler, 2000), but will not completely abolish them, due to the immobility of the solution layer in contact with the surface.

On the other hand, dissociation rates may be down to two orders lower on solid surfaces than in solution, an effect that may be caused by the relatively high concentration of capture ligands on the solid surface that facilitate faster reassociation, the formation of aggregates (Butler, 2000), and the formation of secondary bonds after initial binding (van Oss et al., 1986). This decrease in dissociation rate will increase the apparent affinity and lower the detection limit. It also allows washing steps, where solution phase kinetics would predict a massive loss in signal.

**Protein microarray applications**

Though protein microarrays are hampered with difficulties and have not yet reached their full potential, many impressive studies have already been published. Some examples will be
described for protein expression, interaction, and enzymatic studies, to illustrate some of the different application areas for protein microarray technology.

**Protein expression analysis**

Two-color expression analysis, similar to gene expression analysis using DNA microarrays, but on directly labeled protein mixtures, has been used to compare relative protein concentrations. For instance, Sreekumar *et al.* (Sreekumar *et al.*, 2001) printed 146 antibodies and monitored protein levels with the Cy3 and Cy5 fluorophores after ionization treatment of LoVo colon carcinoma cells. P53, death receptor 5 and other apoptotic regulators were found to be up-regulated after ionization. Also using direct labeling of protein mixtures, Wingren *et al.* (Wingren *et al.*, 2005) used scFvs as capture ligands to detect human complement factors in human serum down to pM concentrations. His tags on the scFvs allowed the scFvs to be immobilized onto nickel-coated slides without prior purification.

Using sandwich detection with antibodies as capture ligands and secondary detection agents, Gao *et al.* (Gao *et al.*, 2005) constructed an array of 84 antibodies directed against serum proteins and subjected these to sera of healthy patients, newly lung cancer diagnosed patients, and patients with chronic obstructive pulmonary disease. Detection was performed with secondary antibodies and rolling circle amplification. C-reactive protein, amyloid A, and other proteins were found to be up-regulated in the cancer sera. 15 of 24 patients with lung cancer could be diagnosed with the method.

Showing that reversed arrays, with antigen spotted instead of a ligand, can be used for expression analysis, Robinson *et al.* (Robinson *et al.*, 2002) spotted 196 different autoantigens and probed them with patient sera. No signals were obtained for healthy individuals, but disease sera from patients with Sjögren’s disease or diffuse or limited scleroderma showed specific binding to autoantigens CENP B, topo1 and La. Janzi *et al.* (Janzi *et al.*, 2005) spotted an array consisting of ~2,000 serum samples from normal and IgA-deficient individuals and probed them with an anti-IgA antibody that was detected with a fluorescently labeled secondary antibody. The results were in agreement with nephelometry results, suggesting that reversed arrays of printed sera could be used for parallel screens of large patient populations.
**Interaction studies**

Protein microarrays have also been used for protein-protein interaction studies. For example, Zhu et al (Zhu et al., 2001) published an ambitious study where 5,800 expressed yeast open reading frames were printed onto nickel-coated glass slides. The slide was probed with biotinylated calmodulin, and subsequently detected with Cy3-streptavidin. Six proteins known to interact with calmodulin as well as 33 new potential proteins were detected. The slides were further probed with phosphoinositide-liposomes and 150 proteins showed signals higher than background, of which 98 were known proteins, including 45 membrane-associated proteins. Further, Poetz et al (Poetz et al., 2005) used microarrays to rapidly screen Fab fragments to assess specificity and affinity. Antigens and a quantifier, to normalize the antigen signal for concentration, were spotted in microarrays and 62 different Fab fragments in crude bacterial lysates were screened. Dissociation constants for all fragments were determined with Biacore and showed a good correlation to the affinity ranking by the microarrays.

Although protein microarrays are often used for protein-protein interaction they are not limited to this type of interaction. For instance, Boutell et al (Boutell et al., 2004) printed p53 mutants on dextran slides, and evaluated their binding ability of labeled DNA. It was found that mutations in the DNA-binding domain severely limited the DNA binding.

**Enzyme studies**

Peptide microarrays are suitable for enzymatic studies, for instance, to investigate substrate specificities of kinases and proteases. For example, Zhu et al (Zhu et al., 2000) constructed a microarray consisting of 17 peptide substrates that were used to investigate the substrate specificity of 119 yeast kinases through the incorporation of radiolabeled $^{33}$Pγ-ATP. 33 kinases were found to recognize one or two substrates, and 27 were found to recognize poly(Tyr-Glu). In another example, Houseman et al (Houseman et al., 2002) investigated C-Src kinase specificity with a peptide library using SPR, fluorescence and phosphorimaging. For the phosphorylation of one substrate, the effect of three enzyme inhibitors was also evaluated. For proteases, Salisbury et al (Salisbury et al., 2002) demonstrated that a proteolytic fingerprint could be obtained for thrombin with the use of a peptidyl coumarin library with different peptide motifs, which upon proteolytic cleavage showed an increase in fluorescence.
Peptide microarrays are easy to produce using robotic spotting of recombinant and synthetic peptides, which allows site-specific incorporation of immobilization groups and fluorescent labels. In a publication by Pellois et al (Pellois et al., 2002), the parallel in situ synthesis of a peptide microarray, containing natural and synthetic amino acid analogues, was possible using digital photolithography and an optical computer-controlled module. The technique was used to produce peptide microarrays to characterize the antigen epitope of a p53 binding antibody, but could also be used for other peptide microarray applications.
Fluorescence-based ligand assays for protein detection using affibody affinity proteins
Present investigation

The work in this thesis has been focused on the development of different protein detection assays, using affibody molecules as ligands and fluorophores as reporter labels. Various aspects such as ligand affinity, production, labeling, immobilization and domain organization have been investigated in solution phase assays (I, II) or heterogeneous solid phase assays (III, IV).
Fluorescence-based ligand assays for protein detection using affibody affinity proteins

**FRET-based detection using anti-idiotypic affibody pairs (I)**

Homogeneous assay formats that require no washing steps are often desired for rapid diagnostics. In paper I, a solution-based homogeneous assay, that allowed detection of unlabeled target protein only a few minutes after sample addition, was designed and evaluated.

**Principle of the detection system**

Fluorophore-labeled anti-idiotypic affibody pairs (Eklund et al., 2002) were used for protein detection utilising FRET. The anti-idiotypic affibody pairs consist of a target-binding idiotypic affibody and an anti-idiotypic affibody, binding the specificity-determining region of the idiotypic affibody. In the absence of target, the idiotype is bound to the anti-idiotype. In the presence of target, the target and the anti-idiotype will compete for binding to the idiotype, and a fraction of the idiotype will bind the target instead of the anti-idiotype (see figure 14). The target-bound fraction depends on the target concentration, and follows the principle of the three-partite formula previously described.

With the covalent attachment of a donor, 5-iodacetamidofluorescein (IAF), to the idiotype, and an acceptor, tetramethylrhodamine-5-iodoacetamide (TMRIA), to the anti-idiotype, it was speculated that binding of a target could be monitored through a change in FRET if the donor was excited. The excited donor would transfer energy to the acceptor only when the two fluorophores were close in space; in the absence of target. Then, donor (IAF) fluorescence would be low and acceptor (TMRIA) fluorescence high. In the presence of target, donor (IAF) fluorescence would increase and acceptor fluorescence decrease.
Figure 14. Schematic picture showing the principle of the detection assay. In the presence of target protein, the antiidiotypic affibody complexes are dissociated and the efficiency of FRET decreases. $K_d^{\text{AI}}$ is the dissociation constant for the idiotypic affibody (I) binding to the antiidiotypic affibody (AI) and $K_d^{\text{T}}$ is the dissociation constants for the idiotypic affibody binding to the target protein (T). (Taken from Figure 1 in paper I).

**Results**

To test this theory, two anti-idiotypic affibody protein pairs, $Z_{\text{Taq}}$/anti-$Z_{\text{Taq}}$ and $Z_{\text{IgA}}$/anti-$Z_{\text{IgA}}$, were used for the detection of Taq DNA polymerase and human IgA, respectively. A cysteine residue was incorporated between the first and second amino acid in all affibody molecules using site-directed mutagenesis. The cysteine residues were used for thiol-selective labeling of the idiotypes, $Z_{\text{IgA}}^\text{Cys}$ and $Z_{\text{Taq}}^\text{Cys}$, with IAF, and the anti-idiotypes, anti-$Z_{\text{IgA}}^\text{Cys}$ and anti-$Z_{\text{Taq}}^\text{Cys}$, with TMRIA.

$Z_{\text{IgA}}$(IAF)/anti-$Z_{\text{IgA}}$(TMRIA) were titrated with human IgA and IgG in buffer and a concentration-dependent response could be observed for incubation with IgA, but not for the control protein IgG, as an increase in IAF emission and a decrease in TMRIA emission. The emission ratio of 515 nm / 573 nm, corresponding to the emission maxima of IAF and TMRIA, respectively, is shown in figure 15. The dynamic range of the IgA measurement was about two orders of magnitude, and the limit of detection of 500 nM would be sufficient for detection of IgA in normal human plasma levels (Janzi *et al.*, 2005).
Furthermore, the affibody ligand combination $Z_{taq}$ Cys(IAF)/anti-$Z_{taq}$ Cys(TMRIA) was used to test if a system with lower dissociation constant ($K_d$) for the binding of the analyte protein would give a lower limit of detection. The $K_d$s for the idiotypic pairs were similar: 0.9 $\mu$M for $Z_{igA}$/anti-$Z_{igA}$ and 0.7 $\mu$M for $Z_{taq}$/anti-$Z_{taq}$, but the $K_d$ for $Z_{taq}$ binding to $Taq$ DNA polymerase was 25 nM compared to the $K_d$ of 0.5 $\mu$M for $Z_{igA}$ binding to IgA; i.e. a 20-fold lower. Titrations of $Taq$ DNA polymerase and an IgG control, showed a concentration-dependent response for $Taq$ DNA polymerase but not for IgG, and the limit of detection was improved from 500 nM for IgA to 50 nM for $Taq$ DNA polymerase (see figure 15).

Figure 15. Relative fluorescence emission ratio of donor/acceptor (515/573 nm) for titration of the fluorescent-labeled anti-idiotypic affibody complexes with increasing concentration of target (—) and control protein (- - -). (A) Titration of $Z_{igA}$(IAF)/anti-$Z_{igA}$(TMRIA) with IgA (—) and IgG (- - -). (B) Titration of $Z_{taq}$(IAF)/anti-$Z_{taq}$(TMRIA) with $Taq$ DNA (—) polymerase and IgG (- - -). (C) Titration of $Z_{taq}$(IAF)/anti-$Z_{taq}$(TMRIA) in 25% human plasma with $Taq$ DNA polymerase (—). (Taken from Figure 3 in paper I).

To further challenge the system, the same titrations of $Taq$ DNA polymerase were performed in a background of 25% human plasma. Interestingly, the response curve was very similar to that obtained from titrations in buffer. Also interesting was the fact that the affibody molecules were produced as fusion partners to an albumin-binding domain to facilitate purification. This domain is likely bound to the albumin, which is present at high concentrations in the plasma,
but the complex presumably formed between the albumin binding domain and albumin did not interfere with the detection system.

**Theoretical aspects of the detection system**

The three-partite system governing the relation between idiotype, anti-idiotype and target was used to simulate how different concentrations and dissociation constants would influence the amount of complex formed and the assay sensitivity. Some fundamental principles could be deduced: i) The concentration of the anti-idiotypic pair must be in the same range as the concentration of the analyte one wishes to study. ii) The dynamic range of the assay can be shifted using higher or lower concentrations of the anti-idiotypic pairs. iii) The lowest feasible concentration of the anti-idiotypic pair is decided by the dissociation constant of the anti-idiotype binding to the idiotype. At low concentrations relative to the $K_d$, the anti-idiotypic pair will be significantly dissociated and present in only a very low fraction of complexes, and the sensitivity of the assay will decrease, since the change in fluorescence will be too small to be measurable compared to the background fluorescence contributed by the unbound anti-idiotypic pair members. Thus, the dissociation constant of the idiotype binding to the anti-idiotype limits assay sensitivity. iv) The dissociation constant of the idiotype to its target limits the assay sensitivity. Since the anti-idiotype and the target are competing for the idiotype, the dissociation constant for the binding of the idiotype to its target must be sufficiently low for the target to efficiently compete with the anti-idiotype. v) Even if the dissociation constant of the idiotype-target is many orders of magnitude lower than that of the anti-idiotypic pair, limit of detection will not be decreased correspondingly, see i) and iii). At low concentrations of analyte, even if all analytes are bound, the concentration of the anti-idiotypic pair will be too high in relation to the analyte concentration to create a measurable change in FRET. vi) Practically, the assay will also be limited by the sensitivity of the fluorescence spectrometer since a certain number of fluorophores are needed for fluorescence to be detected at all.

**Further development**

The limit of detection of an assay using an idiotype with high affinity and an anti-idiotype with a lower affinity might be decreased by physically linking the two ligands with a linker of suitable
length. Then, the local concentration of the anti-idiotypic pair will increase, which should result in an intramolecular complex formation even at concentrations far below the $K_d$ of the anti-idiotypic pair. When the idiotypic affibody has a high affinity for its target protein, the target can still be bound and complexes be dissociated, and detection of the binding will thus be possible. This allows the anti-idiotypic complex concentration to be matched to the target concentration, and the fractional change upon binding becomes measurable. Silverman et al used a related approach by fusing two binders against different epitopes on the same antigen with a linker, so that apparent affinity was increased from the increase in local concentration (Silverman et al., 2005).

**Triple-labeled, synthetic affibody molecules for specific protein detection (II)**

Non-competitive assays are generally better than competitive assays at detecting very small amounts of analytes, as previously discussed (see background). Therefore, a non-competitive, label-free, and homogeneous assay based on chemically synthesized affibody molecules was considered, that also utilizes FRET as a reporting principle for quick detection of target.

Earlier, Karlström et al (Karlström et al., 2001) constructed an assay based on the Ig-binding B domain of Staphylococcal protein A, dually labeled with two environment-sensitive fluorophores; 5-(2-aminethylamino)-1-naphthalenesulfonic acid (EDANS) and 6-(7-nitrobenzofurazan-4-ylamino)-hexanoic acid (NBDX), constituting a FRET pair, for the concentration-dependent detection of the Fc fragment of IgG. However, the labeling reaction of recombinant protein in solution was not completely specific and a heterogeneous labeling mixture was produced that was subsequently purified with HPLC. In a follow-up study, affibody molecules directed against other targets were subjected to the same labeling procedures, however, the difficulty in attaining homogeneous products led to the insight that alternative synthesis routes would be preferable.

**Principle of the detection system (II)**

The fluorophores EDANS and NBDX, constituting a FRET pair, were incorporated on opposite sides of the binding surfaces of the IgG-binding $Z$ domain and the IgA-binding $Z_{\text{IgA}}$ affibody,
respectively. It was theoretized that upon target binding, fluorophore displacement or changes in the local environment would induce a detectable change in the relative emission of the two fluorophores, and in this way, target detection and quantification would be possible. Ligands would be constructed with chemical synthesis to allow site-specific incorporation of the fluorophores instead of the previously used production route based on labelling of genetically engineered proteins produced by recombinant means.

**Chemical synthesis of triple-labeled affinity ligands**

Fmoc solid phase peptide synthesis was used to synthesize the full-length, 58 amino acid, Z domain, binding IgG, and the affibody molecule $Z_{IgA}$, directed against human IgA. First, the Z domain was synthesized, and an unexpected -18 Da mass shift was observed when the product was analyzed by MS. Therefore, the synthesis product was subjected to trypsin treatment and the digest peptides were analyzed by MS/MS. The side reaction could be mapped to the Asp-Asn residues in positions two and three, which presumably had undergone aspartimide formation. The Asp in position two was therefore replaced with Glu in following syntheses, and no further dehydration side products were observed. Next, after the synthesis of $Z_{IgA}$, many truncated peptides were identified with MS. It seemed that where trityl-protected amino acids were involved, incomplete acylation was often a result. Therefore, a new coupling scheme involving double couplings of selected amino acids were used, which minimized side products, and a yield of 20-30%, corresponding to an average coupling efficiency of 97-98%, was obtained. This was sufficient to allow further construction of the biosensors. Two orthogonal protecting groups, Alloc and Mtt, introduced as side chain protecting groups during synthesis, could be specifically removed to allow the introduction of fluorophores and an immobilization tag (biotin). In short, this was performed with the removal of the Fmoc group at Val$^{1}$ and the subsequent coupling of NBDX. The Alloc group at Lys$^{58}$ was removed with Pd$^{0}$-catalysed allyl transfer, and biotin was coupled. Last, the Mtt group at Lys$^{23}$ was cleaved with dilute acid treatment (1% TFA) and EDANS was coupled. This was followed by HPLC purification and characterization of the synthesized product.
Fluorescence-based ligand assays for protein detection using affibody affinity proteins

**Characterization of the synthetic, labeled affinity ligands**

The synthetic double-labeled $Z^{\text{NBDX, EDANS}}$ and $Z_{\text{IgA}}^{\text{NBDX, EDANS}}$ proteins were compared to recombinantly produced His-tagged Z and $Z_{\text{IgA}}$ proteins in circular dichroism (CD) studies and in biosensor studies based on surface plasmon resonance (SPR) (Biacore). In the CD studies, the helicity of the synthetic proteins were found to be similar to that of the recombinant proteins, as in the biosensor studies, the dissociation constants of the recombinant and the labeled synthetic affibody molecules were similar for binding to their respective targets. Taken together, this suggested that the synthetic proteins retained their native-like fold and affinity after synthesis and labeling.

**Protein detection**

Fluorescence spectroscopy was carried out using excitation at 337 nm, and emission spectra for EDANS and NBDX were collected. The $Z^{\text{NBDX, EDANS}}$ and $Z_{\text{IgA}}^{\text{NBDX, EDANS}}$ proteins were titrated with IgA and IgG, and the ratio of the emission peaks for EDANS (480 nm) and NBDX (520 nm) was calculated and plotted (see figure 16). Both the $Z^{\text{NBDX, EDANS}}$ and $Z_{\text{IgA}}^{\text{NBDX, EDANS}}$ proteins showed a target-specific response, and a limit of detection of 100 nM.

Figure 16. Relative fluorescence emission ratio of donor/acceptor (480/520 nm) for titration of the dually fluorophore-labeled synthetic protein with increasing concentration of target (—) and control protein (– - -). (A) Titration of $Z^{\text{NBDX, EDANS}}$ with with IgG (—) and IgA (– - -). (B) Titration of $Z_{\text{IgA}}^{\text{NBDX, EDANS}}$ with IgA (—) and IgG (– - -). (Taken from Figure 4 in paper II).
The dually fluorophore-labeled $Z^{\text{NBDX, EDANS}}$ protein was also labeled with a biotin tag at Lys$^{58}$. The triple-labeled protein was immobilized via the biotin moiety onto a streptavidin biosensor surface and was subjected to 50 nM concentrations of IgG and IgA. Binding ability was retained and an IgG-specific response was observed.

**Theoretical aspects of the assay**

The assay described does not rely on competition for detection as the previously described assay (paper I). Still, the detection limit is only about 100 nM, even higher than the dissociation constant for the binding between Z and IgG (20 nM). The reason is the background fluorescence of the fluorophores in unoccupied biosensor molecules that will make small changes in FRET undetectable, and the relatively high concentration of labeled biosensor proteins used that makes the assay insensitive to low concentrations of analytes. However, lowering the concentration of labeled proteins produced signals too weak to be measured by the fluorescence spectrometer, and therefore the assay was limited by the insensitive equipment. However, even if more sensitive equipment is used, at concentrations of the biosensor proteins below $K_d$, only a fraction of the labeled biosensor will be bound. The background fluorescence of unoccupied biosensors will thus limit assay sensitivity.

**Further development**

Due to its small size of only 58 amino acids, solid phase peptide synthesis is available for the production of affibody molecules, as shown in this study. This is in contrast to antibody-based scaffolds, even the small scFvs of ~ 250 amino acids, which are too big to allow this production route. Chemical synthesis of affibody molecules allows specific incorporation of natural and unnatural amino acids, fluorophores, linkers, immobilization tags and other organic building blocks during or after synthesis. About three to four days are needed for the complete synthesis of an affibody molecule, not including purification and characterization of the synthesis product. Synthesis yields are relatively high, and amino acids can be substituted during synthesis without the need for cumbersome cloning work. For faster synthesis of affibody ligands, the third helix of the affibody ligand scaffold can be pre-made in a large batch since it is constant for all affibody molecules, and be used as starting material for synthesis of the two helices containing the specificity-determining positions.
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The introduction of the biotin moiety in this paper, and the subsequent attachment of the biosensor to a streptavidin coated surface, indicated that the affibody could be used for microarray-based detection on a streptavidin-coated slide, or on other surfaces using other immobilization tags and coupling chemistries.

Affibody ligand microarrays (III)

The stability of affibody molecules to strong bases (Nord et al., 2000), strong acids (paper II), lyophilization (paper I and II) and rapid refolding characteristics of the related B-domain (Arora et al., 2004) as well as the high-yield recombinant production and flexibility of chemical synthesis, make affibody molecules attractive as capture ligands in protein microarrays. Previous work on surface immobilization of antibodies and antibody fragments by Peluso et al (Peluso et al., 2003) and Vijayendran et al (Vijayendran et al., 2001), suggested that capture ligands based on antibodies were best immobilized by directed coupling with their binding epitope oriented away from the surface, compared to random attachment that creates a heterogeneous population of binding ligands. In the first work on affibody molecules as capture ligands in protein microarrays, which is the basis for paper III, random and specific immobilization techniques for chemically synthesized affibody molecules with different handles were investigated, to see if specific immobilization would give better surface activity also for affibody molecules.

Synthesis of affibody molecules with immobilization handles

Affibody molecules were synthesized with standard Fmoc chemistry using an automated peptide synthesizer. An orthogonal Mtt protecting group introduced as protection for the side chain of lysine in position 58 was used to facilitate the site-specific incorporation of cysteine and biotin, that would act as alternative selective handles for directed immobilization. The two affibody molecules $Z_{Taq}$ and $Z_{IgA}$, directed against Taq DNA polymerase and IgA, were used to evaluate the immobilization concepts. For $Z_{Taq}$, five versions were created: $Z_{Taq}^{Cys}$, with cysteine coupled to the Lys$^{58}$ side chain amino group; $Z_{Taq}^{Bio}$, with biotin coupled to Lys$^{58}$; $Z_{Taq}^{AhxCys}$, with aminohexanoic acid (Ahx), a six carbon linker, incorporated between the Lys$^{58}$ side chain amino group and cysteine; $Z_{Taq}^{AhxBio}$, with the Ahx linker and biotin; and unmodified $Z_{Taq}$ for random amine coupling. Due to the small size of the affibody scaffold, it was speculated that the affibody...
ligands might benefit from a linker that gives them rotational freedom and that place them out from the surface for easier capture of the target. The Ahx-linker was therefore introduced to act as this spacer between the surface and the affibody. For $Z_{IgA}$, three versions were created: unmodified $Z_{IgA}$, $Z_{IgA}^{Cys}$, with cysteine, and $Z_{IgA}^{Biotin}$, with biotin.

**Evaluation of random and directed immobilization**

The target binding activity of the different variants was evaluated on biosensor surfaces by surface plasmon resonance (SPR) (Biacore), and on microarray slides with fluorescently labeled IgA and *Taq* DNA polymerase. For the Biacore experiments, low amounts of affibody were immobilized onto carboxymethyl dextran via amine coupling (random immobilization) and thiol coupling (for directed cysteine immobilization), or on streptavidin-dextran (for directed biotin immobilization) and probed with *Taq* DNA polymerase. After subtraction of response values corresponding to buffer effects and unspecific binding, the response for each construct was normalized to the amount of immobilized affibody. The results are shown in figure 17. A directed immobilization via a cysteine residue produced the highest responses. The introduced spacer improved binding ability for both the cysteine and biotin versions. For the biotin versions, the signal was more than doubled at low concentrations of target when a linker was used between the affibody and biotin. One can speculate that the affibody is constrained by the binding to the streptavidin, and that an even longer spacer could be used to increase binding further. The biotinylated affibody without a linker produced the poorest response of all affibody variants.

![Figure 17. Column chart showing the specific activity of five different affibody variants when subjected to three concentrations of *Taq* DNA polymerase (100, 410, and 1000 nM) in the Biacore biosensor analysis. $Z_{Taq}$ was immobilized by amine coupling to CMD slides, $Z_{Taq}^{Cys}$ and $Z_{Taq}^{AhxCys}$ were immobilized by thiol coupling to TD slides and $Z_{Taq}^{Biotin}$ and $Z_{Taq}^{Ahxbiotin}$ were immobilized onto streptavidin-coated slides. (Taken from figure 2 in paper III).](image-url)
Dextran-coated slides were also used for the protein microarrays, to allow an as equal comparison as possible. The $Z_{\text{Taq}}$ and $Z_{\text{IgA}}$ affibody variants were spotted onto carboxymethyl dextran slides (unmodified variants) and thiol dextran slides (cysteine variants) with a contact arrayer in triplicates in 16-40 arrays on each slide. The biotin variants were immobilized on avidin-coated dextran slides, but these slides did not produce any signals, and streptavidin-coated slides without a dextran layer were therefore used instead for the biotin variants. Arrays of affibody molecules were separated with a silicone mask and titrated with Cy3-labeled target proteins in buffer, seen in figure 18. The affibody molecules coupled by amine and thiol coupling showed similar target intensities and limits of detection; 3 pM and 10 pM for Cy3-IgA on amine and thiol slides, respectively, and 90 pM and 30 pM for Cy3-\text{Taq} DNA polymerase on amine and thiol slides, respectively. The biotin-immobilized affibodies showed lower signal intensities and higher limits of detection. For both cysteine and biotin immobilization, the linker increased the signal intensity, indicating that more target analytes were bound.
Figure 18. Relative fluorescence of $Z_{Taq}$ and $Z_{IgA}$ spots on microarray slides, incubated with increasing concentrations of Cy3-labeled human IgA (A–C) or Cy3-labeled Taq DNA polymerase (D–F). An inset graph in each panel shows the signal at the concentration calculated as the limit of detection. (A,D) $Z_{Taq}$ (– - -) and $Z_{IgA}$ (—) immobilized by amine coupling to CMD slides. (B,E) $Z_{Taq}$ Cys (– - -), $Z_{Taq}$ AhxCys (– –), and $Z_{IgA}$ Cys (—) immobilized by thiol coupling to TD slides. (C,F) $Z_{Taq}$ biotin (– - -), $Z_{Taq}$ Ahxbiotin (– –), and $Z_{Ig}$ Abiotin (—) immobilized onto streptavidin slides. (Taken from figure 4 in paper III).

**Theoretical considerations**

Using chemical synthesis, site-specific incorporation of affinity handles for immobilization onto activated thiol dextran and streptavidin surfaces was possible. The great flexibility in chemical synthesis also allowed the introduction of a spacer moiety between the affibody molecule and
the affinity tag, which increased binding activity for both biotin and cysteine variants, in the subsequent evaluation of the biosensor and the protein microarray platforms.

In the biosensor studies, the specific binding activity against target proteins could be determined by normalizing the responses against the amount of immobilized affibody molecules. On this platform, using specific thiol coupling to create a homogeneous surface of oriented affibody molecules was found to give the best results, compared to both specific immobilization with biotin and random immobilization using amine coupling.

For the protein microarrays, the results were largely in agreement with the biosensor results. The spacer increased the amount of bound fluorescently labeled target protein for both cysteine and biotin variants, and both thiol and amine coupling showed better performance than the biotin-immobilized constructs. However, the affibody molecules immobilized by amine coupling and thiol coupling on the microarray slides were able to bind similar amounts of target, in contrast to the biosensor results where the thiol coupling was significantly better. A possible reason for this could be that the surfaces of the biosensor chips and the microarray thiol dextran slides were different. On the biosensor surfaces, carboxymethylated dextran that was further modified with thiol reactive groups prior to coupling was used, whereas coupling to the microarray slides was performed via thiol groups directly attached to the dextran layer. It is also possible that a different amount of reactive groups and more unspecific binding to the thiol dextran slides may have led to the similar results for the amine and thiol coupled affibody molecules in the microarray experiments.

Interestingly, the limit of detection for IgA was lower than for Taq DNA polymerase although the affibody binding the later has a lower dissociation constant; 25 nM for Z\textsubscript{Taq} compared to 0.5 µM for Z\textsubscript{IgA}. The reason is likely an avidity effect caused by two identical epitopes being present on the IgA molecules that allow the simultaneous binding of two Z\textsubscript{IgA} ligands.
**Multidomain affibody microarrays (IV)**

In this paper, the presentation format for affibody molecules as capture ligands was further investigated. Recombinantly produced multimeric proteins, consisting of up to four identical N- to C-terminally fused affibody molecules, were spotted and evaluated for their binding ability to fluorescently labeled target or unlabeled target in a sandwich format, with antibodies as secondary detecting agents.

**Multidomain constructs**

Two different affibody molecules directed against insulin, Z\textsubscript{Ins}, and IgA, Z\textsubscript{IgA}, produced as multimeric constructs of 1-4 (Z\textsubscript{Ins}) and 1-3 (Z\textsubscript{IgA}) domains, were immobilized with amine coupling or directed immobilization via a C-terminal cysteine onto carboxymethyl or thiol dextran slides in triplicates in 16 arrays/slide. Arrays were separated with a silicone mask, and after incubation with fluorescently labeled target in buffer, signal intensities corresponding to the amount of captured target protein were compared. Amine coupling on carboxymethyl dextran slides or directed immobilization with the C-terminal cysteine on thiol dextran slides produced similar results (see figure 19 for thiol dextran target titrations). For both the Z\textsubscript{Ins} and Z\textsubscript{IgA} ligands, a gradual increase in intensity was observed when going from monomeric to trimeric constructs, with the most dramatic effect when going from monomeric to dimeric ligands. Use of the trimeric and tetrameric Z\textsubscript{Ins} constructs however, gave similar results. The increased capture ability for multimeric constructs was likely due to an increased local concentration of capture domains, facilitating rebinding, as well as a spacer effect similar to that which was observed for the synthetic affibody constructs with the Ahx spacer (in paper III). The avidity effect of multiepitope targets bound to multidomain affibody constructs, such as IgA (discussed in paper III) to Z\textsubscript{IgA}\textsubscript{2-4}, is likely also a cause for the increased binding of targets to the multidomain constructs. To allow a fair comparison between presentation formats it was important that the number of domains was equal in all spots. A general protein stain and a secondary antibody binding the affibody molecules were used to verify this.
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Figure 19. Relative fluorescence intensities after incubation with dilution series of Cy3-labeled human IgA (100 pg/ml – 100 ng/ml) and insulin (100 pg/ml – 100 ng/ml) over multivalent affibody molecules, containing one to four domains. The mean of three dilution series on triplicate spots on three slides is plotted with error bars of one standard deviation. (Taken from figure 3 in paper IV).

Dimeric affibody ligand microarrays

Six dimeric affibody molecules directed against IgA, IgG, IgE, insulin, Taq DNA polymerase and TNF-α, were immobilized onto thiol dextran slides via their C-terminal cysteines to evaluate specificity and sensitivity. The dimeric constructs were used since the greatest change in intensity had been observed when going from monomeric to dimeric constructs. Arrays containing the six affibody dimers were subjected to titrations of Cy3-labeled target in buffer (see figure 20). No cross-reactivity was observed for any of the affibody molecules, and limits of detection were good: 10 pg/ml (70 fM) for IgG, 100 pg/ml (600 fM) for IgA, 3.3 ng/ml (20 pM) for IgE, 330 pg/ml (60 pM) for insulin, 1 ng/ml (10 pM) for Taq DNA polymerase and 330 pg/ml (20 pM) for TNF-α (Table 1). The limits of detection were thus improved for IgA and Taq DNA polymerase using the dimers compared to the results from the microarrays with the synthetic monomers in paper III.
Figure 20. Relative fluorescence intensities after incubation with dilution series of Cy3-labeled human IgA (33 pg/ml – 10 ng/ml), TNF-α (100 pg/ml – 10 ng/ml), IgG (3.3 pg/ml – 1 ng/ml), insulin (100 pg/ml – 10 ng/ml), IgE (330 pg/ml – 100 ng/ml), and Taq DNA polymerase (100 pg/ml – 33 ng/ml) over dimeric affibody molecules. The mean of two dilution series on triplicate spots on two slides is plotted with error bars of one standard deviation. (Taken from figure 4 in paper IV).

**Sandwich detection**

Comparison of unlabeled and fluorescently labeled insulin and IgA (optimised for microarray detection) by biosensor (Biacore) experiments showed that only 20% and 40%, respectively, of their potencies after labeling were retained, indicating that labeling procedure had damaged the proteins. By using a secondary detecting agent, labeling is circumvented and extra specificity and sensitivity is added, as two recognition events are needed for detection and the signal can be
amplified with more fluorescently bound secondary detecting agents. The disadvantage is that a suitable secondary binder is not always available. Here, the two dimeric affibody molecules directed against TNF-α and IgA were titrated with unlabeled targets that were subsequently probed with target-specific antibodies, in turn detected with fluorescently labeled streptavidin or a fluorescently labeled secondary antibody. For TNF-α, the limit of detection was decreased to 33 pg/ml (2 pM) using the sandwich format. However, for IgA, the limit of detection was increased to 330 pg/ml (2 pM), possibly due to higher background signals or partially overlapping binding epitopes for the capture affibody and the target-specific antibody sandwich partner.

### Complex background mixtures

The sandwich format was also used for detection of protein in the complex protein solutions human serum and plasma. Affibody capture arrays were incubated with 1:100 diluted IgA-deficient or normal sera, and IgA was detected with a biotinylated IgA-specific antibody, followed by fluorescently labeled streptavidin. A 1,000-fold stronger signal was observed for the normal serum compared to the IgA-deficient serum, showing that affibody capture microarrays could be used for specific detection of IgA in human sera. Titrations of TNF-α was carried out against a background of 1:100 diluted plasma. However, the detection limit was considerably higher than for experiments performed with the target protein in buffer, 3.3 ng/ml (200 pM) compared to 33 pg/ml (2 pM). For the plasma experiments, blocking reagents were found to be crucial for achieving good signals. Non-specific binding to the dextran surface, mass transport issues, competition from cross-reactive high-abundant proteins, or degradation or binding of the target proteins by the serum or plasma are some possible explanations for the increase in limit of detection, however, further studies are needed to investigate this.

### Theoretical considerations

The better capture ability for the multimeric Z_{Ins} and Z_{IgA} constructs could be caused by increased rebinding of targets, resulting in lower apparent off-rates. The increased binding ability is likely also caused by a spacer effect from the placing of the affibody domains away from the protein-repelling dextran layer.

To investigate to what extent the spacer helps facilitate target binding, multidomain constructs
with an N-terminal binding domain linked to non-binding domains immobilized via a C-terminal cysteine could be evaluated side by side with multidomain constructs consisting of only binding domains. This could be performed for targets with multi-epitopes and only one epitope so that avidity effects from a single multidomain construct could be determined.

The detection limits for TNF-α in the plasma were not sufficient for relevant diagnosis in human plasma. It would be interesting to use a multidomain construct with affibody molecules binding different epitopes. If binding could take place simultaneously, avidity effects would cause an apparent increase in affinity, as was shown by Silverman et al (Silverman et al., 2005) for multivalent avimers.
Conclusions and future perspectives

The work in this thesis has been centered on the development of solution and solid phase assays for biomolecule detection using affibody affinity ligands. Recombinant and synthetic production routes have been used for the construction of specifically modified biosensors and ligands. In papers I and II, two different solution-phase assays for detection of unlabeled proteins were developed, utilizing a change in relative fluorescence for detection. In papers III and IV, different presentation formats in protein capture microarrays were evaluated, including random and specific immobilization and the use of genetically fused multidomain constructs. The small size of affibody molecules has allowed great flexibility in incorporation of chemical groups, utilizing solid phase peptide synthesis. Taken together, this has showed that affibody molecules are well suited as ligands in various assay formats, for sensitive and specific detection of biomolecules also in complex, clinically relevant, samples.

The key concepts, homogeneous and label-free, which papers I and II built upon, are important for rapid diagnostics with a minimum of user intervention, and avoiding many of the problematic issues of analyte labeling. Future assays in diagnostics could benefit from total analytical systems where only analyte addition is needed prior to read-out. This would grant the possibility of wide-spread use by physicians and home-consumers.

Just as radiolabels have largely been replaced by fluorophores, fluorophores will one day be replaced by more sophisticated methods. The development of label-free detection methods,
using inherent biophysical properties for analyte detection, will likely gain interest as sensitivity is improved, because after all, avoiding labels all together would be optimal.

Beyond papers III and IV, for protein microarrays, technological research is direly needed in the coming years. Sensitivity, cross-reactivity, ligand generation, instrumentation sensitivity, etc, are issues that severely limit the possible applications. However, protein microarrays have come a long way in the last decade, and will continue to develop, hopefully rapidly, in the coming years. Focused arrays for detection of small groups of biomarkers will be developed many years before the advent of futuristic million spot microarrays for whole total-omics analysis, if they ever see daylight.

Hopefully, when the ligand assay field for massive parallel quantification has developed and matured, it may become possible to routinely cluster the expression profiles of individuals under different stages in life with bioinformatic tools so that deviations from normal patterns can used as diagnostic tools to detect very early stages of disease and unwanted health states. Hopefully by then, suitable therapy will developed that can be used to address any problems detected. And after that, further into the future, fully automatic analytical systems capable of detecting diseases and administrating suitable therapy without user intervention could be in use. Though this is taking ligand assay technology to the verge of science fiction, a hundred years ago, most people would have smirked or laughed at the idea of satellite-TV and microwave ovens.
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