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Affibody ligands in immunotechnology applications

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Cover pictures
Above: Model of the structure of a tetrameric affibody-β-galactosidase fusion protein. Below: Immunohistochemical staining of IgA deposition in glomeruli using an affibody-β-galactosidase immunoconjugate and a fluorescently labled antibody, respectively.

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Abstract

Recent development of methodology for the construction and selection from protein libraries have opened up for alternative routes for the generation of affinity reagents for immunotechnology applications, obviating the need for immunization. Diverse repertoires (libraries) of potential affinity proteins has been constructed based on antibody fragments or other proteins, later used for the identification of desired members by various selection principles capable of providing a physical coupling between genotype and phenotype.

This thesis describes the development and use of non-immunoglobulin affinity proteins denoted affibodies as alternatives to antibodies in different immunotechnology applications. A 58 aa IgG Fc binding three-helix bundle domain Z, derived from staphylococcal protein A has been used as framework for library constructions, in which the face of the molecule involved in the native binding activity has been engineered by combinatorial protein engineering. Recruting 13 surface-located positions for simultanenous substitution mutagenesis, using degenerated oligonucleotides for library assembly at the genetic level, two libraries differing in the choice of codons were constructed to serve as general sources of novel affinity proteins. The libraries were adapted for display on *E. coli* filamentous phage particles allowing *in vitro* selection of desired variants capable of binding a given target molecule. In selections using human IgA as target, several new IgA specific affibodies could be identified. One variant Z_{IgA1}, was further investigated and showed binding to both IgA1 and IgA2 human subclasses as well as to secretory IgA. This variant was further demonstrated uesful as ligand in affinity chromatography purification for recovery of IgA from different samples including unconditioned human plasma. Affibodies of different specificities were also fused to other protein domains to construct fusion proteins of relevance for immunotechnology applications. Using Fc of human IgG as gene fusion partner, "artificial antbodies" could be produced in *E. coli* as homodimeic proteins, where the antigen binding was confered by N-terminally positioned affibody moieties of different valencies. One area of application for this type of constructs was demonstrated through specific detection of the target protein by western blotting. Exploiting the uncomplicated structure of affibody affinity proteins, gene fusions between affibodies and the homotetrameric reporter enzyme β-galactosidase were constructed, which could be produced as soluble proteins intracellularly in *E. coli*. The potential use of such recombinant immunoconjugates in immunotechnology was demonstrated in ELISA dot-blot and immunohistochemistry, where in the latter case IgA depositions in the glomeruli of a human kidney biopsy could be specfically detected with low background staining of surrounding tissues. In a novel format for sandwich ELISA, the possible advantage of the bacterial origin of the affibody class of affinity proteins was investigated. As a means to circumvent problems associated with the presence of human heterophilic antibodies in serum, causing bakground signals due to analyte-independent crosslinking of standard capture and detection antibody reagents, assay formats based on combinations of antibody and affibody reagents for capture and detection were investigated and found to of potential use.

Keywords: phage display, combinatorial, affinity, IgA ligand, immunohistochemistry, affibody-fusions

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List of publications

This thesis is based on the following publications, which will be referred to by their Roman numeral:

I. Nord, K., Gunneriusson, E., **Ringdahl, J.**, Ståhl, S., Uhlén, M. and Nygren, P-Å. (1997) Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain. *Nature Biotechnology*, **15** (772-7).

II. **Rönnmark, J**., Grönlund, H., Uhlén, M. and Nygren, P-Å. (2002) Human immunoglobulin A (IgA)-specific ligands from combinatorial engineering of protein A. *Eur J Biochem*. **269** (In press)

III. **Rönnmark, J**., Hansson, M., Uhlén, M. and Nygren, P-Å. (2002) Construction and characterization of affibody-Fc chimeras produced in *Escherichia coli. J Immunol Meth* **216** (199-211)

IV. **Rönnmark, J.**, Kampf, C., Asplund, A., Höidén, I., Uhlén, M. and Nygren, P-Å.
IgA-specific affibody-β-galactosidase immunoconjugates produced as soluble fusion proteins in the *E. coli* cytosol. Manuscript

V. Andersson, M., **Rönnmark, J**., Nygren, P-Å. and Ahlborg, N.
Inclusion of a non-antibody component in capture two-site ELISA for quatification of human serum proteins without interference by heterophilic serum antibodies. Manuscript

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Introduction

1. Historical perspective

Immunotechnology is a field combining immunology and biotechnology and relates to applied aspects of principles and cellular and molecular reagents derived from the immune system. Of central importance for many of the different disciplines of the field, is the availability of antibodies, capable of specific and predictable antigen binding allowing their use in numerous applications including detection, quantification, bioseparation and therapy. One of the earliest references in the field of antibodies is written by Thucydides in 450 BC where he noticed that only people that had recovered from the plaque could nurse the sick without catching the disease a second time. Several hundred years later, in the 15th century, Chinese and Turks made attempts to induce immunity by inhaling dried crusts from smallpox. Edward Jenner's work in the 18th century and Pasteur's experiments a hundred years later marked the beginning of the discipline immunology. The responsible agent for immunity was discovered in 1939 when Tiselius and Kabat managed to separate, by electrophoresis, serum into several fractions. One of the fractions called the γ-globulin (now immunoglobulin) contained antibodies. The three-dimensional structure of an antibody Fab fragment resolved in 1973, and in 1992 the structure of an intact antibody was determined (Poljak et al., 1973; Harris et al., 1992).

An increased understanding of the underlying genetic principles of the immunesystem together with an ever increasing amount of methods provided by recombinant DNA technology, has provided a rich tool box for the isolation of relevant genes. This has allowed for design and production of tailor made reagents for use in immunotechnology applications. Further, progress in the development of methods for the construction and use of protein libraries has in later decades opened up for new opportunities in the field of immunotechnology. It is now possible to harvest the immune repertoires of for example naïve or immunized donors and using them in various formats for the coupling of genotype and phenotype. Such libraries have allowed for the isolation and identification of useful reagents by powerful *in vitro* selection principles, like the phage display technology first described in 1985.
As a further development, the progress in protein library technology has promoted the exploitation of repertoires of also non-antibody proteins as alternative sources of selective binding reagents of relevance for immunotechnology applications. Here, a general introduction to the field of immunotechnology will follow, with an emphasis on applications involving antibodies or antibody-binding reagents and technology for the generation of useful reagents. In later chapters, this thesis describes efforts to investigate the potential of a novel class of reagents (affibodies), developed using combinatorial protein engineering principles, for use in immunotechnology applications.

2. Generation and purification of antibody reagents for use in immunotechnological applications.

The field of immunotechnology includes many different disciplines based on the use of cells and molecules of the immune system. In this overview, the focus will be on applications based on the use of antibodies and antibody-binding affinity reagents. Such applications include for example detection, localization and quantitation of antigens and antibodies in different samples, purification of antibodies and other molecules as well as therapy applications. As mentioned in the introduction, the source of reagents can today vary significantly, but has traditionally been in the form of polyclonal or monoclonal immunoglobulins isolated from the blood of immunized animals or supernatants from hybridoma cell cultures, respectively, having exploited an immune system of a mammal for their generation. Such antibodies, or fragments thereof, can either be used as native antibodies or as conjugated to other groups (including enzymes, biotin and fluorophores) facilitating different assays. Today, also recombinantly produced reagents are available.

2.1 Antibody classes and structure

In general terms most antibodies share a common overall structure (Fig. 1) based on the assembly of two pairs of identical protein chains, denoted heavy (H) and light (L) chains, respectively (Goldsby et al., 2000). Variability lies in defined regions on both types of chains, called VH (variable heavy chain) and VL (variable, light chain), respectively. Within those regions hypervariable regions called complementary-determining regions (CDR) reside, responsible for the interaction between an antibody and an antigen. The constant parts of the antibody defines different classes of immunoglobulins, so called isotypes and isotype subclasses, responsible for different effector functions in the immune response, and varies in numbers in different species (Spiegelberg, 1989). During the primary response, after a first injection with an antigen into a laboratory animal, the concentrations of antibodies primarily of the IgM isotype are increased. IgM is of a pentameric structure, thus exposing ten antigen-binding sites to the surroundings, which increases the affinity to the antigen through avidity effect. This multimeric form is very efficient in precipitating foreign molecules, but might not be an ideal isotype for immunotechnology applications. Therefore, the animal is usually boosted with the antigen after a period of time, inducing a secondary response, involving the stimulation of memory B-cells to secrete antibodies of IgG isotype, which is an antibody of monomeric structure, and more convenient for use in different applications. Beside the IgM and IgG isotypes there are also IgA, IgD and IgE antibodies in serum. IgA is like IgM involved in the first line of defence and is mainly of monomeric structure, but is also present in the form of dimers in the serum, connected by a J-chain. During secretion to mucosal tissues, were most of the IgA content is comparted, two IgA monomers become further connected by a protein chain denoted secretory component. IgE antibodies are the serum components responsible for allergic reactions causing symptoms like asthma, hay fever, hives and anaphylactic shock. The IgD isotype is together with IgM the major membrane bound immunoglobulins expressed on mature B cells, but is also present in serum.

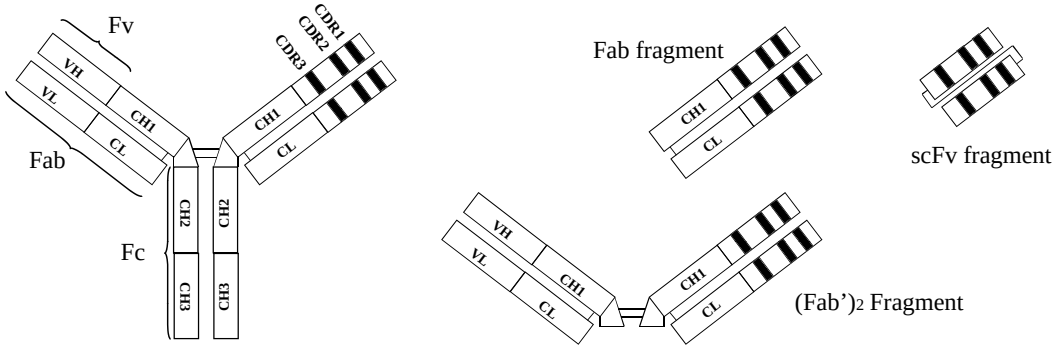


Fig. 1. A schematic view of the general structure and domain nomenclature of a human IgG1 antibody. Also shown are different antigen binding subfragments possible to obtain by proteolytic cleavage or by recombinant means, including Fab, $F(ab')_2$ and scFv fragments.

As an alternative to whole antibodies, fragments of antibodies have been used in several immunotechnological applications. Traditionally, enzymatic cleavage with either papain or pepsin has been used to fragmentize antibodies. Papain cleavage results in Fab and Fc fragments whereas pepsin digests the Fc domain at several sites resulting in a homodimeric Fab fragment ($F(ab')_2$), see Fig. 1. Interchain disulfides holding the different chains together can be broken by reducing agents, resulting in proteins of approximately 50 kDa (heavy chain) and 25 kDa (light chain) sizes. Although the characteristics of the different domains of an immunoglobulin has been known for a long time, the three dimensional structure of an antibody molecule was not known until 1973, when the structure of an antibody Fab fragment was solved, and it was not until 1992 that the structure of an intact antibody was determined (Poljak et al., 1973; Harris et al., 1992).

2.2 Polyclonal antibodies and different isotypes

The general working principle for the immune system is to discriminate between self and non-self, which means that antibodies can be raised against other foreign molecules than direct pathogens. This fact has been extensively exploited for the generation of antibodies to relevant antigens for use in immunotechnology applications. Normally, a typical antigen used for immunization will have more than one 'antigenic determinant' i.e., more than one part (epitope) that will induce the production of different antibodies when injected into a laboratory animal. Hence, the produced immune serum will contain a cocktail of different antibodies directed towards the same antigen. Such preparations, denoted polyclonal antibodies, can be refined by different methods to obtain antibodies of different purities and

compositions, with regard to isotype/subclass type and antigen binding characteristics (see section 2.6).

Traditionally different animal species has been used for polyclonal antibody generation. The choice of animals like rabbit, mouse, rat, hamster, goat, guinea pig and lately hen, depend on several parameters, such as the amount of antibody required and the phylogenetic distance between the donor of antigen and the recipient (Hanly et al., 1995). The small animals, like mouse and hamster, are often used in generation of monoclonal antibodies, see below, while the larger animals are primarily used for polyclonal antibody generation. Rabbits are so far the most used animals for polyclonal antibody generation, since they are easy to keep and handle and can be bleed repeatedly in a safe way. For some purposes rat, hamster and guinea pig could be a better choice. Generation of anti-mouse protein antibodies could be more efficient in those species than in rabbit, due to the larger phylogenetic distance. If larger amounts of antiserum are needed, larger animals like goats, horses and sheep could be used. Those animals are easy to handle and can be bleed without anaesthesia, but requires special housing facilities. An alternative to the mammalian species could be to generate antibodies in eggs from immunized chickens. Chickens provide a great phylogenetic distance from mammals and is particularly useful for polyclonal antibodies raised towards homologous intracellular proteins, highly conserved between mammalian species, or when effector functions of mammalian immunoglobulins could interfere with the antibody used. The large quantity of chicken antibodies (IgY) in egg, improved purification protocols and the non-invasive collection of eggs has made the choice of chicken an interesting alternative to the classical animals.

2.3 Monoclonal antibodies

Although a polyclonal set-up of antibodies is invaluable, and some times a preferred reagent type, in some immunotechnology applications, including research, diagnostics and therapy, a more precise control of antibody quality, specificity and properties is desired. In 1975, Köhler and Milstein presented a method for preparation of so called monoclonal antibodies (Köhler and Milstein, 1975), which has become one of immunotechnology's key technologies. They were able to generate a so-called hybridoma cell by fusing a B-cell producing antibody of a single specificity (clone) with a myeloma cell. The resulting cell obtained the combined properties of being possible to culture and at the same time secreting antibodies of defined specificity. The monospecificity and homogeneity of such preparations together with the possibility to produce more of the antibody on demand in a reproducible manner has made monoclonal antibodies attractive reagents. Today, monoclonals can be made using hybridoma technology from a variety of different species. Most common are hybridomas from rodents like mouse and rat, but other nonrodent animals have also been used, for example hamster, monkey and horse (Groves and Morris, 2000). Alternatively, transfection of B-cell lines with viruses can also be used to obtain an immortal B-cell lineage (Roome and Reading, 1984).

For human immunotherapy applications, monoclonal antibodies of human origin would be ideal reagents to prevent adverse human anti-mouse-antibodies (HAMA) immune responses upon administration. However, work with human B-cell lineages

for monoclonal production has been used with limited success. Alternative strategies have involved protein engineering efforts to humanize murine antibodies into more human-like proteins (see also section 3.4). Recently, the use of transgenic mice (XenoMouse) (Green, 1999), containing human antibody genes, allowing for production of human monoclonal antibodies has also been investigated. However, the resulting glycosylation patterns for such antibodies are the same as for mouse antibodies which has been suggested to be one of the reasons why mouse monoclonal antibodies have a limited half-life in human (Borrebaeck, 1999). However, alternative systems are under development and are discussed below in chapter 4.

2.4 Recombinant antibodies

As mentioned above polyclonal antibodies are not always suitable in immunotechnological applications and there is also an ethical issue on using animals for antibody production. Monoclonal antibody production using hybridomas is one way of circumventing the problem with a heterogeneous mixture of polyclonals. However, this route of production does not allow for an engineering of the antibody protein at the genetic level, to yield variants of the antibody more suitable in different applications. These and other considerations have driven efforts to isolate the antibody encoding genes from hybridoma allowing the use of other host cells for their production by recombinant means. Recombinant antibodies and fragments thereof have been produced in several different host cells including bacteria, yeast, mammalian, insect and plant cells (Larrick et al., 1998; Verma et al., 1998).

Considering parameters, like complexity of the antibody construct, post-translational modifications and production scale-up, different systems could be evaluated. Mammalian cell culture have traditionally been used for large-scale production of whole antibodies, but production in plant cells has also been described (Giddings et al., 2000). In the late 1980´s several groups described the production of functional antibody fragments in *E. coli*, including the monovalent antigen-binding Fv (Skerra and Plückthun, 1988), single chain Fv (scFv) (Bird et al., 1988) and Fab fragments (Better et al., 1988). Fab and Fv fragment assembly relies on spontaneous association of separately expressed domains, whereas in scFv VH and VL domains are connected by a peptide linker, which also reassures equal production of both moieties.

An example of a further engineered antibody fragment format are diabodies, corresponding to divalent scFv dimers formed by two scFv proteins engineered with very short connective linkers promoting interprotein VH-VL (Holliger et al., 1993). Such divalent fragments could be of a single antigen specificity, increasing the binding avidity effect by the multimerization, or they could be used for the generation of bispecific reagents (Koelemij et al., 1999; Kriangkum et al., 2001). More recently, triabodies and even tetrabodies have been described using similar protein engineering principles (Todorovska et al., 2001). Multivalent constructs could also be obtained by fusion to other domains capable of spontaneous multimer formation (Plückthun and Pack, 1997; Kriangkum et al., 2001).

In general, due to the requirement for correct disulfide formation for biological activity, a preferred route for *E. coli* antibody fragment production has been to secrete the proteins to the bacterial periplasm. However, also intracellular production has

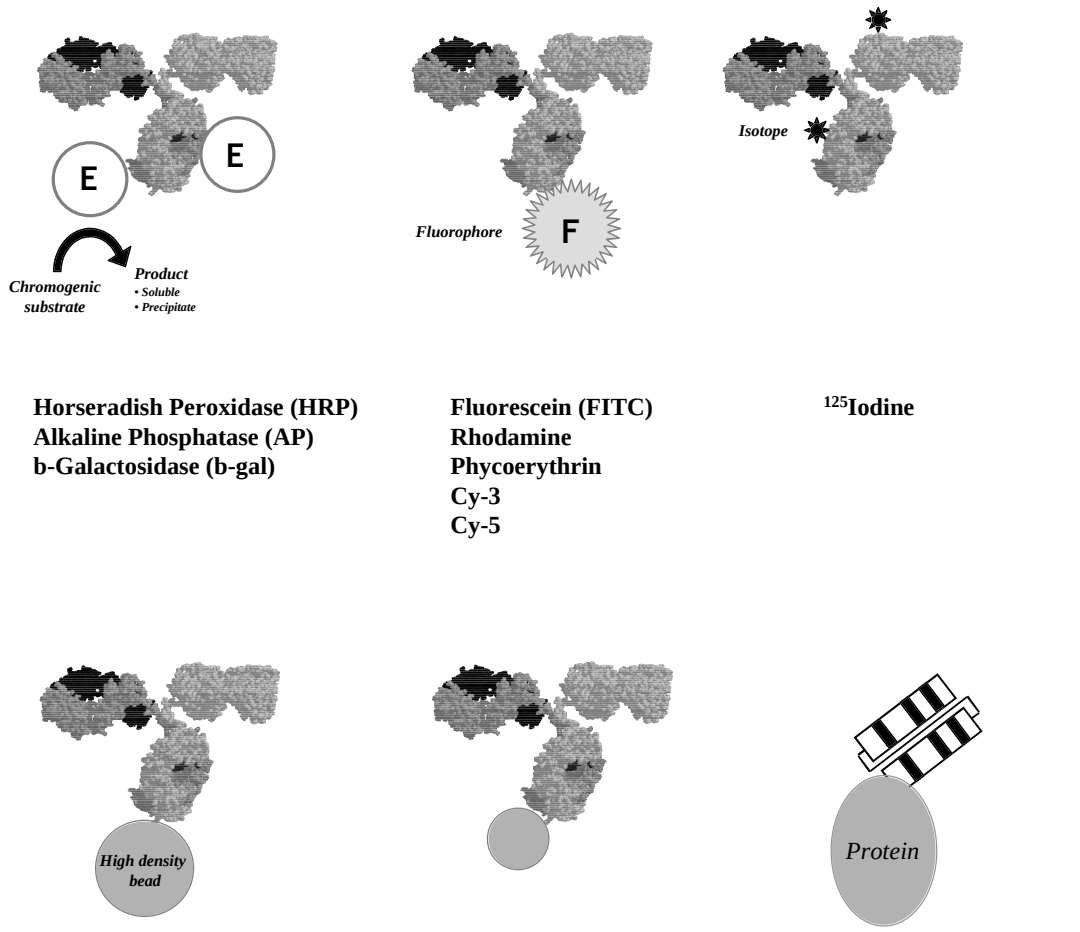
frequently been utilized, which in most cases has resulted in the formation of inclusion bodies from which soluble antibody fragments can be obtained refolding procedures. However, some progress on antibody scaffold engineering facilitating the intracellular production of soluble and active antibody fragments have been described (Martineau and Betton, 1999; Jermutus et al., 2001). In addition, the development of engineered *E. coli* host strains, resulting in a less reducing cytoplasm has also been described to result in a facilitated intracellular expression of soluble antibody fragments (Bessette et al., 1999; Levy et al., 2001). Recently, the first report on soluble expression of whole antibodies in *E. coli* was described (Simmons et al., 2002). The production of non-antigen binding regions of antibodies, such as complete Fc regions, have also been described using bacterial expression systems (Jendeberg et al., 1997).

As will be discussed later, the recombinant route of production offers the possibilities of protein engineering, including strategies to novel types of immunoconjugates (see chapter 2.5) and to convert monoclonal antibodies into more human-like proteins (see chapter 3.4).

2.5 Antibody conjugates

Antibodies are often linked to other molecules, isotopes or particles recruited as reporter or effector groups, for use in *in vitro* or *in vivo* applications, including diagnostic and therapeutic techniques (chapter 3). Whole antibodies or fragments conjugated with some other molecule are called "bifunctional antibodies" (Fig. 2), not to be mixed up with the term "bispecific antibodies". Historically, such linking has been made by chemical coupling principles, using for example cross-linking reagents such as SPDP or glutardialdehyde. Alternatively, compounds could be preactivated by for example N-hydroxysuccinimide esters or by iodoacetamide chemistry for coupling to primary amines or cysteines, respectively, in the antibody protein. More recently, also gene fusion strategies have been employed, obviously limited to conjugates between antibodies and proteins.

Antibodies conjugated with enzymes like calf intestine alkaline phosphatase, horse radish peroxidase and *E. coli* β-galactosidase are of great importance in methods like ELISA and immunoblotting (see later chapters). Antibodies could also be labeled with a radioactive compounds such as ^{125}I, an isotope of iodine, often used in RIA (radio immunoassay). Fluorescent organic molecules like fluoresceine isothiocyanate (FITC) or Cy5, or fluorescent proteins like phycoerythrin and green fluorescent protein (GFP), frequently used in cell sorting (flow cytometry) applications, have also been used. In the field of therapy, antibodies have been labeled with isotopes or cytotoxins for cancer treatment (Green, 1999; Garnett, 2001), Antibodies, as well as other proteins, are also often biotinylated to facilitate subsequent binding to streptavidin (or avidin) conjugated molecules via the strong biotin-streptavidin interaction.



Horseradish Peroxidase (HRP)
Alkaline Phosphatase (AP)
b-Galactosidase (b-gal)

Fluorescein (FITC)
Rhodamine
Phycoerythrin
Cy-3
Cy-5

^{125}Iodine

Gold
Ferritin
Wolfram

Biotin

Green fluorescent protein (GFP)
AP
b-gal

Fig. 2. Schematic view of different types of antibody conjugates, including antibodies chemically conjugated with enzymes, fluorescent groups or proteins, radioactive isotopes, high density particles, biotin or genetically fused to a different protein.

However, chemical coupling procedures between proteins can lead to partial inactivation of both the antibody and reporter protein and one have also to consider batch-to-batch variations, which has motivated the investigation of gene fusion strategies for more reproducible linking of antibodies and reporter proteins. This requires that both the antibody fragment and the reporter protein can be functionally expressed using a common expression route. Several studies have described the use of bacterial alkaline phosphatases (AP) as reporter enzyme fusion partner to antibody fragments, suitable for expression as periplasmic fusion proteins in *E. coli* (Ducancel et al., 1993; Weiss and Orfanoudakis, 1994; Kerschbaumer et al., 1997). For example,

a fusion between a scFv antibody to *E. coli* alkaline phosphatase was successfully used for the detection of a plant pathogen in a sandwich ELISA (Kerschbaumer et al., 1997).

To date, no antibody-enzyme gene fusion conjugates containing the two widely used reporter enzymes horse radish peroxidase or β-galactosidase have been described. This is probably due to that both these reporter enzymes are intracellular proteins, making production of disulfide-dependent antibody-enzyme fusions difficult. However, as described earlier, progress both in antibody framework (Martineau and Betton, 1999; Jermutus et al., 2001) and host cell engineering (Bessette et al., 1999; Levy et al., 2001), such fusions could prove possible to produce. In fact, a first *E. coli* produced HRP fusion protein for use in immunotechnology applications was recently described (Grigorenko et al., 2001). This fusion protein (HRP-human heart fatty acid binding protein) was refolded from inclusion bodies using a protocol, which included a reconstitution of the enzyme with the heme group necessary for activity. The *E. coli* β-galactosidase is a tetrameric intracellular enzyme, not possible to secrete into the bacterial periplasm, and therefore difficult to use as reporter in gene fusion conjugates in connection with antibody fragments. Fc fragments of antibodies have also been recruited as fusion partners for the development of one type of immunoconjugates. In such fusion proteins, denoted immunoadhesins, the Fc moiety has been exploited as partner to a variety of other non-immunoglobulin protein domains, including adhesion molecules, extracellular receptor domains or soluble ligands for different reasons (Chamow and Ashkenazi, 1996) (see also chapter 6).

2.6 Affinity purification of antibodies

Depending on the application, antibody reagents need to be of different purities. Whereas relatively crude preparations of for example rabbit polyclonal antibodies can be used for research purposes in Western blotting procedures, other applications such as therapy sets significantly higher demands on purity. As for any protein, conventional purification schemes including combinations of for example precipitation, ion exchange, size exclusion chromatography and hydrophobic interaction chromatography (HIC) can obviously be applied for antibody purification and are not discussed in detail here. However, many antibody purification protocols utilize general ligands of both proteinaceous and non-proteinaceous nature useful for affinity chromatography for the recovery of antibodies.

2.6.1 The receptins protein A, G and L

Some bacterial species are equipped with cell surface displayed proteins capable of interacting with mammalian host proteins, like albumin, fibronectin and immunoglobulins. The biological significance is not fully investigated, but a proposed explanation might be that the bacteria becomes more host-like and can escape the hosts immune defence (Sauer-Eriksson et al., 1995). Such structures are denoted bacterial receptors, or more correctly "receptins" since they, unlike the receptor proteins, have been found to induce a secondary intracellular effect upon binding to mammalian proteins (Kronvall and Jönsson, 1999). The immunoglobulin binding receptins bind their targets through non-immune interactions, involving other regions

than the CDRs, such as Fc and CH1. These binding activities make them interesting as general antibody binding tools in immunotechnology applications. Three examples of immunoglobulin binding receptins that have found widespread use in immunotechnology are staphylococcal protein A, streptococcal protein G and peptostreptococcal magnus protein L.

Protein A (SPA) is a cell wall anchoring anchored protein on *Staphylococcus aureus* consisting of five homologous immunoglobulin binding domains of α-helical structure followed by a cell wall spanning/anchoring region. Each domain interacts with the CH2-CH3 hinge region on Fc (Deisenhofer, 1981) of different species (Langone, 1982), including three of the four human IgG subclasses (IgG1, 2 and 4) (Fig. 3). Each of the wild type protein A domains can also bind to the VH region of certain antibodies (for example the human VHIII family), recruiting a different molecular face of the domain than involved in the Fc binding activity (Graille et al., 2000), (Fig. 3). Due to its characteristics, immobilized protein A has been widely used for affinity recovery of IgG (Fahrner et al., 1999). In reverse formats, wild type or engineered domains of protein A have been frequently used as gene fusion partners to facilitate purification of recombinant fusion proteins by IgG affinity chromatography (Ståhl and Nygren, 1997). One engineered protein A domain, denoted Z (Nilsson et al., 1987), has been used as scaffold structure for construction of combinatorial protein libraries from which novel affinity proteins, affibodies, have been selected (see chapter 5).

Streptococcal protein G (SPG) is a protein with somewhat broader immunoglobulin binding properties than protein A. Protein G from the Streptococcus G148 contains three immunoglobulin domains which bind to all human IgG subclasses and to IgG of several other species, including mouse and rabbit (Björck and Kronvall, 1984; Åkerstrom et al., 1985; Guss et al., 1986), but it seems to lack binding to the other isotypes IgA, IgM and IgD (Björck and Kronvall, 1984). Protein G binds to Fc regions at a site overlapping with the binding site of SPA (Kato et al., 1995; Sauer-Eriksson et al., 1995), but can also bind to CH1 regions of certain antibodies via a different mechanism (Derrick and Wigley, 1992) (Fig. 3). Protein G also contains regions capable of binding to human serum albumin (HSA) which have been exploited as affinity gene fusion partners to recombinant proteins (Nygren et al., 1988; Ståhl and Nygren, 1997).

The *Peptostreptococcus magnus* protein L (PL) consists of up to five domains (depending on isolate) that bind to VL regions of kappa light chains (Fig. 3), from several species, including human (Nilson et al., 1992), and has been used for affinity recovery of various antibodies (Nilson et al., 1993). The binding specificity of Protein L makes it interesting for purification of antibody fragments devoid of Fc domains, such as scFv constructs.

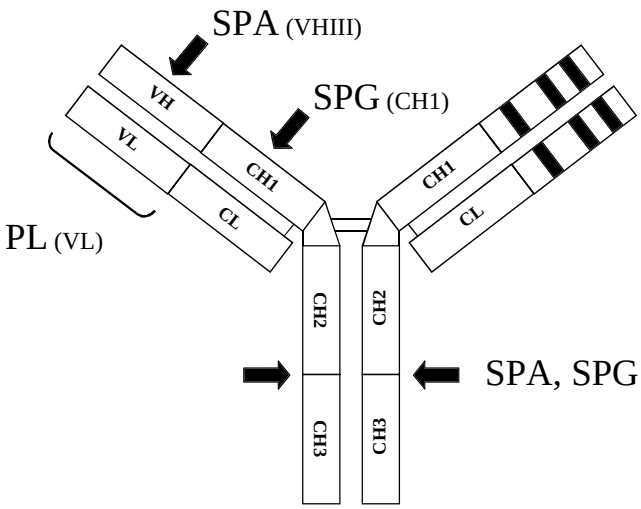


Fig. 3. Binding sites for the immunoglobulin binding proteins staphylococcal protein A (SPA), streptococcal protein G (SPG) and peptostreptococcal protein L (PL).

2.6.2 Lectins

Lectins are carbohydrate binding proteins of different saccaride specificities and can be isolated from different sources including bacteria, plants and animals (Vijayan and Chandra, 1999). A lectin used for antibody purification is Jacalin (Roque-Barreira and Campos-Neto, 1985), isolated from jackfruit seeds, which has been used for affinity recovery of IgA1 (but not IgA2), due to the presence of O-linked sugars, in the hinge region of IgA1 (Skea et al., 1988). A different lectin, the rabbit mannan binding protein (MBP), has been used for purification of mouse, human and bovine IgM (Nevens et al., 1992).

2.6.3 Non-proteinaceous ligands

Motivated by the possibility to develop more robust affinity chromatography media capable of withstanding harsh industrial column sanitation procedures, efforts have been made to develop alternatives to protein ligands for antibody purification. Recently a low-molecular-weight synthetic ligand that mimics a motif on protein A domain B know to interact with the Fc portion of IgG was developed (Li et al., 1998) and later refined (Teng et al., 1999). This synthetic compound was designed with knowledge of the crystallographic structure of the interaction and with help of molecular modelling. The ligand has been shown to be useful for the purification of

IgG from human plasma at high yield and purity (Teng et al., 1999). Recently new chromatographic matrices using synthetic peptidomimetic molecules as ligands have been put on the market (KAPTIV, Technogen, Italy). One such described ligand was identified by screening of a synthetic peptide library (Fassina et al., 2001), and showed binding to several immunoglobulin isotypes.

3. Applications

The capability of specific antigen recognition has made antibodies invaluable as analytical tools and there are a vast number of different applications using whole antibodies, conjugated antibodies or antibody fragments. Synthetic antibody fragments and non-immunoglobulin ligands, developed by combinatorial methods (see ch. 4) can often be directly applied to the same methods as antibodies. Enzyme linked immunoassays and radioimmunoassays, extensively used for over 30 years, as well as immunoblotting assays are methods used for detection and characterization of diverse molecules. Other applications for antibodies are for example in immunohistochemistry and flow cytometry analyses, and in affinity purification of antigens. Antibodies have also made progress in the field of therapy, although there are still only a few antibody based drugs on the market. Here, a brief description of some immunotechnology application will be made.

3.1 Specific recognition and affinity

In many immunotechnology applications, the affinity between the antigen binding reagent and its target is considered. This affinity can be regarded as the sum of all noncovalent interactions between an epitope on the ligand (L) and the ligand-binding site on the target (T) molecule and can be described by the equation

$$L + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LT$$

were k_1 is the association rate constant [M^{-1} s^{-1}] and k_{-1} is the dissociation rate constant [s^{-1}]. The ratio of k_1/k_{-1} is the association constant K_a [M^{-1}], the equilibrium constant for the above reaction and could be calculated from the molar ratio of bound ligand-target complex to unbound ligand and target as follows.

$$K_a = \frac{[LT]}{[L][T]}$$

Sometimes the affinity is expressed as the dissociation constant K_d (=$1/K_a$) which is the inverse of the K_a and has the unit [M].

Low-affinity complexes have K_a values typically between 10^3 and 10^5 M^{-1} and high affinity complexes can have values up to 10^{11} M^{-1} or higher.

Different L-T complexes of identical K_a values can still have radically different characteristics if also the kinetics of the interactions are considered. This can be illustrated by a comparison of simulated sensorgrams for three different complexes characterized by a common K_a of $1x10^7$ M^{-1}, but with different association and

dissociation rate constants (on and off-rate kinetics) produced using the program Biasimulation (Fig. 4). One of the complexes is characterized by fast on- and off-rate kinetics ($10^6/10^{-1}$) (Fig. 4A), the second has somewhat slower on- and offrate kinetics ($10^5/10^{-2}$) (Fig. 4B), and the third has still lower on-and off-rate kinetics ($10^3/10^{-4}$) (Fig. 4C).

Depending on the application, different properties of the interaction could be desired. For instance, a ligand intended for use in affinity chromatography could be considered more attractive if it showed a relatively slow dissociation rate for its target, enabling extensive washing of the column to remove impurities (B, C). For use as an on-line sensoring ligand an interaction characterized by both a fast on-rate and a fast off-rate could be ideal, since it would show a rapid response to the presence of a target analyte and be regenerated quickly as soon as the target analyte is not present (A).

The above considerations are based on the assumption of single-ligand single-target-binding interactions. If possibilities for multiple interactions between ligand and target are present, as for di- or multivalent ligands binding to repetitive or surface immobilized targets, avidity effects must also be considered.

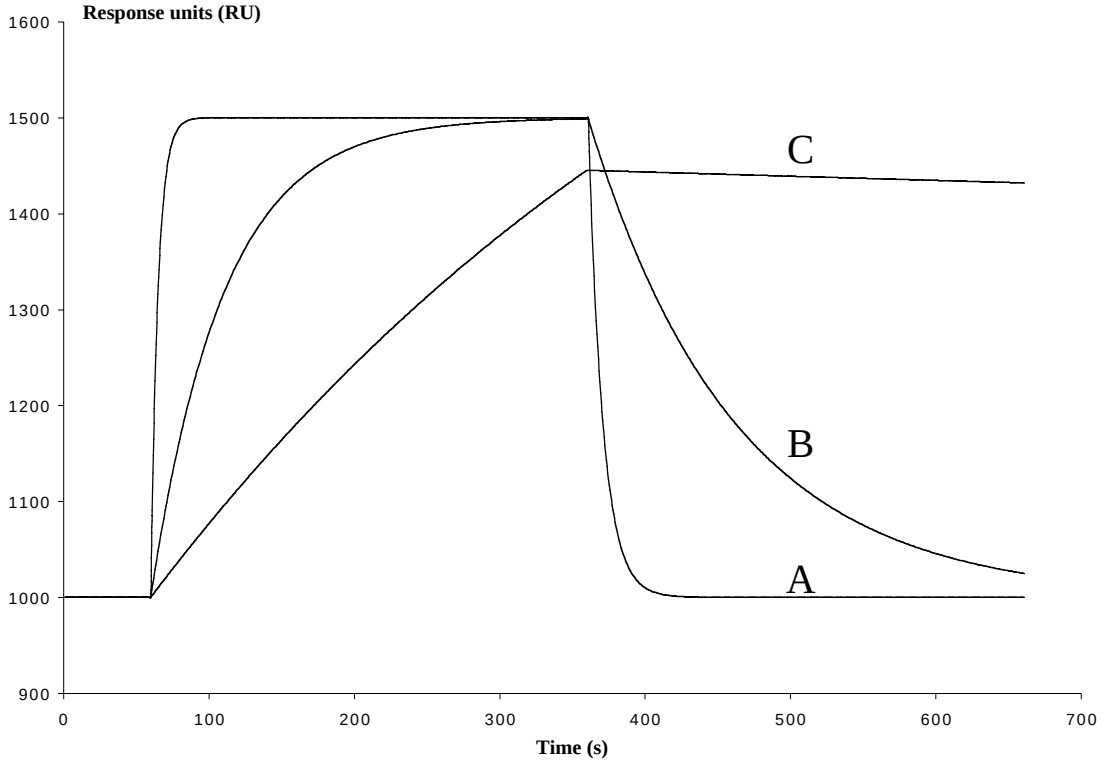


Fig. 4. Simulated Biacore sensorgrams of three different ligand-target interactions with a common K_d of 10^{-7} (M), but characterized by different on- and off-rate kinetics.
A: $k_{on}=10^6$ [M^{-1} s^{-1}], $k_{off}=10^{-1}$ [s^{-1}]
B: $k_{on}=10^5$ [M^{-1} s^{-1}], $k_{off}=10^{-2}$ [s^{-1}]
C: $k_{on}=10^3$ [M^{-1} s^{-1}], $k_{off}=10^{-4}$ [s^{-1}]

3.2 Detection

3.2.1 Solid phase immunoassays

In solid phase immunoassays, as the name implies, a solid phase is used as support for performing detection and/or quantification of analytes (including antibodies) using immunoglobulin-based detection principles. There are several different set-ups for the performance of a solid phase immunoassay, based on the attachment of either the antibody or the antigen to an immunosorbent material. The different assay formats can be divided into three classes; antibody capture assays, antigen capture assays or two-antibody sandwich assays (Fig. 5), where either the reagent or the analyte reaction is observed. An assay could be done in antibody or antigen excess, with antibody or antigen competition. If the analyte or molecule used for detection is radioactive it is called a radioimmunoassay (RIA) and if it is an enzyme it is called an enzyme linked immunosorbent assay (ELISA), first described by Swedish scientists in 1971 (Engvall et al., 1971) and lately reviewed in (Porstmann and Kiessig, 1992; Butler, 2000). Commonly used enzymes in ELISAs are alkaline phosphatase, horse radish peroxidase and β-galactosidase, attached to either monoclonal or polyclonal antibodies. Conjugated secondary antibodies are often used for detection, partly to attain an increased sensitivity and partly for economic reasons.

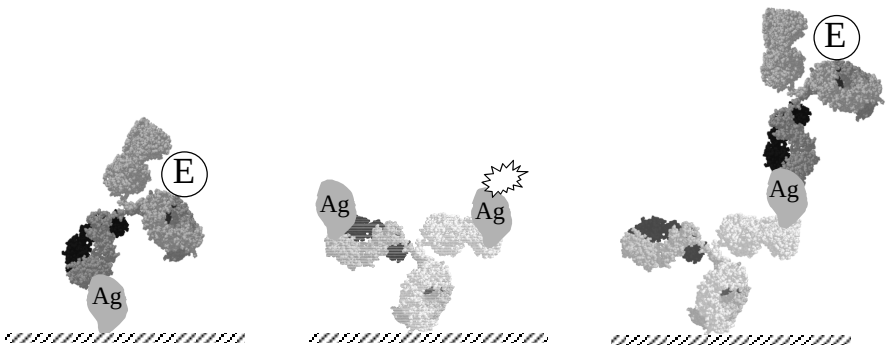


Fig. 5. Different formats for ELISA. Antibody capture, antigen capture and two-antibody sandwich assays.

One set-up for antibody detection would be to immobilize a pure antigen in a microtiter well followed by addition of either pure or impure antibody sample, which is detected either by a second antibody or another immunoglobulin binding protein, for example enzyme conjugated protein A (Pain and Surolia, 1979). This system could also be applied to detection of purified antigen, for example using competing labeled antigen of known concentrations in the sample with detection antibody, or for analyses by immobilization of crude test sample, followed by a detection antibody.

The sandwich technique using two antibodies is probably one of the most valuable variations of ELISA (Fig. 5 C). A first analyte specific antibody is immobilized to the well and used for capture and another antibody, directed to a different site of the analyte, is used for detection. Due to that, the assay involves the specific recognition by two different antibody reagents, of which the first serves as a capture of analyte also from low concentration samples. This technique could be used for absolute amount measurements in an unknown sample. However, the format requires two antibody reagents directed to two different sites on the analyte, which can not always be found for small molecule analytes.

Berson and Yalow developed Radioimmunassay (RIA) for detection of antigen in the 1960´s (Berson and Yalow, 1968). The principle of RIA is the competitive binding of unlabeled and radiolabeled antigen to a high-affinity antibody (Fig. 5 B). Gamma-emitting isotopes, such as ^{125}I or beta-emitting, such as ^{3}H, could be used to label the antigen. For example, a test sample, with unlabeled antigen of unknown concentration, is added in increasing amounts to a solution of antibody saturated with labeled antigen. Decreasing amounts of radiolabeled antigen bound in the presence of test sample is measured, either by precipitation or by using a solid phase carrier for the antibody. By using a standard curve previously generated using samples of known concentrations, the concentration of antigen in the test sample is determined.

3.2.2 Immunoblotting

Although immunoblotting could be regarded as a variant of solid phase methods, one generally speaks of it as a method of its own. Blotting methods could be an alternative to enzyme immunoassays if concentrations of antigen are very low, present in an impure sample or if additional information besides concentration is desired, such as molecular size. One commonly used method is Western blotting (Laemmli, 1970; Burnette, 1981), which also could be useful if the antigen is insoluble, difficult to label or easily degraded (Goldsby et al., 2000). The method includes the separation of proteins or peptides of different molecular weight by SDS-polyacrylamide gel electrophoresis followed by transfer of the separated molecules from the gel to a membrane support, often nitrocellulose or polyvinylidene fluoride (PVDF) membrane, either by heat or electroblotting after which remaining binding sites on the membrane are blocked. Localization of the bound antibody could be determined using for example an enzyme conjugated secondary antibody reagent. To avoid problems with antibody or enzyme deactivation during chemical coupling, there are some indirect systems. For instance, the PAP technique accounts for crossreactivity of a secondary antibody also recognizing a peroxidase-anti peroxidase complex. The peroxidase reaction is than used for localization of the antigen. The separation step often results in denatured sample proteins (some renaturation can

occur at the membrane) and the antibody used for detection has to be able to recognize such epitopes; thus polyclonal solutions are often used. Another similar but simpler method is a so-called dot blot assay. A non-denatured sample could be directly applied on a membrane to detect the presence of an antigen in the test sample. Such an assay is not a quantitative method, but could be a quick method for detection of for example expressed proteins in a cell culture.

3.2.3 Localization of analytes in tissues and cells

Knowledge of cell structure, organisation and function and changes of those in diseases could be acquired by using *in situ* methods like immunohisto- and immocytochemistry on tissues and cells, respectively. Thin slices of cell or tissue are normally fixated to a solid support such as glass plates, with for example formalin, often resulting in more or less denaturation of the proteins. Labeled specific ligands, traditionally antibodies are thereafter applied onto the tissue layer and inspected using microscopy. Immunohistochemistry gives a broader analysis of for example depositions of proteins or antigens in different tissue compartments whereas immunocytochemistry is a method for analysing details within one cell, for example structure and localization of different organelles. The ligands could be conjugated with fluorophores or enzymes like AP, β-gal, HRP and glucose oxidase (Ishikawa et al., 1983; Wilson and Walker, 2000) or genetically fused with for example green fluorescent protein (GFP) (Casey et al., 2000). One advantage with enzyme labeled ligands for immunohisto/cytochemical procedures is the possibility to use an ordinary white light microscopy, where also chemical counterstaining of organelles could be seen. One drawback could be background staining due to endogenous tissue enzymes and other labels could therefore be preferred. Fluorescent molecules or proteins like fluorescein, rhodamine, phycoerythrin and Cy5 should be used, rather than enzymes, in immunocytochemistry, since diffusion of chromogenic substances would interfere too much in the smaller cell samples. A laser microscope is used for images, and several differently labeled ligands applied at the same time could be detected since different fluorophores emit light of different wavelengths. It is also possible to get a three-dimensional localization of target proteins in the cell, by confocal techniques. Subcellular details on cells, not able to determine using conventional microscopy could be visible using ligands conjugated with an electron dense reagent, such as gold or ferritin and HRP products, visualized an electron microscopy.

By the addition of conjugated antibodies directed to cell surface proteins, positive cells with desired characteristics could be distinguished from background cells. Two applications are cell sorting with a fluorescence-activated cell sorter (FACS) (Herzenberg and De Rosa, 2000) and immunomagnetic cell sorting (MACS) (Thiel et al., 1998). In the beginning of the 1970´s FACS was mainly used for characterization of B-cells and hybridomas (Herzenberg and De Rosa, 2000), but now FACS could also used for other applications for sorting protein expressing cells as well as sorting artificial cell-like compartments, e.g. oil-emulsions and synthetic beads, with the only requirement of a fluorescent molecule being attached to it. Sorting by MACS is performed with antibodies conjugated to small magnetic particles. In FACS, cell-containing droplets of salt buffers are sorted by an electric field, but in MACS a magnetic device carries out the sorting, allowing for a faster throughput.

Antibodies could also be immobilized on chip surfaces, for various applications. One simple approach would be to produce a chip with few antibodies for analyses of several samples and a more complex application could be to use chips with thousands of ligands arranged in an array format. Such protein chips have been suggested for important tools for more global analyses of the proteome (Borrebaeck, 2000; Templin et al., 2002).

3.2.4 Precipitation methods

An important property of many antibody preparations is their ability to form a complex, precipitate, with their antigens, present in solution (immunoprecipitation) or on cells (agglutination). The mechanism driving the formation of such insoluble complexes is concentration dependent and precipitation occurs only in a narrow interval, called the zone of equivalence (Wilson and Walker, 2000). Agglutination could be used for detection of either ligand or antibodies in test samples. One method is to use agarose gels for immunodiffusion in either a single diffusion way where the antigen sample is applied in a well on an antibody containing gel, or in double diffusion (Ouchterlony assay) where both antibody and antigen are applied in separate wells on the gel. Diffusion creates a concentration gradient and at the zone of equivalence precipitation lines will occur. In immunoelectrophoresis test sample proteins are separated by electrophoresis, either in an antibody containing gel or the antibody will be added for diffusion after separation.
In immunonephelometry, a method for immunoglobulin concentration determinations, the light scattering of antibody complexes in samples prepared by immunoprecipitation is measured (Ackerman and Rosevear, 1979; Wilson and Walker, 2000).

3.3 Purification

Immunoaffinity chromatography (IAC) has become a popular one-step purification method based on the affinity between an antibody, or antibody-like molecule, and its ligand, reviewed in (Hage, 1998; Stevenson, 2000; Subramanian, 2000). The antibody, could rather be an affinity purified monoclonal than polyclonal, since a monoclonal antibody has a defined affinity and generation of it is reproducible. The affinity should be high enough for efficient attachment, but low enough to enable elution without destroying ligand or target. Optimal antibody association equilibrium constants (K_a) values for IAC has been described to be in the range 5×10^7 to 5×10^8 M^{-1} (Jack, 1994), but typical antibody-antigen K_a values are in the range of 10^8 to 10^{12} M-1 (Hage, 1998). Immunoaffinity ligands are usually immobilized onto matrixes like silica, agarose or sepharose and coupling often involve reaction with the carboxyl or amino groups on the antibody. The support could be activated with for example N,N-carbonyl diimidazole, cyanogen bromide or N-hydroxy-succinimide (Stevenson, 2000). Another approach would be to use biotinylated antibodies on a streptavidin support or a matrix with immobilized immunoglobulin binding molecules, like protein A or G, but the latter could result in significant ligand leakage from the column during elution. Commonly used elution principles involves lowering the pH with acetic or alkaline solutions or addition of chaotropic salts. A variant of immunoaffinity is so called weak affinity chromatography (WAC) described by

(Ohlson et al., 1988). A weakly binding antibody, $K_a<10^4$ M^{-1}, is here used to create a true chromatographic process, in contrast to the immunoaffinity chromatography just described which is rather an on /off process.

However, antibodies in general can be quite fragile to low pH elution conditions and proteases. Neither are they suitable in combination with harsh matrix cleaning with solutions like sodium hydroxide, common in industrial cleaning-in-place procedures. Alternatively, other protein libraries could generate more appropriate ligands with suitable characteristics (see later chapters).

3.4 Therapy

Monoclonal antibodies for treatment of cancer has been considered being the ideal "magic bullets", theoretically capable of sparing normal tissue while attacking only rapidly proliferating disease cells. Direct use of antibodies for therapy by gamma globulin injections has been carried out for several years, but lately the generation of more specified antibodies has been improved and whole monoclonal antibodies are now on the market for cancer therapy of metastatic breast cancer (Stebbing et al., 2000) and colorectal cancer (Welt and Ritter, 1999). These are of IgG isotype, as most of today's antibodies in clinical trials (Glennie and Johnson, 2000), but recent studies also point out the possible use of antibodies of IgA type (Dechant and Valerius, 2001). IgA is an efficient recruitment of neutrophils and it is also actively transported into mucosal secretions, which could improve the targeting of for example luminal cancers. Although the field of cancer therapy is very extensive, antibody based drugs for treatment of other diseases like rheumatoid arthritis, Crohn´s disease and different virus diseases, are approved or in clinical trials, reviewed in (Glennie and Johnson, 2000).

The first attempts with antibody therapy were performed with murine antibodies, which whose efficacy appears to be hindered by an immune response denoted human anti-mouse antibodies (HAMA), preventing repeated treatment. As mentioned earlier, the production of human monclonal antibodies by hybridoma technology has been unsuccessful. Hence alternatives like chimeric or humanized antibodies have been constructed, where portions like Fab regions or only CDR regions, respectively, of murine antibodies are grafted into a human antibody framework (Clark, 2000; van Dijk and van de Winkel, 2001). However, fully human antibodies would be the goal and as described earlier, transgenic mice (XenoMice®) expressing fully human antibodies (Green, 1999) has opened up the possibility for efficient hybridoma production of human antibodies.

A number of potential mechanisms has been identified to be related with antibody treatments (Glennie and Johnson, 2000), such as blocking cell-to-cell signalling by binding, for example, soluble growth factors and cytokines or receptors. Upon binding, the free Fc part could signal to activate the complement system, resulting in lysis of the cell, and could also recruit cellular effector cells, like natural killer (NK) cells (Clynes et al., 2000). Radiolabeled antibodies for radioimmunotherapy using antibodies labeled with yttrium or iodine isotopes are also being developed and tested in clinical trials (Goldenberg, 2001). Indirect use of antibodies in the field of therapy for example tumor localization, so called tumor imaging are also explored (Chester et

al., 2000; Kalofonos et al., 2001). During the last decade, novel techniques for the generation of fully human antibodies have been developed, based on protein library technologies (see chapter 4).

4. Novel technologies for development of binding proteins

In recent years, there has been a rapid development of different technologies for construction, expression and handling of large collections of proteins. Progress on various systems for physical linking the genotype and phenotype of each member in such libraries has made *in vitro* and *in vivo* selection by function, as well as selection for specific characteristics, possible. This includes the construction of so called display libraries of both antibody fragments and other proteins, which has been evaluated for generation of novel affinity ligands for applications including immunotechnology. Such ligands could serve as alternatives to antibodies generated by immunization of animals.

Several novel techniques for displaying libraries has been presented over the last years, starting with the *in vivo* phage display technology developed by Smith in 1985 (Smith, 1985). Here, bacteriophages are used for surface exposure of molecules by genetic insertion of foreign proteins into the native viron. The bacteriophage is thus providing a link between phenotype and genotype. Today, the *E. coli* filamentous phage M13 is most commonly used, but other systems based on for example phage λ or T7 has been reported, reviewed in (Benhar, 2001). The single-stranded DNA molecule, encoding all phage proteins, is surrounded by an envelope consisting of several thousand identical α-helical coat proteins (protein VIII) and a few minor proteins (protein III), responsible for infection of host cells, are capping the end of the phage (Marvin, 1998). Most systems are based on fusions to the minor coat protein using either whole phage dsDNA vectors or phagemid DNA, containing an expression cassette and signals for packing of DNA. The latter requires that helper phages are used for infection of phagemid containing cells, to provide all other phage proteins needed for phage particle formation (Fig. 6). When using whole phage systems every coat protein will be fused to the foreign protein, whereas a phagemid system does not provide more than a few fusion proteins among the native coat proteins.

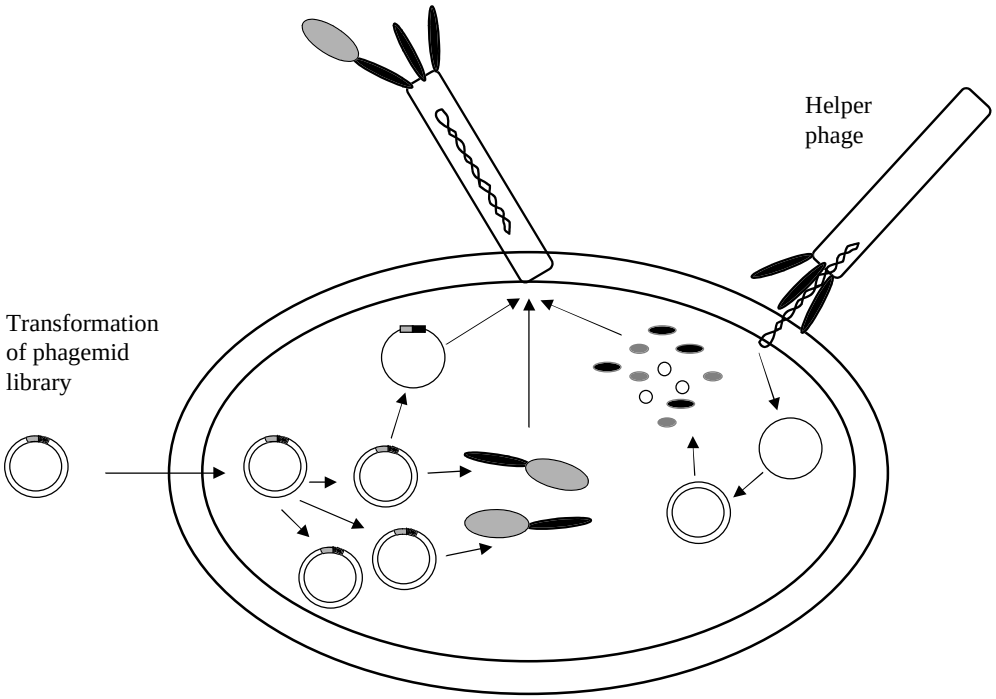


Fig. 6. Phage particle formation using a phagemid system. A transformed phagemid vector provides protein III fusion proteins and the helper phage all other proteins necessary for assembly of new phage particles.

The basic procedure (Fig. 7) for selecting library members from a phage display library involves repetitive cycles of exposure of the library to a desired target followed by the use of recovered phage particles for reinfection of host cells (Smith and Petrenko, 1997). By that, the subpool of phage clones containing potential ligands is amplified for a new round of selection. This scheme is typically repeated four to five times. Such selection, or biopanning, can be performed towards targets for example on solid supports, present as labeled molecules in solution or present on fixed cells and tissue sections. Elution of interacting phage clones can be performed using methods, such as incubation with acidic or basic solutions solutions, target competition or enzymatic cleavage at introduced cleavage sites (Hoogenboom et al., 1998). The outcome of the selection procedure is a mixture of ligands of diverse characteristics and analyses of true target-interacting variants can be performed by different means, including ELISA assays using phage particles or soluble proteins produced directly from the vector used during selection (phagemid systems), or after subcloning to an expression vector.

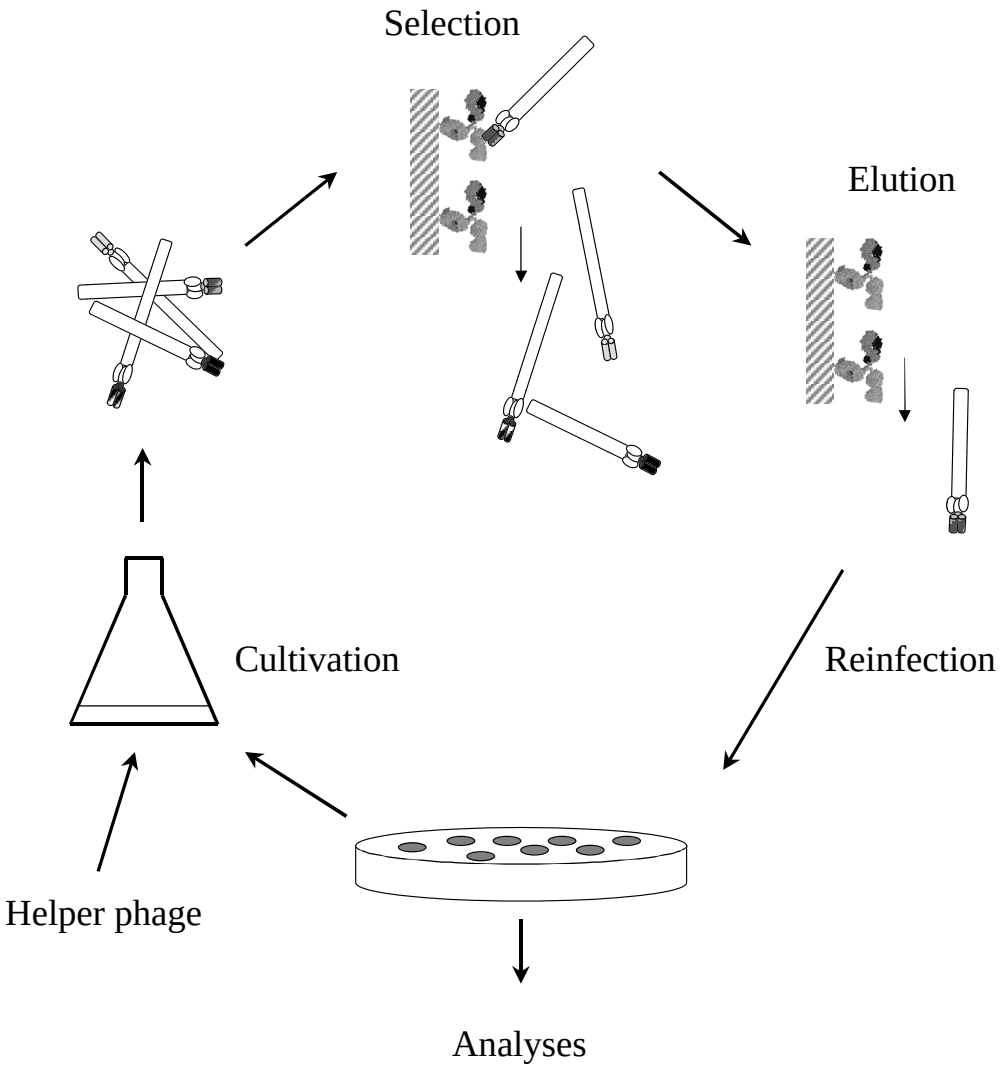


Fig. 7. The basic procedure for one round of selection using a phagemid based system.

An often discussed parameter is the size of the constructed library. It is obviously desired that a full sampling of the theoretical number of possible library member variants be obtained during library construction. Depending on the situation, this number is not always known but generally, the larger the library (i.e. containing more variants), the more likely it is that variants having desired characteristics can be isolated. However, the construction involves the transformation of bacterial cells with DNA, which in many cases sets technical hurdles for how large libraries can be constructed. Most described libraries are in the range of 10^6-10^9 variants, but larger libraries have also been described reaching over 10^{10} members (Vaughan et al., 1998). Protein classes for which libraries have been constructed and functionally been displayed on phage include for example cytokines, receptors, enzymes, protease inhibitors, antibody fragments, DNA binding proteins, cDNA encoded proteins (Crameri and Blaser, 1996) and several different peptides (Clackson and Wells, 1994; Benhar, 2001).

Numerous antibody libraries have been constructed since the first library was described in 1989 (Huse et al., 1989), using different sources for the antibody encoding genes, including molecular harvesting of genes encoding variable regions from natural (immune or naïve) donors or by in vitro cloning of "man-made" (or synthetic) repertoires (Griffiths and Duncan, 1998; Skerra, 2000). Immune libraries could for example be created from fragments encoding the heavy and light chain variable regions of Ig genes from spleen B-cells of an immunized donor, resulting in repertoires biased towards the set of antigens to which an immune response were induced, giving raise to a small library of high affinity antibodies. The use of immunized human donors is limited, but animals like mouse, chicken, rabbits and camels (Arbabi Ghahroudi et al., 1997; Griffiths and Duncan, 1998) has been used for immunization. More diverse libraries have been obtained from the naïve set-up of antibody encoding genes from a non-immunized donor. Such libraries thus facilitate the identification of antibodies to several different antigens, without the need for immunization, which is of special interest for human antibody libraries.
The third group of libraries are the synthetic antibody libraries, where the antibody encoding genes have been constructed artificially for example through the grafting *in vitro* of synthetic CDR encoding fragments into a set of designed antibody frameworks (Krebs et al., 2001). Similar principles have been used for the grafting of natural CDR encoding regions into predefined framework fragments (Söderlind et al., 2000).

Libraries of non-immunoglobulin proteins/peptides have also been used for the development of affinity reagents. Such approaches include libraries of random peptides, typically between 4 and 40 residues in length which could either be of linear or cyclic formats, through the formation of disulfides between introduced cysteins flanking the variegated window (Smith and Petrenko, 1997). Such libraries have been used for variety of applications, including antibody epitope mapping analyses (Irving et al., 2001) and identification of peptides serving as antagonists to interleukin-1 (IL-1) or agonists to the EPO and TPO receptors (Dower, 1998), capable of binding to cell-surface markers like integrins (Brown, 2000), peptides targeting different organs (Pasqualini and Ruoslahti, 1996), peptides having potential use in targeted drug delivery applications (Signore et al., 2001) or peptides for use as affinity ligands in bioseparation (Huang et al., 1996; Krook et al., 1998).

Another approach for design of combinatorial libraries has been to use proteins as framework, where a suitable number of surface located amino acids has been subjected to mutagenesis. Depending on the choice of starting structure, ligands selected from such libraries could have several advantages compared to peptide or antibody fragments in different applications. Compared to flexible peptides, proteins could be expected to have a more organized structure, contributing to higher proteolytic stability also making it more likely to maintain its binding activity when fused to other proteins or after immobilization on a solid support. A protein could also be expected to present hydrophobic residues without significant structural changes. In addition, selection of ligands with higher affinity would be expected, due to smaller loss of entropy upon binding to the target (Ladner, 1995).

Some considerations should be made when choosing a protein as a scaffold (framework) for construction of combinatorial libraries (Nygren and Uhlén, 1997; Skerra, 2000). Ideally the protein should be monomeric, not depending on disulfides

for its structure, relatively small to facilitate engineering and possible to produced in prokaryotic systems (Nord et al., 1997). Further, it would be desirable if the protein has a high tolerance to amino acid substitutions and is compatible with the selection system intended to use. Several different proteins have been used as scaffold for library design during the past years, differing in size, structure and origin. Surfaces of different topologies has been subjected to combinatorial mutagenesis (Table 1) (Nygren and Uhlén, 1997; Skerra, 2000; Ladner and Ley, 2001). Some examples includes anticalins (ca. 18 kDa), derived from a butterfly bilin-binding protein in which residues in the bilin binding cavity have been addressed for engineering (Beste et al., 1999), human fibronectin-variants (25 kDa), in which surface-exposed loops have been engineered (Koide et al., 1998), small fungal disulfide-stabilized cellulose binding domains (ca. 4 kDa), denoted "knottins" where different parts of the wedge-shaped protein has been addressed (Smith et al., 1998; Lehtiö et al., 2000) and the bacterial three-helix bundle Z domain (6 kDa) where positions located to the immunoglobulin binding face of the domain have been engineered (Nord et al., 1995; Gunneriusson et al., 1999; Hansson et al., 1999; Nord et al., 2000; Nord et al., 2001; Eklund et al., 2002) (I-V).

Table 1. Examples of scaffold engineering approaches.

Scaffold protein (origin)	**Size**	**Structure**	**Binding activity of wild type protein**	**Engineering principle**	**References**
Lipocalin *(Pieris brassicae)*	174 aa	β-barrel	bilin	Replacement, loops	(Beste et al., 1999; Schlehuber et al., 2000)
CTLA-4 (human)	16 kDa	Ig domain-like	CD80/CD86	Replacement, loops	(Nuttall et al., 1999; Hufton et al., 2000)
Z domain *(S. aureus)*	58 aa	Three-helix bundle	Ig (Fc)	Replacement, α-helices	(Nord et al., 1997; Gunneriusson et al., 1999; Hansson et al., 1999; Nord et al., 2000)
Cellulose binding domain *(T. reesei)*	36 aa	Knottin	Cellulose	Replacement, β-turn/β-sheet	(Smith et al., 1998; Lehtiö et al., 2000)
Fibronection (FN3 unit) (human)	94 aa	Ig domain-like	Cell-cell interactions	Replacement, loops	(Koide et al., 1998)

Abbreviations: Ig, Immunoglobulin, Fc, fragment crystallizable, aa, amino acids, *T. reesei*, *Trichoderma reesei*, *S. aureus*, *Staphylococcus aureus*, bp, base-pairs.

As alternatives to the phage display technology several other techniques for the construction and handling of protein libraries have been presented, including the use of other microrganisms (Benhar, 2001; Samuelson et al., 2002) such as *E. coli* bacteria (Fuchs et al., 1991; Lu et al., 1995) and yeast (Boder and Wittrup, 1997). In addition to such systems, alternative principles for coupling of genotype and phenotype have been presented, obviating the need for transformation of cells for expression of library members, which facilitates the construction of large libraries. Such systems, denoted *in vitro* display technologies, instead rely on the translation of library member encoding mRNAs in cell extracts prepared from for example bacteria or rabbit reticulocytes, depending on the system design. Such systems include ribosomal display (Hanes and Plückthun, 1997), mRNA display (Roberts and Szostak, 1997) and covalent display (FitzGerald, 2000), where a genotype-phenotype coupling is achieved via mRNA-ribosome-protein, mRNA-puromycin-protein and DNA-protein complexes, respectively. This class of systems also include the use of artificial cell-like compartments based on oil-water emulsions for the physical linkage of corresponding DNA-protein pairs (Tawfik and Griffiths, 1998; Doi and Yanagawa, 1999). A number of various other systems for selection of proteins with desired binding characteristics from libraries have also been described including plasmid display, recruiting DNA-binding proteins as library member fusion partners (Cull et al., 1992; Speight et al., 2001), periplasmic expression library (PECS) technology relying on the diffusion of labeled targets into the periplasm of ligand expressing bacterial cells allowing flow cytometry screening (Chen et al., 2001) and protein contact assay (PCA), relying on target-ligand interaction dependent reconstitution of the enzyme activity of a fragmented enzyme necessary for survival in selective media (Pelletier et al., 1999; Mössner et al., 2001). Further, affinity ligands have also been selected from libraries of oligonucleotide libraries (Gold et al., 1995). Such ligands denoted aptamers are thus composed of nucleotide building blocks rather than amino acids, and have been shown to be selectable to various target proteins including nucleic acid binding proteins such as polymerases and transcription factors, non-nucleic acid binding proteins such as growth factors, as well as small organic molecules such as ATP and theophylline (Gold et al., 1995; Green et al., 1995; King et al., 1998; Jhaveri et al., 2000).

Present investigation

5. Construction of and selection from affibody libraries (I and II)

5.1 The Z domain scaffold

The work in this thesis is based on the immunotechnological use of affinity ligands (denoted affibodies) selected from protein libraries based on the so-called Z domain, utilized as scaffold for library constructions. This Z domain is a 58 aa non-cystein protein consisting of three anti-parallel α-helices forming a bundle structure. The Z domain corresponds to an engineered variant of the native B domain of staphylococcal protein A, in which an Asn_{28}-Gly_{29} dipeptide sequence, sensitive to fusion protein cleavage by hydroxylamine, has been changed into an Asn-Ala sequence. In addition, an alanine residue at position one has been changed into a valine (Nilsson et al., 1987). This Z domain has been frequently used as affinity fusion partner for the production of either Z or ZZ-fusion proteins in a number of different host cells (Ståhl and Nygren, 1997). Utilizing the IgG binding capacity of the Z domain, such fusion proteins have been possible to recover and immobilize using IgG-containing matrices or surfaces (Ståhl and Nygren, 1997). The Z domain has a high solubility, which has been exploited to decrease problems with protein aggregation during protein refolding procedures (Samuelsson et al., 1991). All the five native SPA domains show binding to Fc regions of IgG (Moks et al., 1986) and to antibodies of different isotypes from a number of animal species (Langone, 1982). In addition, these domains also possess a binding activity towards VH regions of certain subpopulations of antibodies containing VH regions belonging for example to the human VHIII family. Recently, data from X-ray diffraction analysis of the co-crystal complex between the D domain of SPA and a Fab fragment, has shown that the Fc and VH binding activities are structurally separated and recruit residues of helices one and two or two and three, respectively (Graille et al., 2000). Assuming a similar situation also for the other homologous SPA domains, the considerable lower VH binding demonstrated for the Z domain (Jansson et al., 1998) can be explained by the Gly_{29}-Ala substitution, corresponding to the introduction of a larger amino acid in the protein-protein contact surface.

5.2 Libraries of the Z domain for phage display selection of novel ligands

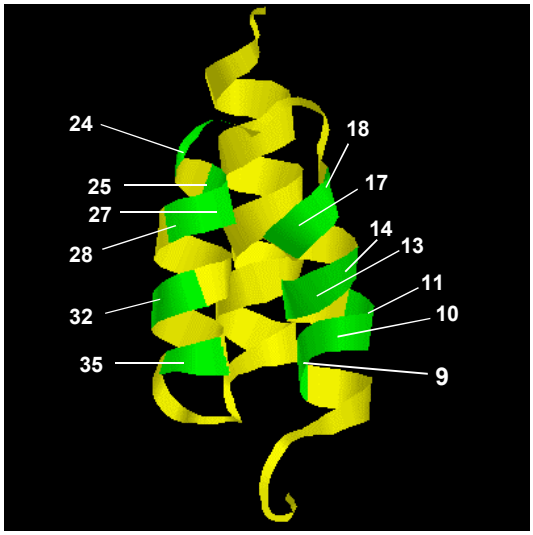
The features of the Z domain discussed above, suggested that the domain could be an ideal scaffold for a combinatorial protein engineering approach, directed to surface located residues, to obtain variants of the Z domain having desired binding specificities, other than the IgG Fc binding activity of wild type domain. For selection of the location and number of amino acid positions to be included for variegation, the co-crystal structure between the B-domain of SPA and Fc of human IgG1 determined by Deisenhofer in 1981 was investigated (Deisenhofer, 1981). Since it was desired that entirely new binding specificities should be obtained by the approach, positions involved in the native binding interaction were considered for mutagenesis. Literature data suggested that eleven residues of the B domain were involved in the interaction

with IgG1 Fc, located to the first two helices of the domain. Nine of these (Q9, Q10, N11, F13, Y14, L17, N28, Q32 and K35) were located to α helical regions at the molecular surface and therefore included in the mutagenesis. Isoleucine 31, although suggested to be involved in the IgG Fc binding, was not considered based on earlier described substitution mutagenesis studies of the Z domain showing this residue to be of structural importance (Jendeberg et al., 1995). In addition, the F5 position also suggested to be involved in the binding, but located outside the helical regions, was excluded. However, four other additional residues in this region of the domain (H18, E24, E25 and R27) were included based on their location at the molecular surface. Thus, totally thirteen amino acid positions, all located in the first two helices of the three-helix bundle Z domain, were included in the combinatorial protein engineering strategy (Fig. 8).

A solid phase library assembly method was used for construction of gene pools encoding variegated helix 1/2 regions. This method had earlier been evaluated in pilot library constructions and been found to work efficiently (Nord et al., 1995). Using paramagnetic streptavidin-coated beads as solid support for a first biotinylated double stranded anchor gene fragment, two single-stranded oligonucleotides each encoding one of the two variegated helices were pre-ligated in solution to one another with the aid of a bridging complementary oligonucleotide, before added to the beads for ligation to the anchor fragment. A third double stranded segment was finally ligated to the bead-immobilized construct. The bead immobilized assembly product was subsequently employed as PCR template for amplification yielding material of sufficient quantity for further cloning work (Fig. 9).

Two different libraries were constructed, based on the use of two different degenerate codons at the thirteen positions chosen for randomization. In one of the libraries (Zlib-1), NN(G/T) codons were used whereas in the second library (C/A/G)NN codons were used (Fig 8). The NN(G/T) codon includes 32 codons, including codons for all 20 amino acids and the TAG amber stop codon. Although the (C/A/G)NN codon includes a larger number of combinations (48 of the 64 possible codons), fewer amino acids are encoded by this codon. In addition, no stop codon is included and cysteins excluded, which is considered an advantage to avoid unwanted dimerizations between affibodies during selection and analyses. However, codons for the aromatic amino acids Tyr, Trp, Phe are excluded as well.

The theoretical number of affibody variants possible to obtain using the NN(G/T) codon at 13 positions in Zlib-1 is 20^{13}=8.2 x 10^{16}, encoded by 3.7 x 10^{19} (32^{13}) genetic variants. A similar calculation for the Zlib-2 library yields 16^{13}=4.5 x 10^{15} variants encoded by 7.2 x 10^{21} (48^{13}) genetic variants. However, on the protein level, these large numbers of clones are difficult to generate using selection systems dependent on transformation of cells. Further, a stoichiometric limitation lies in the amount of anchoring DNA fragments used during gene library assembly. In both cases approximately 15 pmol fragment were immobilized onto the beads, corresponding to approximately 2x10^{10} fragments which could serve as starting points for the same number of variants.



Zlib-1

NN(G/T)

TTT TTC TTA TTG	Phe Leu	TCT TCC TCA TCG	Ser	TAT TAC TAA TAG	Tyr STOP STOP	TGT TGC TGA TGG	Cys STOP Trp
CTT CTC CTA CTG	Leu	CCT CCC CCA CCG	Pro	CAT CAC CAA CAG	His Gln	CGT CGC CGA CGG	Arg
ATT ATC ATA ATG	Ile Met	ACT ACC ACA ACG	Thr	AAT AAC AAA AAG	Asn Lys	AGT AGC AGA AGG	Ser Arg
GTT GTC GTA GTG	Val	GCT GCC GCA GCG	Ala	GAT GAC GAA GAG	Asp Gln	GGT GGC GGA GGG	Gly

Zlib-2

(C/A/G)NN

TTT TTC TTA TTG	Phe Leu	TCT TCC TCA TCG	Ser	TAT TAC TAA TAG	Tyr STOP STOP	TGT TGC TGA TGG	Cys STOP Trp
CTT CTC CTA CTG	Leu	CCT CCC CCA CCG	Pro	CAT CAC CAA CAG	His Gln	CGT CGC CGA CGG	Arg
ATT ATC ATA ATG	Ile Met	ACT ACC ACA ACG	Thr	AAT AAC AAA AAG	Asn Lys	AGT AGC AGA AGG	Ser Arg
GTT GTC GTA GTG	Val	GCT GCC GCA GCG	Ala	GAT GAC GAA GAG	Asp Gln	GGT GGC GGA GGG	Gly

Fig. 8 Above: A representation of the Z domain and the thirteen solvent exposed residues that were chosen for random mutagenesis in the construction of the Z libraries. **Below**: Codon representations of the two different degenerated codons used in the libraries.

After PCR amplification, PCR products were purified, cleaved and ligated to an expression cassette in the phagemid vector (pKN-1) (Fig. 9) designed for monovalent display of affibody variants, fused to a truncated version of protein III, on filamentous phage particles. This vector already contained a gene fragment encoding the non-variegated helix 3 of the Z domain. Also included in the expression cassette, in front of an amber stop codon preceding the protein III gene, was a gene fragment encoding a 46 amino acids albumin binding domain derived from streptococcal protein G, useful for convenient affinity recovery of selected affibodies after selection and subsequent expression in a non-suppressor strain using the same vector used during selection. The expression from the cassette was driven by the *E. coli lac* promoter, and an *E. coli* Omp A-signal peptide was recruited for periplasmic localization of the gene products.

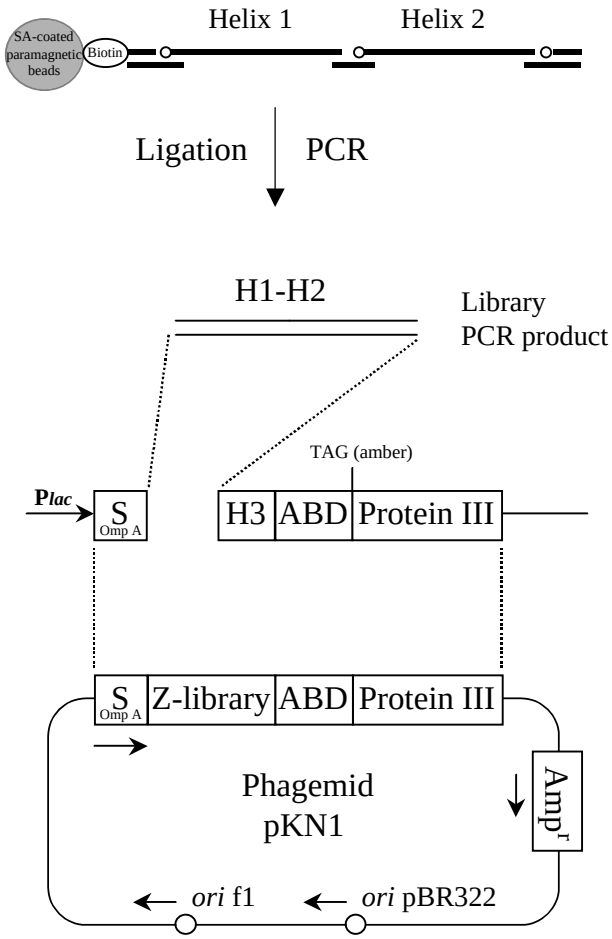


Fig. 9. Construction of the phagemid libraries. A pool of genes encoding randomized helices 1 and 2 were assembled using streptavidin coated paramagnetic beads as solid supports, PCR amplified and ligated into the phagemid vector in frame with the gene encoding the third unvariegated helix.

Transformation of 50 aliquots of the ligation mixture onto separate agar plates yielded approximately 4 x 10^7 and 4.5 x 10^7 transformants, respectively, which were separately pooled to yield Zlib-1 and Zlib-2, respectively. The two libraries were subsequently used for selections to three different target proteins (human insulin, human recombinant apolipoprotein A-1 and *Thermus aquaticus* DNA polymerase) after preparation of phage stock solutions using standard helper phage-based protocols. In these selections, the Zlib-1 library was used for selections to all three targets whereas the Zlib-2 library was only used for selection towards the apolipoprotein A-1 target. To all three target proteins used in these first selections, affibody ligands could be identified which showed affinities (K_d) for their respective targets in the range of 3 x 10^{-5} - 1 x 10^{-6} M. For the apolipoprotein A-1 target, affibodies were identified from both libraries. An analysis by circular dichroism of

three selected affibodies showed that these had secondary structure contents in parity with the wild type Z domain used as scaffold for the library constructions. Taken together, these initial selection experiments indicated that the two libraries had the potential to serve as general sources of new affinity ligands to desired target proteins.

5.3 Selection of affibodies to human IgA

As discussed earlier, the wild type staphylococcal receptin protein A (SPA) has been extensively used as an immunotechnology tool for the detection and purification of immunoglobulins due to its capability of binding to immunoglobulins of IgG isotype. The availability of similar reagents, each specific for one of the other human isotypes would constitute a valuable collection of immunotechnology tools useful in various applications. In fact, among other identified bacterial receptins some have been described to bind other isotypes than IgG, including IgD and IgA (Kronvall and Jönsson, 1999). In the study performed here, the focus was to investigate if the constructed Z-libraries could be used to identify affibody variants capable of selective recognition of human IgA.

IgA is the most abundant immunoglobulin isotype in humans and is mainly located in mucosal surfaces and is also present at high levels in plasma, approximately 1-3 mg/ml (Mestecky and McGhee, 1987). There are two subclasses of IgA, (IgA1 and IgA2) differing in the hinge region between CH1 and CH2 (Mestecky and McGhee, 1987). In addition, when IgA is actively transported to the mucosal surface, linkage of the secretory component and the J-chain with two IgA molecules results in secretory IgA (sIgA). Dimers linked only by J-chain is also present in plasma, but to a much lesser content than monomeric IgA. IgA is believed to play an important role in the defence against infections in mucosal tissues (Lamm, 1997), but IgA also recruits neutrophils efficiently, thus could be of major interest in therapy, for example, in dental care (Ma et al., 1998), in passive protection against viral diseases (Weltzin and Monath, 1999) or in cancer treatment (Dechant and Valerius, 2001). IgA has, as well as other immunoglobulins, been recombinantly expressed in a range of different systems (reviewed in (Chintalacharuvu and Morrison, 1999)), for example, in Chinese hamster ovary and COS cells, insect and plant cells as well as in transgenic animals.

For selection experiments, two myelomas generated IgAs of subclass one, was *in vitro* biotinylated for subsequent solid phase selection on streptavidin coated beads. The myelomas was used alternatively during selections (Fig. 10) of the Z libraries, with aim to direct variants to constant parts of the IgA molecule. In the first cycle, the libraries were applied separately and in the following four cycles a mixture of both was used.

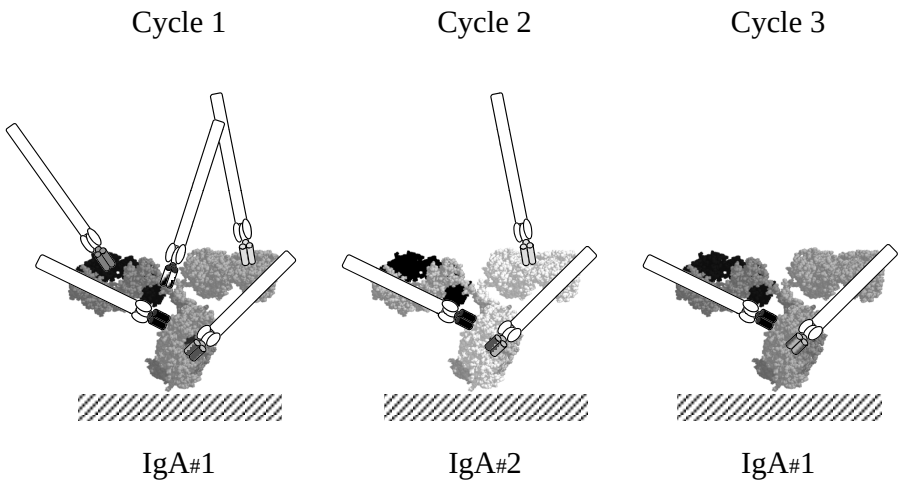


Fig. 10. The principle for the biopanning against human IgA (V). Efforts were made to select phage variants recognizing constant domains by using different myeloma IgAs as targets in an alternating fashion.

5.4 Binding characteristics of selected ligands

Ten randomly picked clones after the fifth selection cycle were used for DNA sequencing analysis of their respective variegated regions. Two different variants were represented twice, and six were unique but with some similarities in some of the variegated positions (II, Fig. 1). Two clones showed aa patterns used for randomization in Zlib-1 (Z_{IgA6} and Z_{IgA7}) and the other clones originated from Zlib-2. All eight variants were expressed as ABD fusion proteins in a non-suppressor *E. coli* strain and purified on HSA sepharose from periplasmic fractions. Purified proteins were thereafter analyzed for IgA binding using biosensor technology. After indirect immobilization onto sensor chip surfaces coated with human serum albumin (HSA) IgA binding was investigated using injections of myeloma IgA (II, Fig. 2). Five of the eight variants were shown to bind IgA, indicating that the selection had worked, and three of those were further analysed for affinity determinations. One of the IgA-binding affibody ligands (hereafter called Z_{IgA1}) was shown to have a K_d value of 0.5 x 10^{-6} M and was chosen for further studies. Biosensor binding studies showed no detectable interaction between Z_{IgA1} and the other human immunoglobulin istotypes (IgG, IgM, IgD and IgE) whereas polyclonal IgA, secretory IgA, composed of two IgA moieties connected by the J-chain and the secretory component, and the samples of the two subgroups IgA_1 and IgA_2 were all binding to the Z_{IgA1} affibody. This indicated that the Z_{IgA1} affibody recognized a constant part of the IgA molecule

accessible also in the more complex IgA forms. Since both subgroups were recognized by Z_{IgA1}, the hinge region differing both in length and composition between IgA_1 and IgA_2, was presumably not significantly involved in the binding. These findings suggested that the IgA ligand could be useful as a general immunotechnology tool for IgA detection and purification.

5.5 Affinity recovery of human IgA from complex samples

The Z_{IgA1} affibody was chosen for further studies, involving chromatographic recovery of human IgA from different samples. This would contribute to the assessment of the binding selectivity of the affibody in regard to possible background binding to other proteins than immunoglobulins. For a first experiment a divalent $(Z_{IgA1})_2$-ABD ligand was constructed and produced followed by covalent immobilization onto a NHS-activated Sepharose medium recruiting primary amines in the ligand. The strategy to use a divalent ligand could be advantageous from several perspectives. A divalent ligand contains two binding moieties which increases the likelihood that at least one moiety of the ligand is biologically active after the covalent coupling procedure. Due to the symmetric nature of the IgA target protein, a divalent ligand could also result in avidity effects during chromatography. In addition, it is possible that a beneficial effect on the presentation of the immobilized ligand to the surrounding solution is obtained if a larger ligand is used (spacer effect).

An *E. coli* total cell lysate spiked with myeloma IgA to a concentration of 0.1 mg/ml was applied onto the column followed by washing and elution. The eluted fraction was shown to contain protein of sizes corresponding to the heavy and light chain of IgA, with no other major bands (II, Fig. 5). These findings encouraged further studies using human plasma as sample. For this experiment, a new ligand was designed since the ABD moiety of the $(Z_{IgA1})_2$-ABD ligand used previously would result in co-purification of serum albumin present at high concentrations in plasma. Thus, a $(Z_{IgA1})_2$-His_6 ligand was constructed containing a hexahistidyl tag facilitating its purification from *E. coli* cultures.

This new affinity column was exposed for unconditioned human plasma and an analysis by SDS-PAGE of the eluate showed that proteins corresponding to expected sizes of light chains and IgA heavy chains were recovered (II, Fig. 7). The human plasma, column flow-through and eluted proteins were further analysed for IgA, IgG and IgM isotype contents by immunoprecipitation (nephlometry). Results from those analyses indicated efficient depletion of IgA from the plasma sample and showed high concentrations of IgA in the eluted protein fraction. In contrast IgG and IgM concentrations were high in flow-through fractions, but could not be detected in the eluted fractions by this method. However, a more sensitive Western blot analysis using anti-isotype HRP-conjugated antibodies indicated small amounts of IgG and IgM in the eluate. This could possibly be explained by the presence of natural immunoglobulin-specific autoantibodies in healthy individuals, resulting in the formation of circulating immune complexes (CIC). Such natural autoantibodies have been described to be of IgM, IgG and IgA isotype (Lacroix-Desmazes et al., 1998). In addition, anti-protein A antibodies recognizing the non-variegated parts of protein A-derived affibody ligand structure could also be present in the plasma, as a result of previous bacterial infections of the plasma donor.

In conclusion, two different libraries, Zlib-1 and Zlib-2, based on the SPA-derived Z domain were constructed for display on filamentous M13 phage for selection of novel affinity proteins. Both these libraries were used for selection of ligands towards human immunoglobulin A, resulting in several binding variants. One of the affibodies was shown to bind human polyclonal IgA, IgA_1 and IgA_2 subgroups as well as secretory IgA with no detectable cross-reactivity towards the other immunoglobulin isotypes. This affibody was, in a divalent format, demonstrated useful as an immunotechnology tool for affinity recovery of IgA from spiked *E. coli* lysate and also from unconditioned human plasma.

6. Fusions of affibodies to other protein domains (III, IV)

Earlier studies have shown that wild type or engineered SPA domains can be genetically fused to other proteins with a retained immunoglobulin binding capacity, suggesting that the individual three-helix bundle domains have a structural integrity allowing them to fold relatively independently of a fused protein. For example, it has earlier been shown that the wild type Z domain can be functionally fused at different multiplicities to numerous other proteins for various applications (Ståhl and Nygren, 1997). The Z-domain origin of affibodies thus suggested that fusion proteins, in which affibodies of desired specificities could be employed as discrete affinity modules, should be possible to produce. Two such approaches in the field of immunotechnology have been investigated.

6.1 Affibody-Fc fusion proteins (III)

As discussed earlier, an antibody molecule can be divided into separate regions conferring antigen binding and effector functions, respectively. Whereas the Fab-regions contain regions responsible for antigen binding, the Fc fragment has several characteristics making it important immunologically *in vivo* but also useful in *in vitro* immunotechnology applications. Depending on the isotype, Fc fragments can recruit different effector functions through binding to complement factor C1q or Fc receptors present on for example macrophages, mast cells or neutrophiles, desired for elimination of pathogens but also in some cases undesired such as in allergy reactions. For IgG1, such complement trigging or Fc-mediated effector functions have been shown to rely on Fc glycosylation (Jefferis et al., 1990; Nose et al., 1990; Leader et al., 1991). However, binding to protein A (Jendeberg et al., 1997) or to the neonatal receptor (FcRn), the latter important for escaping rapid *in vivo* clearance by proteolysis and thus contributing to the long *in vivo* circulation half-life of IgG, does not require glycosylation (Tao and Morrison, 1989).

In gene fusion constructs, collectively denoted immunoadhesins, Fc fragments have been employed for production, purification and presentation of N-terminally fused non-immunoglobulin proteins. In the majority of such constructs, the hinge and Fc fragment (i.e. hinge-CH2-CH3 portions) of human IgG1 have been combined with extracellular domains of receptors or adhesion molecules, to create antibody-like molecules with desired binding features for research and potentially therapeutic applications (Chamow and Ashkenazi, 1996). Thus, in such constructs, the "antigen"

binding is conferred by non-immunoglobulin domains of predefined specificities, which are linked to an appropriate effector function and structure in terms of for example multimericity, depending on the choice of isotype. For example CD4 immunoadhesins have been constructed using different Fc isotypes including IgG (Capon et al., 1989), IgE (Krauss et al., 1995) or IgM (Traunecker et al., 1989) resulting in constructs of different multiplicities and capabilities in regard of recruiting effector functions. Immunoadhesins based entirely on human subfragments have been proposed as an alternative to human monoclonal antibodies for immunotherapy applications, requiring however that relevant natural binding proteins can be identified.
Research applications in which the structures of the molecules have been employed, besides a possibility to purify the constructs on protein A/G media, includes for example studies on receptor ligand binding such as between interferon-γ and its receptor (Haak-Frendscho et al., 1993), protein localization (LaRochelle et al., 1995), *in vivo* protein inhibition (Liao et al., 1997) and investigations on ligand binding determinants (Heidaran et al., 1995). A different class of Fc fusion proteins are composed of scFv fragments genetically fused to Fc. Such chimeras have been investigated for use in for example melanoma immunotherapy (Wang et al., 1999) and to investigate mitogenic properties of bispecific reagents (Connelly et al., 1998).

6.1.1 Construction and characterization of affibody Fc chimeras (III)

To investigate the characteristics of immunoadhesins based on the use of affibodies as antigen binding moieties different affibody-Fc fusion proteins were constructed. The Fc fragment denoted Fc3(1) recruited for these constructs was a modified variant of human Fc3 engineered to be functionally equivalent to Fc of IgG1 in terms of staphylococcal protein A binding. This protein had earlier been demonstrated to be possible to express as a functional homodimer in the *E. coli* periplasm (Jendeberg et al., 1997).

Two previously selected affibodies to *Taq* DNA polymerase ($Z_{Taq4:8}$) (Nord et al., 1997, I) and to surface protein G of Respiratory syncytial virus (RSV) (Hansson et al., 1999) (Z_{RSV1}) were used as model affibodies and genetically fused N-terminally to the to Fc3(1), via an introduced seven residues linker region (Fig. 11). The latter affibody was also used as a divalent construct during cloning, potentially resulting in tetravalent affibody-Fc molecules (Fig. 11). For comparison of binding characteristics, mono or divalent affibody-ABD fusion constructs were also constructed. All constructs were expressed by secretion to the periplasm of *E. coli* and affinity purified using either protein A (Fc-binding) or HSA (ABD binding) affinity columns.

SDS-PAGE analyses of affinity purified affibody-Fc proteins (III, Fig. 2) showed that whereas the Z_{RSV1}-Fc3(1) fusion was purified to near homogeneity, the two other constructs showed signs of inter-domain proteolytic degradation, suggesting that the sequences of connective linker regions for this type of constructs could be improved. However, all constructs showed to form homodimers of expected sizes. A series of biosensor binding experiments were performed to characterize the different fusion proteins. First, both the Z_{RSV1}-Fc3(1) and $Z_{Taq4:8}$-Fc3(1) constructs could be demonstrated to bind their respective targets in a specific manner, indicating a

functional presentation of the affibody moieties. In addition, binding avidity studies on different $Z_{Taq4:8}$-containing constructs showed that compared to the presentation of affibodies as divalent "head-to-tail" di-affibody-ABD fusion constructs, the Fc presentation format resulted in more pronounced avidity effects (III, Fig. 4B). A comparison of the divalent Z_{RSV1}-Fc3(1) and tetravalent $(Z_{RSV1})_2$-Fc3(1) constructs showed that the addition of a second affibody on each arm resulted in an increased avidity effect, indicating that two affibodies fused head-to-tail could be functionally presented on each Fc3(1) fragment retaining the binding activities of both constituents after fusion (III, Fig. 4A).

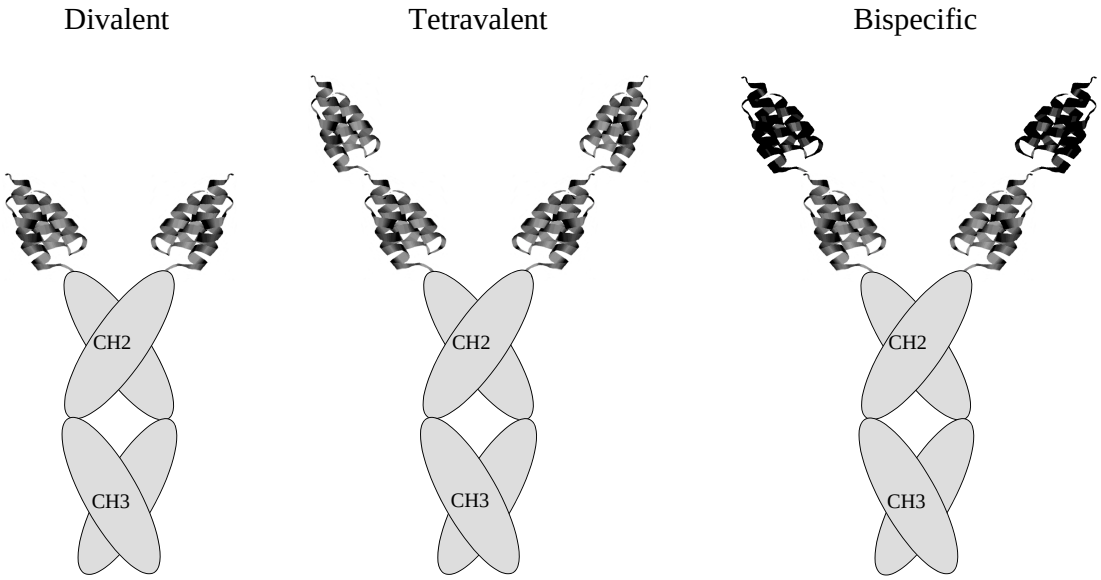


Fig. 11. A generalized picture of affibody-Fc fusion proteins. Divalent (A) and tetravalent (B, C) constructs. Hypothetical structure of bispecific affibody-Fc fusions, using two affibodies in tandem, binding different targets (C).

6.1.2 Western blotting using an affibody-Fc chimera

One immunotechnology application for affibody Fc chimeras could be as easily produced reagents for detection analyses such as Western blot or ELISA analyses. In such settings, the affibody would be recruited as the target recognition moiety and the Fc part as the reporter protein, capable of being bound by a second reagent for amplification of the signal, such as enzyme-labeled antibodies or protein A. To investigate this, the Z_{RSV1}-Fc3(1) chimera was evaluated as antigen recognizing reagent in a Western blotting application. Using a biosensor experimental set-up, it could first be demonstrated that a standard HRP-conjugated anti-human IgG reagent could be used for detection of the Z_{RSV1}-Fc3(1) chimera bound to sensor chip immobilized RSV-derived target protein. Using recombinantly produced RSV antigens of two viral subgroups, either as purified proteins or as spiked into an *E. coli*

lysate, it could be demonstrated that the Z_{RSV1}-Fc3(1) construct corresponding to an *E. coli* produced "artificial" monoclonal antibody reagent could be used for selective recognition of the antigen in a Western blotting format, also in crude bacterial protein samples.

The possibility to produce these different affibody-Fc fusion proteins in *E. coli* as properly assembled homodimers suggests that affibodies as such are well suited for similar fusion strategies. Interestingly, an expression strategy for the production of whole antibodies (exemplified by an α-tissue factor IgG1) in *E. coli* was recently described (Simmons et al., 2002). One of the motivations for the study was the possibility to produce non-glycosylated immunoglobulins for therapeutic applications, where effector functions are not required or even detrimental. However, the serum half-life in chimpanzees for these non-glycosylated antibodies were similar as for antibodies produced by mammalian cell-cultures. Thus, likewise aglycosylated affibody-Fc fusion proteins produced in bacterial expression systems would be interesting to investigate for therapeutic applications where effector functions are not required. The results obtained with the tetravalent $(Z_{RSV1})_2$-Fc constructs suggests that also bispecific reagents could be possible to construct, in which two affibodies of different specificities could be linked in a head-to-tail format before fusion to the Fc fragment (Fig. 11).

6.2. Construction and characterization of affibody-β-galactosidase fusions (IV)

As mentioned earlier (chapter 2.5), antibodies or antibody binding proteins linked by different chemical means to reporter groups such as enzymes including alkaline phosphatase (AP), horse radish peroxidase (HRP) and β-galactosidase or fluorophores, constitute very important immunotechnology tools. Recently, recombinant immunoconjugates produced by genetic fusion between antibody subfragments and enzymes have been described, representing an alternative strategy for the linking of antigen recognizing domains to reporter functions, potentially resulting in a simpler and more reproducible production (Ducancel et al., 1993; Lindbladh et al., 1993; Kerschbaumer et al., 1997).

In this study, the construction, production, purification and immunotechnological use of two different recombinant immunoconjugates between the IgA-specific Z_{IgA1} affibody and the *E. coli* enzyme β-galactosidase (β-gal) was investigated. Successful production of soluble recombinant fusion proteins containing β-gal and different extensions of the wild type five-domain bacterial immunoglobulin binding protein A have earlier been described for use in immunotechnology applications (Strandberg et al., 1990; Chowdhury et al., 1994). In contrast to antibody fragments, protein A domains are devoid of cysteins, and thus have the potential to be unaffected by the reducing environment in the cytosol, which allows for the recovery of soluble and active protein A-β-gal proteins directly from bacterial lysates, without the need for refolding. The successful production of such reagents showed upon the possibility to generate target-specific reagents by the same strategy using previously selected protein A-derived affibody ligands as the recognition modules. Such reagents should have the potential to be useful as "stand-alone" immunotechnology reagents for detection and localization of other proteins than immunoglobulins.

The *E. coli* β-gal enzyme is an intracellular (cytosolic) tetrameric enzyme composed of four identical 116 kDa subunits. Here, a slightly truncated version of the enzyme was used. However, it has been shown that up to 26 amino acid residues at the N-terminus of β-gal can be substituted for other residues without significant loss of enzyme activity, although the quaternary structure seemed to be slightly disturbed (Fowler and Zabin, 1983).

For comparison, both a monovalent and divalent versions of the previously described human IgA-specific Z_{IgA1} affibody was fused to the gene encoding β-gal. To provide for a flexible link between the affibody moieties and the enzyme subunits a heptapeptide linker sequence (Gly-Gly-Gly-Ser-Gly-Arg-Pro) was introduced between the two fusion protein partners. For production, a lacZ-negative *E. coli* strain was used, thus avoiding the interference of endogenously produced native β-gal in the assembly of the fusion proteins. The protein expression was driven via an arabinose inducible promoter and the soluble cytoplasmic protein content from shake-flask cultures were collected as the supernatant after centrifugation of sonicated cells. For purification of the immunoconjugates, the soluble cytoplasmic proteins were applied onto a cation/anion exchange column and fractionated using a stepwise salt gradient. To identify fractions containing β-gal activity, ONPG substrate was used and proteins from such fractions were analysed on an SDS PAGE gel showing that relatively pure proteins of expected molecular weights were obtained by this one-step chromatography procedure (IV, Fig. 3). In general, yields of affibody-β-gal fusion proteins were relatively high, in the range of 200-250 mg/l culture. Biosensor binding studies on purified material showed that the affibody moieties of the produced immunoconjugates had retained IgA-binding capacity, which motivated further investigations on the use of these recombinant affibody-β-gal immunoconjugates in different immunotechnology applications.

6.2.1 Use of affibody-β-gal immunoconjugates in immunotechnology applications

As described earlier, a number of different formats for enzyme linked immunosorbent assays (ELISA) have been described. One simple format for antigen detection and quantification, which was investigated here, is to apply the sample directly to immunosorbent wells followed by subsequent detection using an antigen binding reagent. The reagent could be a suitable immunoconjugate for direct detection or a reagent, which is subsequently recognized by a second reagent. Here, a direct detection strategy comparing various concentrations of the two produced recombinant immunoconjugates Z_{IgA1}-β-gal and $(Z_{IgA1})_2$-β-gal reagents was investigated. Different concentrations of polyclonal human IgA were used to coat a 96-well plate. As controls, polyclonal human IgG and HSA was coated in some wells. The results showed that both immunoconjugates were capable of specific recognition of IgA, with no significant cross reactivity with the other proteins (IV, Fig. 5). Interestingly, the octavalent immunoconjugate showed capability of detecting lower concentrations of IgA (lower limit ≈ 0.13 μg/ml) than the corresponding tetravalent conjugate (lower limit ≈ 0.25 μg/ml), suggesting a higher functional avidity.

The use of the Z_{IgA1}-β-gal and $(Z_{IgA1})_2$-β-gal reagents were also investigated in a dot blot assay were samples were applied onto a nitrocellulose membrane. The samples

used were pure IgA preparations or human plasma (containing IgA at a concentration of approximately 1.5-3 mg/ml), respectively, representing samples of different complexities for an investigation of potential unspecific binding activities. After blocking of the membranes, affibody-β-gal immunoconjugates were added and allowed to bind for 1-3 hours followed by development using an X-gal substrate. As controls, both IgG and HSA samples were used. The results from the dot blot assay showed upon no cross-reactivity with the HSA and IgG controls (IV, Fig. 6). In concordance with the results from the ELISA-experiment, the octavalent immunoconjugate proved to be more sensitive allowing detection of IgA down to approximately 0.2 mg/ml, which was about 5-fold lower than for the tetravalent construct.

The Z_{IgA1}-β-gal conjugate was also investigated for IgA detection in an immunohistochemistry experiment. Thin (0.5 µm) cryo-prepared sections of fresh human kidney biopsy samples from a patient with IgA nephropathy, characterized by depositions of IgA in the glomeruli, were analyzed. Incubating a first sample with the X-gal substrate only showed that no endogenous background β-gal activity could be observed, confirming described advantages with the use of β-gal reporter conjugates in non-paraffin embedded samples (Liu et al., 2000). Using the Z_{IgA1}-β-gal conjugate, intense staining was seen in the glomeruli regions of the sections, with very low background staining of surrounding cells, indicating a specific recognition of deposited IgA. As control, a rabbit α-IgA polyclonal antibody preparation was used for immunofluorescence staining of a similar sample from the same biopsy. Here, intense fluorescence signals corresponding well with the staining pattern obtained using the Z_{IgA1}-β-gal conjugate were observed, indicating that the antibody and the Z_{IgA1}-β-gal conjugate were both capable of recognizing the deposited IgA (Fig. 12).

The IgA-specific affibody Z_{IgA1} has also been produced as fused to *E. coli* alkaline phosphatase (AP) (Rönnmark, unpublished). Here, a periplasmic expression strategy was used. The resulting Z_{IgA1}-AP conjugate was shown to be functional in both biosensor binding studies and ELISA detection of IgA using crude periplasmic extracts from producing cultures (Fig. 13.) However, production levels were relatively low, hampering more detailed studies.

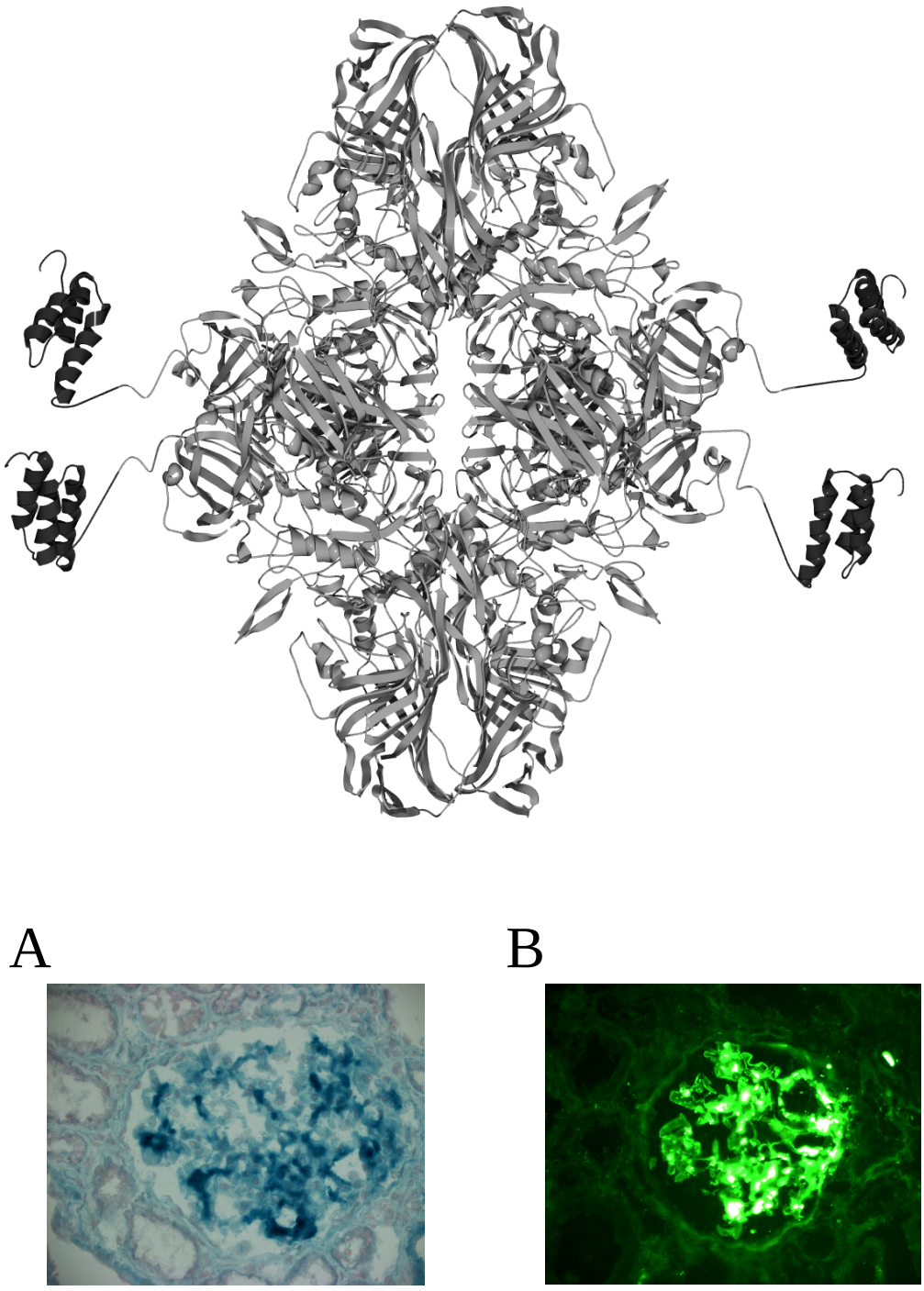


Fig. 12. **Above**: Modelled structure of the tetrameric Z_{IgA1}-β-gal immunoconjugate. Computer graphics image showing a hypothetical structure of a tetramer formed from four Z_{IgA1}-β-gal fusion protein subunits. **Below**: Immunohistochemical staining of IgA deposition in glomeruli using an affibody-β-galactosidase immunoconjugate and a fluorescently labeled antibody, respectively.

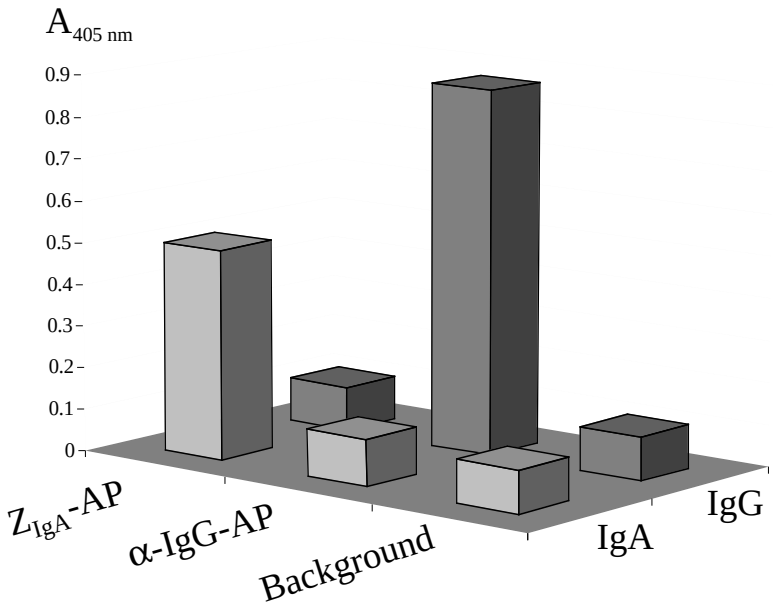


Fig. 13. Results from an ELISA assay using Z_{IgA1}-AP conjugate for IgA detection. Wells were coated with either human IgA or IgG. A commercial rabbit α-human IgG-AP conjugate was used as reference.

7. Affibodies used in sandwich ELISA for analysis of serum samples (V)

A potential problem when measuring analytes by two-site immunoassays in human samples could be an interference from the presence of human heterophilic anti-animal Ig antibodies (HAIA), capable of cross-linking capture and detection antibodies resulting in false-positive signals (Kaplan and Levinson, 1999; Kricka, 1999) (V, Fig. 5). The presence of heterophilic antibodies in human plasma and their interference in immunoassays has been known since the 1970s (Prince et al., 1973; Boscato and Stuart, 1988) and HAIA are found in up to 40 % of sera from healthy individuals. The problem is of special importance if the analyte is present at a low concentration in the serum sample, leading to assays involving less diluted sera (Hennig et al., 2000). One way to circumvent the interference of HAIA in sandwich ELISAs would be to use capturing and detection antibodies from two different species. However, there could still be problems with heterophilic antibodies crossreacting with the two antibodies. A solution to the problem investigated in this study was to use antigen binding proteins of different classes, where the probability to have HAIA interference would be extremely low. A main issue would be to have available binding molecules capable of binding different epitopes on the analyte.

7.1 Capture ELISA for detection of human IgA

To evaluate the possibility to develop a two-site ELISA combining a standard antibody reagent with a non-Ig based ligand; the IgA binding affibody (Z_{IgA1}) described in (II) was used. Criss-cross capture and detection combinations of Z_{IgA1} and IgA-specific goat pAb (pAb_{IgA}) were firstly analysed for reactivity with purified IgA_1, IgA_2 and mixed IgA_1/IgA_2 standards. Z_{IgA1} and pAb_{IgA} reagents were separately immobilized in immunosorbent wells and IgA standards were added in a dilution series followed by detection with either biotinylated Z_{IgA1} or pAb_{IgA} followed by addition of streptavidin-alkaline phosphatase conjugate and substrate. The lower detection level was determined as the lowest standard concentration giving an absorbance value two times the background level.
For the pAb_{IgA}-pAb_{IgA} system, reactivity with the different IgA standards were comparable and the lower detection limit was 0.062 pg/ml (V, table 2) for the IgA_1/IgA_2 standard. The Z_{IgA1}-pAb_{IgA} combination was fully comparable both in terms of reactivity and detection limit. For the reverse system (pAb_{IgA}- Z_{IgA1}) the detection was less sensitive and the last combination (Z_{IgA1}- Z_{IgA1}) were the least sensitive system with a detection limit of 800 ng/ml. Using either the goat anti-IgA pAb reagent or the Z_{IgA1} affibody as capture reagent and biotinylated goat anti-IgA pAb for detection, IgA could be quantified in human serum to similar levels (2.7 and 3.0 mg/ml, respectively). Results from the criss-cross experiments showed that a two-site assay combining the use of an affibody as capture reagent and an antibody reagent for detection was possible to develop. The lower sensitivity in the Z_{IgA1}- Z_{IgA1} combination could possibly be related to the biotinylation of the detection affibody using amine coupling chemistry, possibly recruiting a lysine residue present at one of the randomized positions (II, Fig. 1).

7.2 Interfering antibodies in human serum

The bacterial origin of the Z-derived affinity reagent suggested that the problem of false-positive signals caused by human serum antibodies could be solved by two-site ELISA formats using affibody/antibody reagent combinations. This was investigated using combinations of reagents displaying non-matched specificities (i.e. reagents specific for different analytes). Thus, any signals observed with each system after incubation with human serum should be possible to relate to the presence of cross-linking serum antibodies. Here, two other affibodies, Z_{RSV1} and $Z_{Apo25:4}$ specific for surface protein G of respiratory syncytial virus (Hansson et al., 1999) and human apolipoprotein A-1 (I), respectively, were used. The results showed that all combinations of two antibody reagents tested resulted in different levels of analyte-independent cross-linking, in some cases also after diluting the serum pool 3,125 times.
In contrast, none of the formats in which combinations of antibodies for capture and affibodies for detection were used, or *vice versa*, showed any observable analyte-independent cross-linking (V, Fig. 2B). Noteworthy, although antibody reactivity with non-engineered framework regions of the of *S. aureus* protein A-derived affibody affinity proteins, as a result from an experienced natural exposure to the bacteria, could be expected, the combination of two affibodies displaying different specificities (Z_{RSV} and Z_{Apo}-b) were not cross-linked by human sera (V, Fig. 2B).

A second two-site ELISA assay for quantification of human apolipoprotein was also set up. Here, any complications from analyses of human Ig (HAIA of IgA isotype, anti Z-framework antibodies of IgA isotype) would be circumvented. Also for this assay, functional combinations of affibody and antibody reagents could be found.

8. Concluding remarks

Combinatorial protein libraries based on the Z domain from protein A have been constructed and displayed on phage. In the randomization of the thirteen surface located residues either NN(G/T) or (C/A/G)NN codons, were used; each associated with some limitations related to the inclusion of a stop codon or the exclusion of codon for aromatic residues. In future library constructions, a different approach for mutagenesis not applied here, could be to use oligonucleotides built up from codon triplets, allowing a free design of the variability at the different positions (Virnekäs et al., 1994).

Further, potential use of affibodies as immunotechnological tools were investigated, where affibodies were produced in different formats. One was multimerized and used as ligand in affinity chromatography and that affibody was also fused with an enzyme and used in ELISA and immunohistochemistry, while another affibody was fused to Fc and used in Western blot. An interesting approach for the future would be to construct bispecific conjugates with affibodies in tandem, binding different targets.

All affibodies used in this work have been of micromolar affinities, and it could be expected that affibodies of higher affinities have an advantage in other immunotechnology applications. However, earlier work has shown upon the possibility to obtain affibodies of higher affinities (up to nM range affinities) by affinity maturation principles (Gunneriusson et al., 1999; Nord et al., 2001), suggesting that the library size is a crucial factor. In this thesis and in earlier work, the selection of affibodies to protein targets only have been considered, but it could also be of interest to see if the libraries contains affinity ligands to non-protein molecules, like smaller haptens and carbohydrates.

All together, these findings has shown that the Z libraries withhold individual ligands with specificity to a wide range of proteins and that such affibodies could be used in different formats for some commonly applied immunotechnology methods. Undoubtedly, more applications than demonstrated here could be conceivable and the use of combinatorial approaches in general, has already proven to be of great importance, and will most certainly have an impact on further development of new biotechnological and immunotechnological tools.

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Abbreviations

ABD albumin binding domain
AP alkaline phosphatase
β-gal β-galactosidase
cDNA complementary deoxyribonucleic acid
CDR complementarity determining region
CH constant heavy
CL constant light
C-terminal carboxy terminal
dsDNA double stranded DNA
ELISA enzyme linked immunosorbent assay
Fab fragment antigen binding
FACS fluorescence-activated cell sorting
Fc fragmnet crystallisable
FITC fluorescein isothiocyanate
Fv fragment variable
GFP greeen fluorescent protein
HAIA human heterophilic anti-animal Ig antibodies
HSA human serum albumin
Ig immunoglobulin
IgA immunoglobulin A
IgD immunoglobulin D
IgE immunoglobulin E
IgG immunoglobulin G
IgM immunoglobulin M
K_a association constant
K_d dissociation constant
kDa kilo Dalton
lacZ β-galactosidase gene
mAb monoclonal antibody
mRNA messenger RNA
N-terminal amino terminal
pAb polyclonal antibody
PL protein L
RCR polymerase chain reaction
RIA radioimmunoassay
RSV respiratory syncytial virus
RU resonance unit
scFv single chain fragment variable
SDS-PAGE sodium dodecyl sulfate polyacrylamid gelelectrophoresis
SPA staphylococcal protein A
SPG streptococcal protein G
VH variable heavy
VL variable light
Z engineered protein A domain
Zlib-1 Z library 1
Zlib-2 Z library 2

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