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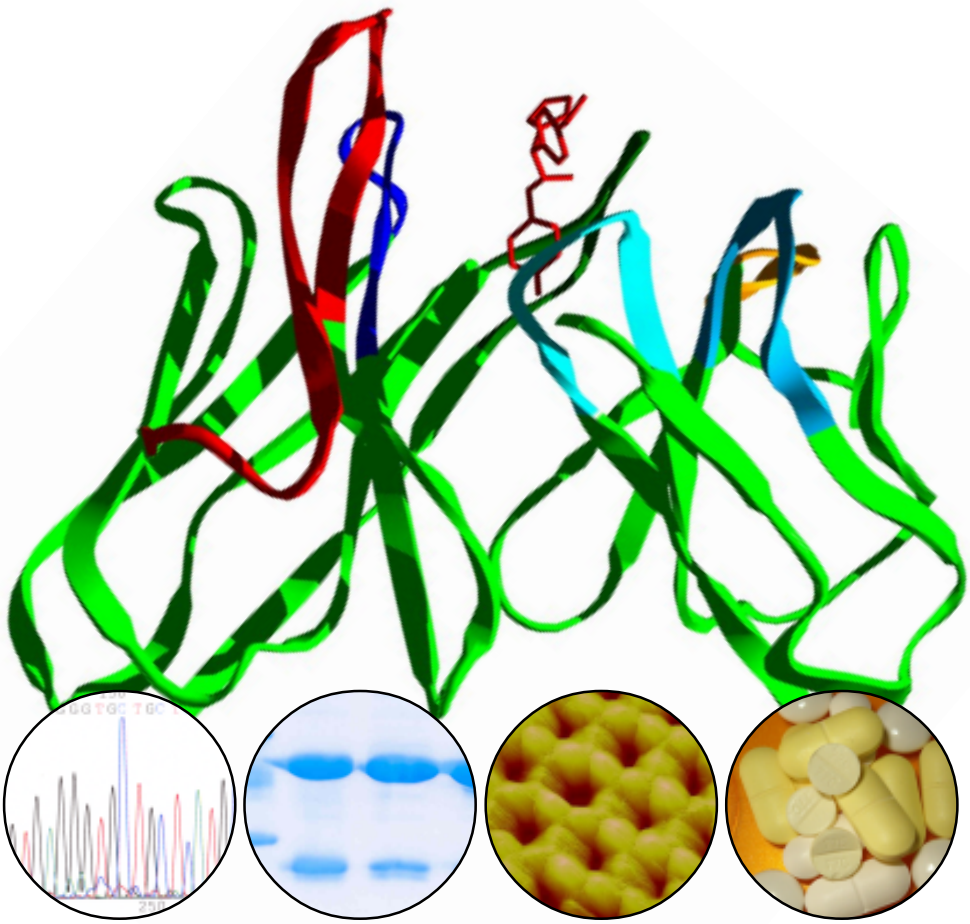

Tarja K. Nevanen

Enantioselective antibody fragments

VTT

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Abstract

Antibodies have a unique ability to bind to a wide variety of different molecules, ranging from large protein antigens to low molecular weight organic molecules. The specificity and affinity of the binding varies between antibodies and can be utilised in many applications. Recombinant antibody fragments have advantages over traditional poly- and monoclonal antibodies in production and immobilisation and in the optimisation of their properties.

Enantiomers of a chiral compound provide a challenge for separation methods and analytics due to their similar chemical and physical properties. Antibodies provide a highly specific way to fractionate enantiomers in both preparative and analytical applications.

In this work two different antibody-based approaches to separate enantiomers of a chiral drug candidate were developed. Antibody fragments were cloned, produced in bacteria and immobilised on a solid affinity support. Repeated affinity purification of enantiomers was achieved in optimised conditions. In the other approach antibody fragments were immobilised inside the nanotubes of an alumina membrane and the bionanomembrane was used to fractionate enantiomers of a racemic mixture.

In addition a sample preparation method, antibody-based solid-phase extraction, was developed in a way that can be applied to high-throughput format. Recoveries were comparable to those reported for non-specific sorbents, but with the advantage of the enantioselectivity. The method was used to extract an enantiomer from a spiked buffer or serum. The preparatory sample treatment protocols usually used for serum, e.g. protein precipitation, were not needed.

A homology model of one of the antibody fragments was constructed and used to design site-specific mutations in order to adjust the affinity of the antibody to be suitable for the preparative and analytical approaches developed in this work. One of the mutants, ENA5His Tyr96Val, had appropriate properties both in preparative and analytical applications.

Preface

This work was carried out in the Protein Engineering group at VTT Biotechnology and it is part of the the research programme "VTT Industrial Biotechnology" (Academy of Finland; Finnish Centre of Excellence programme, 2000-2005, Project no. 64330). I thank Executive Director Juha Ahvenainen, Research Director Hans Söderlund and Research Manager Richard Fagerstöm for providing excellent working facilities. The financial support of the Technology Development Centre of Finland, EU-project BE-1075, Danisco-Cultor, Orion Pharma and Hormos Medical is gratefully acknowledged.

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(Orion Pharma) and Tapani Suortti who spent much time and effort in analysing the enantiomers with HPLC from numerous fractions when I was trying to make antibodies behave in a desired manner. Nana Munck is warmly thanked for the modelling work and for providing endless amounts of pictures of antibody models for various purposes. Oili Lappalainen and Harri Kiljunen are thanked for providing pictures for this thesis and Michael Bailey for revising the language. I am also grateful to Professor Charles Martin (University of Florida) and his group for fruitful collaboration in the field of bionanotechnology. My warm thanks go to Lacromioara Trofin and Punit Kohli for being such lovely hosts during my stay in Florida.

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I wish to thank my sister, Anne, for the joy she and her family have brought to my life. Their company has released me totally from the world of molecules and the trips we have made together are unforgettable. My deep gratitude goes to my parents, Tarja and Antti. I appreciate the support they have given me throughout my whole life.

Most of all I want to thank my family, Anna and Harri. Anna has given me some of the most touching moments of my life. She is also thanked for her practical help and her understanding during this exceptional period in our lives. Harri is deeply thanked for his patience and support for over twenty years. I appreciate that he has not loaded me with additional guilty feelings due to working too much. His kindness, sense of humour and love have carried me through the moments of despair.

Tarja

List of publications

This thesis is based on the following articles, which in the text are referred to by their Roman numerals.

I Nevanen, T.K., Söderholm, L., Kukkonen, K., Suortti, T., Teerinen, T., Linder, M., Söderlund, H. and Teeri, T.T. 2001. Efficient enantioselective separation of drug enantiomers by immobilised antibody fragments. Journal of Chromatography A 925, 89–97.

II Lee, S.B., Mitchell, D.T., Lacramioara, T., Nevanen, T.K., Söderlund, H. and Martin C.R. 2002. Antibody-based Bio-nanotube membranes for enantiomeric drug separations. Science 296, 2198–2200.

III Nevanen, T.K., Hellman, M.-L., Munck, N., Wohlfahrt, G., Koivula, A. and Söderlund, H. 2003. Model based mutagenesis to improve the enantioselective fractionation properties of an antibody. Protein Engineering 16, 1089–1097.

IV Nevanen, T.K., Simolin, H. Suortti, T., Koivula, A. and Söderlund, H. Development of a high-throughput format for analysis of enantiomers using an immunosorbent in 384-well plates. Manuscript submitted to Analytical Chemistry.

Contents

List of abbreviations

α	Selectivity coefficient
AFM	Atomic force microscopy
Ala	Alanine
ATCC	American type culture collection
BSA	Bovine serum albumin
C_8/C_{18}	Chemically bonded silica, 8/18 carbon atom coating
CDR	Complementarity determining region
C_L	Constant domain of the light chain
C_H1	First constant domain of the heavy chain
C_H2	Second constant domain of the heavy chain
C_H3	Third constant domain of the heavy chain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli* -bacteria
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA5His/ENA11His	Codes for two enantioselective antibody fragments
Fab	Immunoglobulin fragment of the V_HC_H1 and V_LC_L
Fc	Immunoglobulin fragment of the C_H2/C_H3 of both heavy chains
FDA	Food and Drug Administration
Gln	Glutamine
HPLC	High-performance liquid chromatography
IC_{50}	Inhibitory concentration for 50% decrease in activity
IDA	Iminodiacetic acid
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMAC	Immobilised metal affinity chromatography
K_D	Dissociation constant
Leu	Leusine
MS	Mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
Phe	Phenylalanine
r	Correlation coefficient
RNA	Ribonucleic acid

RSD	Relative standard deviation
scFv	Single chain antibody fragment
SDR	Specificity determining region
SDS	Sodium dodecylsulphonate
SPE	Solid-phase extraction
Thr	Threonine
Trp	Trypthophan
Tyr	Tyrosine
UV	Ultraviolet (wavelength)
Val	Valine
V_H	Variable domain of the heavy chain
V_L	Variable domain of the light chain

1. Introduction

1.1 Chirality

Life is dependent on the chirality of molecules. Chirality is a spatial property of a molecule and is determined by the three-dimensional arrangement of its atoms (from the Greek word *cheir* meaning hand, handedness). Amino acids and carbohydrates, the building blocks of proteins and of polysaccharides and DNA, respectively, are chiral compounds. Therefore most biological functions are asymmetric, favouring only one chiral form of the molecule. Chirality is an efficient way to increase the specificity of recognition, binding, catalysis and other biological events.

Applied sciences, e.g. pharmaceutical, forensic, environmental and food sciences, have shown increasing interest in chirality due to the increased exposure of humans to synthetic chiral chemicals such as medical and illegal drugs and pesticides. The stereochemical properties of chiral compounds in foods, fragrances and agrochemicals in both natural and work environments have increasingly attracted attention (Armstrong and Zhang, 2001, Müller and Kohler, 2004). When a chiral compound enters a living organism, a chiral distinction occurs in physiological processes. Different forms of a chiral compound, stereoisomers (Figure 1), have different interactions with the target molecules, such as receptors, ion channels, transporters and enzymes. Although scientists have known the phenomenon of chirality from the middle of the nineteenth century when Pasteur performed his experiments with tartaric acid, deeper awareness was not aroused until 1960s when the first indications of the chiral compound thalidomide as a cause of miss-shaped fetuses emerged.

1.1.1 Enantiomers

Distinctive for an asymmetric chiral atom is that all groups attached to it via a covalent bond are different. Most common is a carbon atom having four substituents and it is used here as an example (Figure 2 a). Organic compounds that have one or more such chiral atoms have stereoisomers due to the different spatial arrangement, configuration, of these groups. A pair of non-

superimposable mirror images of a chiral compound is a pair of enantiomers (form the Greek *enantios*, opposite) (Figure 1).

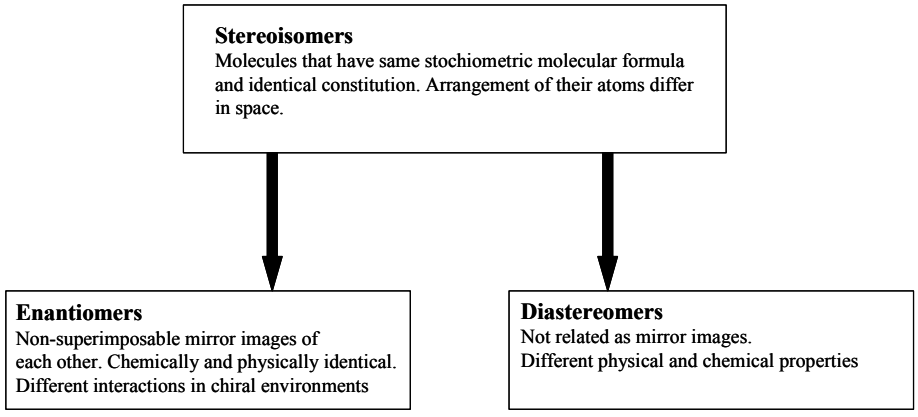
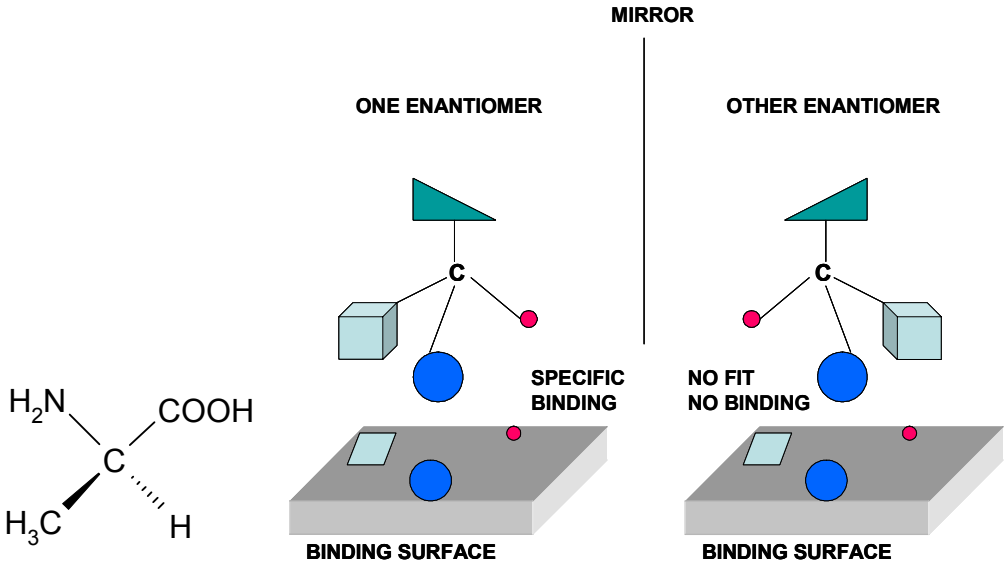


Figure 1. Definitions of stereoisomers and the relationships between them.

The designation (*S*) (lat. *sinister* = left) or (*R*) (lat. *rectus* = right) is used to express the configuration of the groups bonded to the chiral atom. The absolute configuration around the chiral atom is the order of the arrangement of the four different groups. The groups have priorities according to their atomic number. The lowest-priority substituent is placed behind the stereogenic centre and if an imaginary arrow drawn from the highest to the second highest priority group is clockwise then the configuration is *R*, contrary to the *S* configuration. The compound has a maximum of 2^n optical isomers, where n is the number of chiral atoms. Enantiomers have the same physical and chemical properties and they are difficult to separate from each other by any chemical or physical means. They differ only in the way they react with other chiral compounds, such as target proteins (Figure 2 b), and how they rotate polarized light.



a. **b.**

Figure 2. a) A chiral carbon atom with four different substituents: the amino acid alanine as an example. b) Enantiomers are non-superimposable mirror images of each other. The spatial arrangement of different substituents varies between enantiomers and therefore only one of the enantiomers is a perfect fit with the binding surface of the target protein. (Modified from Guzman and Stubbs, 2001)

Configuration does not reveal the light rotating direction and therefore a full description of the chiral compound often also includes a light rotating characteristic d/l or +/-, respectively. The enantiomer which rotates the light to the left is designated by (–) or l (levorotatory), or to the right (+) or d (dextrorotatory). When the solution has equal amounts of enantiomers it is optically inactive. A mixture having an equal amount of enantiomers is called a racemate. Enantiomeric excess ranging 0–100% is used to describe the relative amounts of enantiomers in the mixture from racemate to pure single enantiomer, respectively.

1.1.2 Chiral compounds in humans: the example of drugs

In most cases the enantiomers of a drug differ in their activities in the human body. Enantioselective differences are not only due to the interactions between the drug and its pharmacological receptor but also to its pharmacokinetic properties including absorption, unspecific protein binding, transport, metabolism and excretion.

Figure 3 illustrates the complexity of the different routes of the two enantiomers. After administration the two enantiomers may differ in both their route and their rate of absorption and distribution. The enantiomers may also differ quantitatively in their action with the pharmacological receptor. The active enantiomer is called a eutomer and the less or non-active enantiomer a distomer. Enantiomers may also have qualitative differences and thus distinctly different biological activities. Many of the side effects of chiral drugs are caused by the distomer, which has activity towards another target. Some examples of the effects of distomers are listed in Table 1.

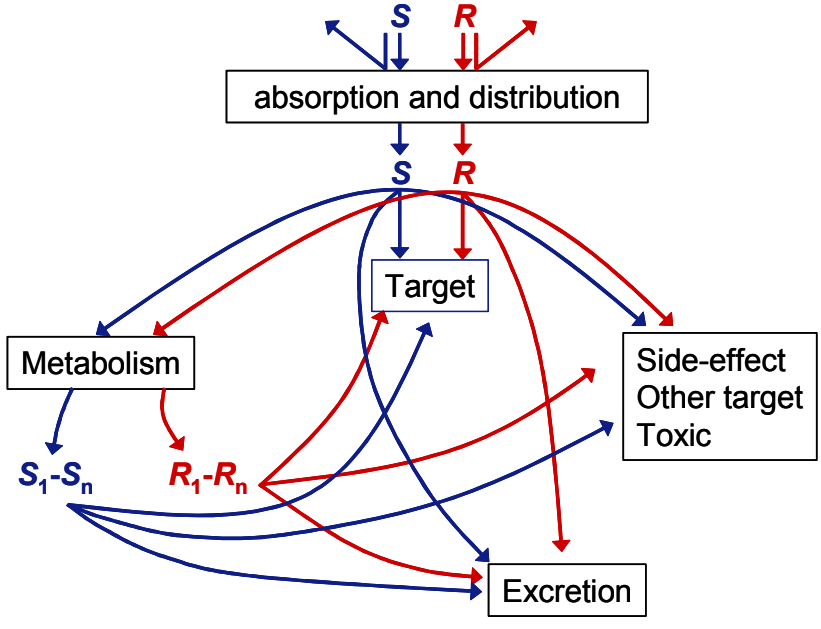


Figure 3. A graphical illustration of the possible complexity of the action of the two enantiomers S and R in vivo. In addition to the different sites of action, their efficiency can vary and competition between the enantiomers may occur. S_1-S_n and R_1-R_n reflect the unknown number of metabolites of the S and R enantiomers, respectively.

Table 1. Examples of the effects of distomers of drugs.

EFFECT OF THE DISTOMER	EXAMPLE	REFERENCE
Lower activity	Methoxychlor metabolites Warfarin	Miyashita *et al*., 2004 Takahashi and Echizen, 2001
Opposite activity	Albuterol (*R*)- Bronchodilatory, anti-asthmatic (*S*)- enhances airway hypersensitiveness	Nelson *et al.*, 1998
Separate activity	Dexanabinol (-)- cannabinoid activity (+)- neuroprotective agent, no cannabinoid activity	Knoller *et al.*, 2002
Undesirable activity	ketamine (*S*)-(+)-anesthetic, analgesic (*R*)-(-)- hallucinations, agitation	White *et al*., 1980

The greatest degree of stereoselectivity is introduced into the drug disposition by drug metabolism. Enantioselectivity in absorption and distribution is substantially lower (reviewed by Caldwell, 1995). The administered drug is a foreign substance and metabolism is a way to eliminate it. The mechanism has evolved to remove potentially harmful organic molecules from the body. Low molecular weight compounds are chemically modified to be excreted more efficiently. Usually excretion is enhanced by increasing the solubility of the compound. The same enzyme may metabolise both enantiomers but with different rates, or the enantiomers may be metabolised by different enzymes (reviewed by Rentsch, 2002). In some cases the metabolite may have even higher activity than the drug itself and much interest has arisen concerning the use of the metabolites as drugs. On the other hand metabolism may be harmful, e.g. by increasing the toxicity of the compound. An example of problematic metabolism is racemic ibuprofen, a non-steroidal anti-inflammatory drug, which is stereospecifically conjugated with glucuronic acids. Conjugation can lead to attachment of the ibuprofen to proteins *in vivo*. Preliminary evidence suggests that such a covalent modification of native proteins by ibuprofen can cause allergic reactions and hepatotoxicity (Ikegawa *et al.,* 2001, Ito *et al*., 2004).

The fact that the behaviour of the enantiomers in racemic mixtures is different than would be expected from their individual properties makes the issue even more complicated. The relationships between eutomers and distomers cannot easily be predicted and they must therefore be experimentally established. For example the side effect of (+)-indacrinone is the retention of uric acid. The other enantiomer antagonises it and the optimal therapeutic profile is obtained with a 1:4 or 1:8 ratio of isomers (Tobert, 1981). There are also cases in which co-

administration of the inactive enantiomer may increase the free plasma concentration of the active enantiomer by reducing its plasma binding, metabolism or excretion (Lloyd, 1997). In such cases it would be advantageous to administer both enantiomers. However, much effort is needed to study the non-racemic mixtures of enantiomers because at the moment the therapeutic effects can only be determined experimentally. Additional costs reduce the viability of such non-racemic mixtures.

In some cases the pure enantiomer undergoes racemisation *in vivo*, as in the example of thalidomide (Triggle, 1997). Although there are indications that the teratogenic activity is related to *S*-enantiomer, the administration of pure *R*-enantiomer would not remove the problem due to this configurational inversion in patients. Unidirectional chiral inversion is also possible, as has been observed with ibuprofen (Ito *et al*., 2004). Stereoselective properties of drugs in general were reviewed by Triggle (1997).

The development of new chiral technologies such as asymmetric synthesis, chiral separations and analytics in the 1980s led to the new guidelines of FDA (Food and Drug Administration, 1992) for chiral drugs. The FDA guidelines state that regardless of whether a racemate or a single enantiomer is the ultimate drug, the chemical, pharmacological, pharmacokinetic and toxicological properties of both enantiomers must be documented. The possible racemisation *in vivo* must also be determined.

Drugs derived from natural sources are usually single enantiomers because of the enzymatic and thus chiral synthesis. Palytoxin, isolated from Hawaiian coral, has tens of chiral centres and represents one of the most extreme examples (Moore and Bartolini, 1981). Stereoselective synthesis of such complex molecules by chemical means can be very difficult, although recent method development of asymmetrical synthesis has been rapid, including enzymatic catalysis. In practice most of the synthetic drugs do not contain so many chiral centres. Of the new drugs approved by FDA (1991–2002), 42% had no chiral centre, 14% were racemates and 44% single enantiomers. Of the single enantiomer drugs 3.5% had only one chiral centre and the rest had multiple centres (Caner *et al.,* 2004). During recent years the number of racemates introduced as new drug entities has been decreasing. Old drugs, however, can still be marketed as mixtures of even several stereoisomers (Goel *et al*., 2004).

Chiral switches are drugs that have already been approved as racemates but have since been redeveloped as single enantiomers. By chiral switching a 3-year extension for the patent protection of the compound can be obtained. This benefit has increased the interest in re-studying the enantiomers of old drugs. For a review and examples of chiral switches see Agranat *et al.* (2002).

According to Caner *et al.* (2004) drug chirality is now a major theme in the design, discovery, development, launching and marketing of new drugs. There is an increasing preference for single enantiomers due to the advantages they provide, such as dose reduction, less side effects and more precise estimation of dose-response relationships. Stereoselective aspects also help to understand the mechanisms of action and make it possible to optimise the safety and effectiveness of the drug.

Proteins, peptides, nucleotides and oligosaccharides are also chiral molecules but they do not exist as racemates *in vivo*. In addition they have different routes of function and elimination from low molecular weight organic compounds and are therefore not included in this thesis.

1.2 Recombinant antibodies

1.2.1 Background

Antibodies (immunoglobulins) are the essential component of the humoral immune response. They are binding proteins produced by B-lymphocytes to eliminate invading foreign macromolecular substances *in vivo*. Antibodies of various species and their characteristics were recently listed by Yau *et al.,* 2003. The unique features of the immune system are ability to distinguish between self and non-self, memory, enormous diversity and specificity of recognition. The diversity and specificity are the most essential attributes for *in vitro* applications of antibodies. The diversity of the immune system *in vivo* is due to the combinatorial assembly of the antibody gene segments and due to the somatic mutations which fine-tune the binding properties of the antibodies. The specificity of the binding originates from the unique structural elements of the antibody proteins, as reviewed below.

Antigens are substances that can induce the production of antibodies *in vivo*. The word hapten is used to describe a small organic compound which in itself cannot elicit the immune response but as a protein conjugate becomes immunogenic. Antibody production *in vivo* is induced mainly by infection or by immunising with an immunogen. After immunisation a set of heterogeneous antibodies can be isolated directly from the serum. These polyclonal antibodies have different affinities and can recognise various sites, epitopes, of the antigen. The lack of reproducibility of the same sera and problems with cross-reactivity and backround binding are disadvantages of the polyclonal antibodies which have been used traditionally in immunoassays and as a research tool. Another route is to produce monoclonal antibodies by utilising hybridoma technology (Köhler and Milstein, 1975). Antibody-producing B-lymphocytes are fused with the myeloma cell line to create immortal cells. Monoclonal antibodies produced by these hybridoma cells have defined affinity and specificity and their production is reproducible. They are widely used in various diagnostic applications although they suffer from the restrictions of the immunisation, e.g. antibodies against self-antigens or toxic compounds are difficult to obtain.

Recent developments in antibody technology, utilising the power of antibody engineering, antibody gene libraries and various selection methods, have provided the possibility to by-pass the immunisation and to obtain specific binders even for challenging antigens and haptens. The high-throughput selection and scaled up production of recombinant antibody fragments in bacteria bring additional advantages over traditional poly- and monoclonal antibodies. Antibody engineering, display and selection technologies are reviewed in sections 1.2.5 and 1.2.6.

1.2.2 Structural elements of antibodies

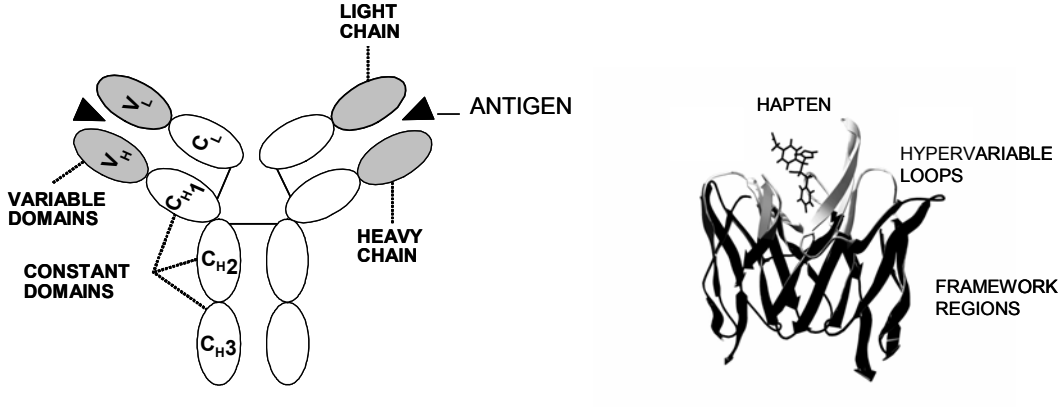
Four polypeptides, two identical heavy chains and two identical light chains, form the basic structure of an antibody. One light chain is joined to one heavy chain and the Y-shaped structure is formed by linking the two heavy chains together as drawn schematically in Figure 4 a. Both heavy and light chain polypeptides fold into globular domains having about 110 amino acids in each domain. Heavy and light chains have four and two domains, respectively. The amino terminal domains of both chains, called variable domains, are responsible

of the diversity of the specific antigen binding. Constant domains (C_L or C_H1, C_H2, C_H3) have conserved sequences and structures among each sub-class (isotypes are κ or λ for the light chain and γ, μ, ε, δ or α for the heavy chain). The two carboxy-terminal domains of the heavy chain have different effector functions which are important for the cellular immune response *in vivo*. Hitherto no functional differences between the light chain isotypes have been observed.

Immunoglobulins are classified according to the isotypes of the heavy chains into five major classes: IgG, IgM, IgA, IgD and IgE. In the primary response, after the first immunisation or infection, the IgM antibodies are the most abundant. IgM antibodies are pentamers of five Y-shaped immunoglobulin units. They usually have low intrinsic affinity for the antigen but due to their multivalency and avidity effect they are able to bind the antigen efficiently. In a secondary response, after subsequent exposure to immunogen, the antibodies of the IgG class become predominant. IgG antibodies have higher affinity for the antigen than IgM antibodies and due to their smaller size they diffuse more readily into extravascular spaces to neutralize the antigen. The dimeric IgA immunoglobulins appear e.g. in saliva, tears and in secretions of the gastro-intestinal tract. Their task is to prevent microbes from adhering to the mucosal cells. The lowest amounts of immunoglobulins present in sera belong to the IgE class. They recognize e.g. allergenic epitopes and induce the symptoms of allergy. The physiological role of IgD is currently not well understood.

Each variable domain (V_H or V_L) has four conserved framework sequences forming the barrel-like structure of two antiparallel β-sheets (Poljak *et al.,* 1973), a tertiary structure called immunoglobulin fold. The three hypervariable loops H1, H2, H3 and L1, L2, L3 for heavy and light chains, respectively, (Kabat and Wu, 1971) are located between these framework sequences in each chain (Figure 4 b). An antigen binding site is a combination of these six loops (complementarity determining regions, CDRs) of two variable domains. Framework regions have mainly a structural role in antibodies, although sometimes a framework residue may be in direct contact with the antigen (Sheriff *et al.,*1987, Wedemayer *et al.,* 1997). Generally the contribution of the framework residues to the affinity is indirect, produced by supporting the structure of the CDRs (Lamminmäki *et al.,* 1999, Daugherty *et al.,* 2000, Kusharyoto *et al.,* 2002) or by affecting the correct folding (de Haard *et al.,* 1998). Decrease in affinity may be caused by a single amino acid change in the

framework region (de Haard *et al.,* 1998, Caldas *et al.,* 2003). The importance of framework regions to the binding properties of an antibody has become evident in humanisation studies in which the insertion of CDRs of murine origin into a human framework without further engineering have caused decreased affinity (Foote and Winter, 1992, Saldanha *et al.,* 1999, Caldas *et al.,* 2003).



A. **B.**

Figure 4. a) Schematic illustration of an antibody domain structure, with IgG antibody as an example. The amino terminal variable domains V_H and V_L form the antigen binding site. b) A ribbon diagram model of the variable domains – hapten complex. Framework areas are black, the loop structures responsible for antigen binding are grey (modelled and picture generated by Nana Munck, VTT Biotechnology).

Hypervariable loops are responsible for the specific recognition of the antigen. They vary in length and have high sequence diversity. The distribution of lengths of CDRs varies between species, in particular the CDR3 of the heavy chain. CDR-H3 loops range from 1–19 and 2–28 residues in mouse and in human antibodies, respectively (Collis *et al.,* 2003). The length of the CDRs makes a great contribution to the combining site topography, although the surface shapes cannot always be directly concluded from the length of the CDRs. A cavity type binding site topography is often observed in anti-hapten antibodies in which the small molecule is to be buried into the cavity, whereas groove and planar binding site topographies are typical for e.g. peptides and proteins, respectively (Padlan and Kabat, 1988, Wang *et al.,* 1991, MacCallum *et al.*, 1996).

Despite the high variability in sequence the hypervariable loops, except CDR3 of the heavy chain, have canonical structures. (Chothia and Lesk, 1987). The length of the loop and certain conserved structure determining residues at CDRs and at the framework region determine the main-chain conformation of the hypervariable loop and thus the canonical structure of the loop. The critical amino acids at CDRs are important in packing and hydrogen bonding or have suitable torsion angles and they are conserved in the canonical class to which they belong. (Chothia and Lesk, 1987, Chothia *et al.,* 1989, Martin and Thornton, 1996, Al-Lazikani *et al.,* 1997) The hypervariable loops can be combined randomly but in practice only a fraction of the possible combinations of canonical structures of CDRs are frequently used in germline antibodies (Vargas-Madrazo *et al.,* 1995). Recently some sequence-structure relationships have also been found for the most variable CDR3 of the heavy chain (Morea *et al.,* 1997, 1998, Shirai *et al.,*1998, Oliva *et al.,* 1998), which has the greatest variability and impact on binding of antigen in many antibodies. CDR-H3, together with CDR-L3, has a central position in the binding site. Randomisation of only the CDR-H3 has produced antibodies with different specificities both *in vivo* (Xu and Davis, 2000) and *in vitro,* showing the importance of CDR-H3 for the specificity (Hoogenboom and Winter, 1992, Nissim *et al.,* 1994, deKruif *et al*., 1995, Braunagel and Little, 1997, deWildt *et al.*, 1997).

At the hypervariable region the most variable residues are mostly in contact with the antigen and are called the specificity determining residues (SDRs) (Padlan *et al.,* 1995). Residues in the centre of the antigen binding site are in general crucial for the interaction with antigen (Hawkins *et al.,* 1993, MacCallum *et al.,* 1996, Tomlinson *et al.,* 1996, Jirholt *et al.,* 2001). During maturation, somatic mutations are introduced to SDRs but especially to other residues not directly interacting with the antigen in order to stabilise the SDRs in conformations favouring specific binding (Wedemayer *et al*., 1997). The importance of non-contact residues in *in vitro* affinity maturation has also been verified in mutagenesis studies (Hawkins *et al.,* 1993, Chen *et al.,* 1995, Daugherty *et al.,* 2000, Short *et al.,* 2001). At the binding region the amino acid composition is different from the rest of the variable domain. In general e.g. tyrosines are abundant at the hypervariable regions (Collis *et al.,* 2003). Due to their unique chemical properties, such as their dual nature of being both hydrophobic and able to participate in hydrogen bonding, they are able to interact with a variety of antigens. One recent statistical study suggested that the differences in residue

composition of the CDRs of the antibodies depend to some extent on the type of antigen, e.g. histidines and asparagines are over-represented in hapten binders (Collis *et al.,* 2003).

Comparisons of the crystal structures of antibodies with or without the bound antigen suggest in many cases a structural adaptation of the antibody conformation in the binding event. The conformation of antigen may also change as a result of binding, even in small haptens (Ramsland *et al.,* 2001). Structural changes in antibodies can be absent (Burks *et al.,* 1997) or restricted only to subtle adjustments of side chains in CDRs, and then the binding can be characterised as a "lock-and-key"-mechanism (Arevalo *et al.,* 1993, Braden *et al.,* 1996, Wedemayer *et al.,* 1997, Valjakka *et al.,* 2002). An "induced-fit"-mechanism has been used to describe the binding with larger structural adaptation such as conformational changes in loops or alterations in the relative dispositions of variable domains (Bhat *et al.,* 1990, Stanfield *et al.,* 1993). The third potential mechanism of binding is based on conformational isomerism of the antibodies in solution. Antibodies may have different conformational stages in solution equilibrium and the ones having the appropriate conformation bind the antigen (Foote and Milstein 1994). Conformational diversity can cause multispecificity, where a single antibody is able to bind two different antigens or haptens depending on its conformational stage (James *et al.,* 2003).

1.2.3 Recombinant antibody fragments

The domain structure of the antibodies (Figure 4 a.) makes them especially suitable for genetic engineering. Fab-fragments consist of the intact light chain (V_LC_L) and the variable (V_H) and the first constant domain (C_H1) of the heavy chain. A disulphide bridge links the chains covalently together. The first report of the production of a recombinant Fab-fragment in bacteria was published 1988 by Better *et al.* Even smaller fragments of antibodies having only the variable domains have been made. They have been connected by non-covalent forces between the varible domains, Fv (Skerra and Plückthun, 1988) or by covalent linkages such as a linker polypeptide chain in scFv (Bird *et al.,*1988, Huston *et al.,* 1988) or by a disulphide bridge in dsFv (Glockshuber *et al.,* 1990). Covalent linkages prevent the concentration-dependent dissociation of the Fv domains observed in low concentrations (Glockshuber *et al.*, 1990). External factors such

as ionic strength, pH and the presence of the antigen can have an effect on the stability of the V_H-V_L-interaction (Arndt *et al.,* 1998). Depending on the length of the linker between variable domains, single-chain antibodies can also form dimers and trimers, thus creating multivalent or bispecific binding (Holliger *et al.,* 1993 Alfthan *et al.,* 1995, Pei *et al.,* 1997, Kortt *et al.,* 1997, Arndt *et al.,* 1998).

Fab-fragments and single chain antibodies are the most widely used antibody fragments in various *in vitro* applications. Reduced size has made it possible to express antibody fragments in various heterologous hosts (section 1.2.4) and to construct antibody libraries displayed in different ways (section 1.2.5). Small size is also advantageous in structure determination and in applications in which dense immobilisation of binding sites is preferred. Antibody fragments usually have the same intrinsic binding affinity as the monoclonal antibody (Bird *et al.,* 1988, Glockshuber *et al.,* 1990, Hemminki *et al.* 1998a). However, especially in scFvs, if the variable chains are reoriented due to removal of the constant domains, some changes in affinity may occur (Huston *et al.*, 1988, Wörn and Plückthun, 2001).

1.2.4 Heterologous expression of antibody fragments

Most often the antibody fragments have been produced in *Escherichia coli* and different expression strategies have been applied. Correctly folded antibody fragments can be produced to the periplasmic space between the inner and outer membranes of *E. coli* (Better *et al.,* 1988, Skerra and Plückthun, 1988, Carter *et al.,* 1992, Pack *et al.,* 1993). The periplasmic space has reducing conditions and chaperones, leading to the formation of disulphide bridges and correct folding of the polypeptides. Production of antibody fragments to the periplasm may lead to leakage or partial lysis of the outer membrane and antibody fragments can then be collected from the culture supernatant. Single mutations to V_H, (Forsberg *et al.,* 1997) or optimised vector and growth conditions have been shown to prevent leakage and lysis of the bacterial cells (Pack *et al.,* 1993, Horn *et al.,* 1996). If the signal sequence, which guides the synthesized polypeptide to the periplasm, is not included then the antibody fragments tend to accumulate in the cytoplasm and may form insoluble aggregates, inclusion bodies (Bird *et al.,* 1988, Proba *et al.,* 1995, Sinacola and Robinson, 2002). Refolding of the

antibody fragments *in vitro* is then necessary and the success of this procedure is antibody-dependent (Sinacola and Robinson, 2002). For a review of expression of antibody fragments in *E. coli* see Plückthun, 1991.

Using optimised expression vectors and high cell density-fermentation high production yields up to 1-3 g/L of the functional antibody fragment have been obtained. (Carter *et al.,* 1992, Pack *et al.,* 1993, Horn *et al.,* 1996). The expression yields of soluble proteins can be improved by increasing the total expression level and also the folding yield, which is connected to the susceptibility of the antibody fragment to proteolytic degradation (deHaard *et al.,* 1998). Co-expression of periplasmic chaperones have in some studies improved the functional expression of the antibody fragment (Bothmann and Plückthun, 1998, 2000, Mavrangelos *et al.,* 2001). The choice of the expression vector, bacterial strain and conditions for cultivation, e.g. media composition and temperature, affect the final yield of soluble active antibody fragment. In addition to these extrinsic factors especially the amino acid sequence of the antibody but also the origin of the antibody and fragment type can have effect on the final yield. Despite identical production vectors and cultivation conditions up to 10-fold differences in production yields of different antibodies have been observed (Knappik and Plückthun, 1995). Even a single mutation can have a dramatic effect on the expression (Dueñas *et al.*, 1995, Knappik and Plückthun, 1995). Critical residues in e.g. turns on the surface or at the domain interface can have a great impact on expression (Knappik and Plückthun, 1995). The importance of the framework regions was shown in one study in which humanisation of an antibody improved the production 10–100-fold compared to the parental murine antibody (Carter *et al.,* 1992). In scFv antibodies the exposed hydrophobic patches may lead to aggregation and necessitate modification for improved yields of soluble antibody (Nieba *et al.,* 1997). Studies to identify the critical positions in antibody frameworks for improved expression have been performed for various antibodies (Table 2). A systematic study with the human variable domains revealed differences in production levels of different germline family consensus domains and different combinations of the domains (Ewert *et al.,* 2003a). Careful consideration is needed if mutations are applied to a different framework, as the studies of deHaard *et al.* (1998) and Kipriyanov *et al.* (1997) have indicated. The change of glutamine to glutamate at position V_H6 caused decrease in productivity of the class IIB antibody without

affecting the affinity, whereas the same mutation for several class IIA antibodies decreased their production levels and also induced loss in binding activity.

Table 2. Some recent examples of antibody fragments engineered for improved production yields. S/M =both single and multiple mutations.

Mutations	scFv/Fab	Origin	Improvement	Notes	References
Single	Fv	human	10 -fold	Engineered turns	Knappik and Plückthun, 1995
Single	scFv	human	25 -fold	V_H84	Nieba *et al*., 1997
Single	scFv	murine	30-fold	V_H6	Kipriyanov *et al.*, 1997
Multiple	scFv	murine	20 -fold		Forsberg *et al*., 1997
S/M	scFv/Fab	murine	0-9 -fold	Mutagenesis study	Hugo *et al.*, 2003
Multiple	scFv	human	2-4-fold	Mutagenesis study	Ewert *et al.*, 2003b

In addition to bacteria other heterologous hosts such as yeast (Horwitz *et al.*, 1988, Shusta *et al.,* 1998, Lange *et al.,* 2001), insect cells (Laroche *et al.,* 1991, Reavy *et al.,* 2000), mammalian cells (Dorai *et al.,* 1994), filamentous fungi (Nyyssönen *et al.,* 1993) and various species of plant (jrecent review Churchill *et al.,* 2002) have also been used to produce antibody fragments. A comparison of bacterial, yeast, insect and mammalian expression systems in the production of antibody fragments was presented by Verma *et al.* (1998).

1.2.5 Antibody gene libraries and selection methods to obtain primary antibodies against haptens

One important factor in the development of antibody-based applications is the efficiency of obtaining suitable antibodies. Antibody gene libraries provide an enormous potential to obtain new binding specificities for various antigens. Fab and scFv antibody phage display libraries have been constructed from immunised or non-immunised (naïve) sources. An antibody library can also be constructed synthetically or combinatorially. A wide variety of antibody libraries (Table 3) have been developed to produce new binding specificities but also to improve properties such as expression (Table 2), affinity, specificity and stability (for some examples see Tables 4–6).

Antibody fragments have been displayed *in vivo* on viruses such as bacteriophages (McCafferty *et al.,*1990, Clackson *et al.,* 1991, Marks *et al.,*

1991, Hoogenboom *et al.,* 1991) and baculoviruses (Mottershead *et al.,* 2000), on bacteria (Fuchs *et al.,* 1991, Georgiou *et al.,* 1997 (review), Daugherty *et al.,* 1998, 2000) and on yeast (Kieke *et al.*, 1997, Boder and Wittrup, 1997, Boder *et al.,* 2000, Feldhaus and Siegel, 2004 (review)). These microorganisms couple the phenotype to the genotype and take care of the amplification and the display of the antibody at the surface. However, the transformation efficiency of the microbes limits the diversity of the antibody library.

Alternatively in *in vitro* display methods the genetic element is attached to the corresponding polypeptide, either non-covalently in ribosome display (Hanes and Plückthun, 1997, He and Taussig, 1997) or covalently in mRNA display (puromycin aided ribosome display) (Roberts and Szostak, 1997). Polymerase chain reaction (PCR)-based mutagenesis or DNA-shuffling (Stemmer, 1994) during selection rounds can be applied to improve the properties of the antibody. *In vitro* display methods are not restricted by the transfection efficiency and libraries having up to 10^{13} clones have been constructed (Hanes and Plückthun, 1997, Roberts and Szostak, 1997). However, possibly due to technical difficulties the use of these *in vitro* display methods is at present not as widespread as *in vivo* display methods. For a recent review see Lipovsek and Plückthun, 2004.

In the search for anti-hapten antibodies phage display followed by bacterial production has been a widely used combination and is therefore briefly described here. The first example of an antibody fragment (scFv) displayed on the surface of a filamentous phage was presented by McCafferty *et al.,* (1990). The genes of the variable regions of an antibody were fused to pIII, one of the phage coat proteins. Fusion protein was displayed on the surface of the phage and the link between phenotype and genotype was formed. Soon the display of active Fab-fragments was also reported (Hoogenboom *et al.,* 1991). The first antibody library (scFv) from immunised mice was constructed and used for selection of specific antibodies by Clackson *et al.,* (1991). Antigen biased libraries from immunised sources have been constructed since then and used to obtain binders for immunogenic antigens such as hapten-protein conjugates (Clackson *et al.*, 1991, Barbas *et al.,*1992, Burmester *et al*., 2001). Immunisation enriches the amount of antigen-specific B-lymphocytes and enhances the transcription of rearranged antibody genes, which facilitates the selection procedure. Libraries of immunised sources usually produce antibodies with

affinities corresponding to the secondary immune response. However, for selection of another antigen a new immunisation and library construction is usually needed. Other forms of preliminary enriched libraries are the patient-specific antibody libraries which have been constructed to obtain human antibody fragments against disease-related antigens of e.g. cancers or allergies (Cai and Garen, 1995, Steinberger *et al.,* 1996, Laukkanen *et al.,* 2003).

Marks *et al.,* 1991 reported the construction of the first naïve antibody library from non-immunised source, making the immunisation procedure and the continual use of animals unnecessary. The affinities of antibodies obtained from naïve libraries are on average in the range of 10^6-10^7 M^{-1}, (Hoogenboom, 1997) corresponding to the affinities usually obtained from primary immune response (Winter and Milstein, 1991). Some examples of non-immunised libraries are presented in Table 3. Naïve libraries are not restricted by the success of immunisation and they can be used to select antibodies for various antigens including non-immunogenic or toxic substances and evolutionarily conserved targets. Increasing the size of the libraries increases the probability of positive clones and the possibility to obtain high affinity antibodies with K_a around 10^9 M^{-1} (Griffiths *et al.,* 1994, Vaughan *et al.,* 1996 de Haard *et al.,* 1999). However, the antibodies selected from naïve libraries as well as from immunized libraries have variability in the whole amino acid sequence, leading to different properties in e.g. stability or expression levels.

In addition to naïve and immunised libraries synthetic and combinatorial antibody libraries have also been constructed to obtain new specificities. Examples are presented in Table 3. Diversity of the antibody genes is not introduced by the immune system but is based on randomisation or design. Random strategies such as error-prone-PCR or mutator strains have been used to give diversity to the whole antibody fragment, and chain shuffling introduces new combinations of variable domains (reviewed by Hoogenboom 1997).

In design-based approaches the synthetic or combinatorial diversity has been targeted to one or several CDRs in one or several frameworks (Table 3). The heavy chain CDR3 has been the frequently used target region for mutagenesis. Mutagenesis of the CDR-H3 alone has been applied to libraries having only one heavy chain (Barbas *et al.,* 1992, Braunagel and Little 1997) or various heavy chains (Hoogenboom and Winter, 1992, Nissim *et al.,* 1994, deKruif *et al.,*

1995). Chowdhury and Pastan (1999) targeted randomisation to hot spots, DNA sequences that are naturally prone to hypermutations. Partial randomisation of a selected antibody may skew the variability towards the specificity of the original antibody and therefore is feasible for affinity maturation (examples in Table 4) or to decrease the cross-reactivity (examples in Table 5), although the protocol has also been used for the selection of totally new specificities (examples in Table 3).

However, randomisation at the nucleotide level has the disadvantage of introducing bias to the sequence, stop codons and non-viable sequences that can cause problems in folding and display. In one synthetic combined HuCAL-library the CDRs were randomised by trinucleotides having optimal codon usage for bacterial expression. This approach introduces extensive sequence and length variability to the selected region without producing unwanted additional stop codons and thus increases the functional diversity of the library. In the HuCAL-library seven heavy and seven light frameworks representing the natural repertoire of human antibodies have been combined and the mutagenised CDRs are attached to these frameworks as prebuilt cassettes (Knappik *et al.,* 2000).

In another type of combinatorial library the vast repertoire of all six CDRs derived from the antibody genes of the B-lymphocytes were individually and simultaneously recombined to an optimal V_H and V_L frameworks (Jirholt *et al.,* 1998, Söderlind *et al.,* 2000, Steinhauer *et al.,* 2002, Azriel-Rosenfeld *et al.,* 2004). The advantages of this set-up are that all the CDRs are of natural origin and the non-functional loops can be avoided. The length, amino acid sequence and the combination of CDRs are varied at the same time. Antibodies have been obtained from the single framework library with typical binding site topographies of cavity, groove and planar binding surface for hapten, peptide and protein antigens, respectively (Söderlind *et al.,* 2000). Libraries based on a single predetermined framework have the advantage that the scaffold can be selected or modified to have optimal properties, e.g. good stability and high production yield.

Table 3. Examples of antibody phage display libraries used for selection of new hapten binders.

	Source	Size	Format		Reference
Naive				**hapten binders for**	
	human	10^7	scFv	phenyloxazolone	Marks *et al.*, 1991
	human	10^{10}	Fab	various haptens	Griffiths *et al*., 1994
	human	10^{10}	scFv	fluorescein, DTPA	Vaughan, 1996
	human	10^8	scFv	oestradiol	Pope *et al*., 1996
	human	10^{10}	Fab	phenyloxazolone	de Haard *et al*., 1999
Synthetic				**Notes**	
	human	10^7	Fab	single framework, CDR-H3 random	Barbas *et al*., 1992
	human	10^7	scFv	49 V_H, CDR-H3 5 or 8 residues random	Hoogenboom and Winter, 1992
	human	10^8	Fab	single framework, CDR-H3 and -L3 random	Barbas *et al*., 1993
	human	10^8	scFv	49 V_H, CDR-H3 random	Nissim *et al*., 1994
	human	10^8	scFv	49 V_H + 7 V_L, CDR-H3 random	deKruif *et al.*, 1995
	human	10^9	scFv	single framework, CDR-H3 random	Braunagel and Little, 1997
	human	10^9	scFv/Fab	7 V_H + 7 V_L, CDR-H3 and -L3 random	Knappik *et al*., 2000
	human	10^9	scFv	single framework, recombined CDRs	Söderlind *et al*., 2000

Most of the reported antibody libraries are in the scFv display format but some Fab display libraries have also been reported (Barbas *et al.,* 1991, 1992, Kang *et al.,* 1991, de Haard *et al.,* 1999, Knappik *et al.,* 2000). Fab fragments are monomeric both in phage display and as soluble proteins, which provides a benefit over single-chain antibodies. Due to the multimerisation tendency of some scFvs the resulting avidity effect may lead to enrichment of the low affinity binders and hampering the affinity ranking of the positive clones.

The proportion of isolated positive clones depends on the library size, antigen, selection conditions and the number of selection rounds. The selection strategy influences the outcome: antibodies with different properties were isolated from the same library by two different selection methods (Lou *et al.,* 2001). Antibody phage libraries and selection methods were reviewed by Hoogenboom, 1997, Hoogenboom *et al.,* 1998, Bradbury *et al.,* 2003 and Bradbury and Marks, 2004.

The common selection strategies for anti-hapten antibodies displayed on a phage use hapten-protein conjugate immobilised to a solid support (McCafferty *et al.,* 1990, Barbas *et al.,* 1991, Marks *et al.,* 1991,) or biotinylated hapten in solution (Hawkins *et al.,* 1992), and binders are caught by a hapten support or by a streptavidin-coated support, respectively. After washing, the eluted phages are

used for infection and the binders are subjected to subsequent rounds of enrichment. Especially the elution step affects the outcome. Elution with soluble antigen has been shown to increase the specificity (Hemminki *et al.*, 1998a, Charlton *et al*., 2001), and the amount of eluting hapten may have an effect on the affinity of the binders selected (Schier *et al.,* 1996, Hemminki *et al.,* 1998b). For other properties than affinity and specificity different types of selection methods have been applied as discussed below.

Screening of antibody libraries in an automated high-throughput format (Hallborn and Carlsson, 2002) can also be used to obtain anti-hapten antibodies. The conjugated haptens can be immobilised for ELISA type screens or the haptens can be modified with e.g. fluorescent label for detection purposes. Screening of an entire primary library would however be impractical and require a large amount of hapten. Therefore small or pre-enriched libraries are preferred for screening. Recently a new method, "single-molecule-PCR-linked *in vitro* expression", has been used for rapid screening of mutant antibodies with improved affinity. 384-well plates were used in all steps and the total time period was less than 10 hours for amplification of the mutants, cell-free protein synthesis and screening of individual clones in ELISA-format (Rungpragayphan *et al.,* 2004). The method is best suited for small libraries used e.g. to fine-tune the affinity or specificity.

1.2.6 Improving the properties of antibody fragments

In some cases fine-tuning of properties is required for better performance of the antibody fragments. Various libraries have been constructed and knowledge-based approaches have been applied to improve the properties of an antibody obtained from hybridoma cell line or from the above-mentioned primary libraries (for examples see Tables 4–6). Here the improvements of the properties relevant for anti-hapten antibodies in *in vitro* applications are reviewed. The optimisation of other properties such as reduced immunogenicity and toxicity or construction of antibody fusion proteins for detection purposes is not included in this introduction.

The antibody-antigen interaction is a combination of steric and electrostatic complementarity between the paratope of an antibody and the epitope of an antigen. Hydrophobic interactions, hydrogen bonds, salt links and van der Waals

interactions may all contribute to the binding. By increasing the number of specific interactions and the size of the buried surface area it is possible to improve the affinity. The binding properties of the antibodies have been improved by single mutations, random mutations to selected residues, random mutagenesis for the whole or some part of the fragment, CDR-walking, insertions in selected CDRs and chain shuffling (examples in Table 4). In general, the knowledge-based site-specific approaches to improve the affinity have not been widely used due to the lack of detailed high resolution structural data of antibodies and to the difficulty in predicting the possible conformational changes occurring during antigen binding (Dougan *et al.,* 1998). Homology modelling has been used successfully to target single residues or areas for mutagenesis (Roberts *et al.,* 1987, Ruff-Jamison and Glenney*,* 1993, Iba *et al.,* 1998, Hemminki *et al.,* 1998a). At present mutagenesis, random or targeted, followed by selection for improved affinity continues to be a convenient method.

Sometimes the association or the dissociation behaviour of the antibody has been the special target for affinity-tuning (Dueñas *et al.,* 1996, Söderlind *et al.,* 2001, Jirholt *et al.,* 2001, Hugo *et al.,* 2003). In many antibody applications the kinetic parameters of the binding are important. In diagnostic and immunoaffinity applications as well as in therapeutic applications and antibody chip technologies a high association rate is crucial. Katakura *et al.,* (2004) developed a kinetic model to improve the isolation of such antibodies. On the other hand the dissociation rate constants are very important in applications such as continuous monitoring and affinity chromatography applications, which prefer a rapid dissociation (Hugo *et al.*, 2003). Some examples of affinity engineering of hapten-binding antibodies are listed in Table 4. As high as femtomolar affinity has been achieved (Boder *et al.*, 2000).

*Table 4. Examples of remarkable affinity improvements of anti-hapten antibodies. * 2–4 amino acid inserts to CDR-H2*

Target area	Hapten	Affinity improvement	Notes	References
12 residues	Phosphotyrosine	10-fold		Ruff-Jamison and Glenney, 1993
5 residues	Cortisol	8-fold	Improved specificity	Chames *et al*., 1998
CDRs	Testosterone	12-fold		Hemminki *et al*., 1998
CDR-H2	Estradiol	12-fold	Improved specificity	Lamminmäki *et al*., 1999*
Whole fragment	Fluorescein	>1000-fold		Boder *et al*., 2000
Whole fragment	Fluorescein	30-fold		Jermutus *et al*., 2001

Specificity requirements for the antibody fragment depend on the application. In general high specificity is preferred and some examples of antibody engineering to decrease cross-reactivity are listed in Table 5. Random mutagenesis to defined areas has been a successful approach for specificity optimisation. High specificity is important for diagnostic antibodies and especially in cases in which the cross-reacting compound may be present in samples at high concentrations. Determination of different steroid hormone levels in human sera samples is a good example of a high requirement of specificity of anti-hapten antibodies (Pope *et al.,* 1996, Hemminki *et al.,* 1998a, Lamminmäki *et al.,* 1999). On the other hand in applications such as affinity chromatography and antibody-based solid-phase extraction the binding of related substances can sometimes be considered advantageous. Cross-reactivity for structurally similar compounds makes it possible to capture all of them simultaneously and to subject the enriched mixture to further identification and quantitation (Houben *et al.,* 1999, Delaunay-Bertoncini and Hennion, 2004).

*Table 5. Examples of specificity engineering of anti-hapten antibodies. Antibody libraries aiming at new specificities are presented in Table 2. *17-α hydroxyprogesterone, ** many clones with different specificities.*

Target area	Hapten	Change in specificity	References
Heavy chain	Estradiol	reduced cross-reactivity	Saviranta *et al*., 1998
CDRs	Testosterone	reduced cross-reactivity	Hemminki *et al.*, 1998 ab
3 CDR	17-OHP*	broadened specificity	Iba *et al*., 1998
SDRs	Digoxin	altered specificities**	Chen *et al*., 1999
CDR-H2	Estradiol	reduced cross-reactivity	Lamminmäki *et al*., 1999
3 CDR + random VH	11-deoxycortisol	altered specificity	Miyazaki *et al*., 1999
5 or 10 residues	Digoxin	altered specificity	Short *et al*., 2001
Whole fragment	Sulphonamides	broadened specificity	Korpimäki *et al*., 2003

Stability is an important requirement for all antibodies, independent of specificity. *In vitro* applications may require antibodies with a long half-life, high stability in organic solvents and resistance to surface denaturation and dry atmosphere. Natural antibody frameworks have very different stability properties (Ewert *et al.,* 2003a). Single amino acids may have a great impact on stability (Honegger and Plückthun, 2001). Two aspects of stability engineering, structure-based framework engineering and CDR-grafting to more stable frameworks, were recently studied by Ewert *et al.,* 2004. CDR-grafting is a way introduce the valuable binding properties of CDRs to a superior framework. Targetted

mutations can be used to refine the stability of an antibody. Mutations affecting stability may influence e.g. the packing of the hydrophobic core, hydrogen bonding or charge interactions. The importance of the disulphide bridges for stability was indicated by several authors (Glockshuber *et al.,* 1990, Proba *et al*., 1997, 1998), although scFv with sufficient stability can tolerate the loss of both disulphide bridges (Wörn and Plückthun 1998b). High temperatures or denaturants have been used to introduce selection pressure for increased stability of antibody fragments (Proba *et al*., 1998, Jung *et al.*, 1999). Examples of successful stability engineering are presented in Table 6. Different aspects of stability engineering of scFv's were reviewed by Wörn and Plückthun (2001). In addition to the protein engineering approach, chemical cross-linking by e.g. glutaraldehyde has been used to stabilise the antibody fragments (Glockshuber *et al.,* 1990, Vuolanto *et al.,* 2004).

The stability of an antibody sometimes correlates with the expression yield (Jung and Plückthun, 1997, Jung *et al*., 1999, Ewert *et al.,* 2003b) and even a single amino acid replacement can improve both stability and expression (Hugo *et al.,* 2003). The balance between the correct folding and aggregation may also depend on a single amino acid (Knappik and Plückthun 1995). The best correlations between stability and production level have been obtained with cytoplasmic expression, whereas the correlation of stability with periplasmic expression is less straightforward, e.g. mutations increasing the periplasmic expression of anti-fluorescein scFv had no influence in stability (Nieba *et al.,* 1997).

Table 6. Examples of stability engineered recombinant antibody fragments. Loop (or CDR) grafting = 1-6 CDR loops of one antibody are transferred to a more optimal framework.

Target area	Origin	Fragment	Notes	References
Loop grafting	murine	scFv	also improved expression	Jung and Plückthun, 1997
Single replacements	murine	scFv		Wörn and Plückthun, 1998a
Single replacements	humanised	scFv	also improved expression and affinity	Jung *et al*., 1999
Whole fragment	human	scFv		Jermutus *et al*., 2001
Six replacement	human	scFv	also improved expression	Ewert *et al*., 2003b
Single replacement	murine	scFv/Fab	L34, also improved expression	Hugo *et al*., 2003

Targetted and knowledge-based engineering has been applied to improve the crystallisation properties of antibodies. Surface mutagenesis was applied to introduce a packing motif to the β-strand of variable domain in order to promote

their crystallisation propensity (Wingren *et al.,* 2003). Immunoreactivity, expression and overall structures of the antibodies were maintained. Rational design of packing motifs promoting crystallization also opens new possibilities for anti-hapten antibody applications, see section 1.3.5.

1.2.7 Specific features of anti-hapten antibodies

Antibodies recognising small haptens typically have concave antigen binding sites compared to the flattened binding sites of antibodies against macromolecules such as proteins, although some exceptions do exist (MacCallum *et al.,* 1996). By analysing 381 sequences of murine and human antibodies with known antigen specificities Vargas-Magrazo *et al.* (1995) concluded that different antigen types prefer different combinations of canonical structures of CDR loops. Some canonical structure classes are specific for certain antigens whereas others, called multispecific classes, are represented in antibodies recognizing different antigen types. Among the specific canonical classes a correlation between the length of the CDR-L1 and CDR-H2 loops and the antigen has been shown: e.g. antibodies against haptens prefer long L1 and H2 loops. In addition there is some correlation between the antigen type and the amino acid composition of CDRs. Certain residues are under– or over–represented in hapten binders. (Collis *et al.,* 2003). In anti-hapten antibodies most of the contacting residues are located close to the centre of the binding site, whereas larger antigens are also in contact with less central CDR residues (MacCallum *et al.,* 1996). The knowledge of the special features of anti-hapten antibodies can be applied e.g. to the design of synthetic or combinatorial antibody libraries for anti-hapten binders.

Due to the fact that low molecular weight compounds are not capable of eliciting an immune response these compounds must be linked to a macromolecule via a linker before immunisation. Furthermore the current selection methods for antibody phage libraries require conjugated hapten. The linking of haptens to proteins or to e.g. biotin can be very problematic, requiring sophisticated chemistry. Due to the linking the anti-hapten antibodies may recognise the hapten and a part of the linker (‘the bridge effect’) (Barbas *et al.,* 1992, Tuomola *et al.,* 2000, Kusharyoto *et al.,* 2002, Monnet *et al.,* 2002). In such cases the antibody may have significantly lower affinity for the hapten alone. In principle

the linking may also distort the structure of the hapten and the antibody therefore recognises the conjugated more strongly than the native form of the hapten. The composition of the linker and the conjugation site of the hapten have an impact on the binding affinity and specificity of the selected antibody. Site-directed mutagenesis to reduce binding to the linker moiety (Kusharyoto *et al.,* 2002) or optimisation of the panning strategy, have been used to increase the specificity for the hapten moiety (Charlton *et al.,* 2001, Kramer, 2002). Specific elution with soluble hapten increased the recovery of hapten-specific antibodies, in comparison to unspecific elution which produced binders against hapten conjugate (Barbas *et al.,* 1992, Charlton *et al.,* 2001). According to Kramer (2002) immunisation with a hapten conjugate produces non-redundant antibodies, making selection of anti-hapten antibodies difficult. In their approach the B-cells of immunised mice were enriched with hapten-coated magnetic beads before constructing the library. Using this approach 75% of the clones were specific binders after three cycles of panning.

1.2.8 Site-specific immobilisation of recombinant antibody fragments for affinity chromatography

In most applications a high density antibody immobilisation is preferred. The importance of the optimal method of immobilisation increases as the size of the antibody fragment decreases. The smaller the antibody fragment, the more crucial it is to have oriented and site-specific immobilisation. Immobilisation should be efficient and stable and occur in conditions compatible with the antibody fragment. Random immobilisation, such as in the case of methods using passive adsorption to a surface or covalent linkage between reactive groups of the immobilisation surface and the lysine residues of the antibody, usually causes decreased binding capacity (Spitznagel and Clark, 1993, Peluso *et al.,* 2003). Immobilisation may take place directly via lysine residues on CDRs, or the antibody can become partially denatured due to multipoint attachment to the support. Random orientation of antibody fragments may cause steric hindrance of antigen binding. However, steric hindrance has less effect on the binding efficiency of low-molecular weight haptens than on the binding of larger protein antigens (Schramm and Paek, 1992, Spitznagel and Clark, 1993).

Denser packing of Fab-fragments and up to 10-fold improvements in antigen binding capacity were obtained by site-specific compared to random immobilisation (Peluso *et al.,* 2003). Various strategies have been developed for site-specific and oriented immobilisation of recombinant antibody fragments. An antibody fragment can be captured in an oriented way on the surface by intermediate proteins such as Protein G or streptavidin (for biotinylated antibodies). However the demands for immobilisation in affinity chromatography differ from those of other antibody applications due to the elution step. Every component and linkage on the immobilisation must also be stable in the conditions used to release the bound molecule. Various non-covalent immobilisation strategies have been reviewed by Burgess and Thompson (2002).

For recombinant antibody fragments a widely used approach is genetically fused immobilisation tags. Introduction of a single cysteine to the carboxy terminus of an antibody fragment has been used for covalent immobilisation (Kipriyanov and Little, 1997). A slightly longer tag for both purification and immobilisation purposes, a histidine tag, provides a versatile approach. Since Hochuli *et al.,* 1987 reported that adjacent histidine residues can be used to immobilise a protein to the metal-affinity chromatography support, various histidine-containing peptides have been fused to recombinant antibodies (Skerra *et al.,* 1991). A tag of six histidine residues is the most widely used. A number of histidines has varied between 2 and 9. Efficient, over 90% immobilisation can be obtained by three or more histidines but the stability of the binding increases as a function of additional histidines (Hochuli *et al.,* 1988). The binding strength and efficiency can also be adjusted by the choice of immobilised metal, because different metals have different binding strengths for a chelate (Cu > Ni > Co = Zn) (Arnold, 1991). For a recent review of immobilised metal affinity chromatography (IMAC) see Ueda *et al.,* 2003. Kinetically stable immobilisation via histidine tags can be achieved by immobilisation to a cobalt support followed by oxidation (Hale, 1995). Oxidised metal affinity support is resistant e.g. to low pH, which broadens the usability of this immobilisation method.

In addition to short peptides whole binding domains, such as cellulose- and chitin binding domains, have been fused to antibody fragments in order to immobilise fusion proteins directly from the crude bacterial lysate to inexpensive and environmentally friendly supports of cellulose and chitin,

respectively (Reinikainen *et al.,* 1997, Linder *et al.,* 1998, Blank *et al.,* 2002, Azriel-Rosenfeld *et al.,* 2004). For a review of different affinity fusion strategies for recombinant proteins see Nilsson *et al.* (1997).

1.3 Applications of enantioselective antibodies

1.3.1 Background

Specific preparative fractionation of enantiomers is needed after chemical synthesis of a chiral compound. Although enantioselective synthesis has been under extensive development, at the early stages of compound development the separation approach still dominates over the asymmetric synthesis Maier *et al.* (2001). Setting up an asymmetric synthesis is time consuming and expensive. Enantiomers can be separated from each other by indirect or direct methods. Indirect methods include a derivatisation step with an enantiomerically pure compound, followed by separation by fractional crystallisation or by conventional chromatography. Later the derivatizing agent is removed and the enantiomer is purified. This method has been widely used in the pharmaceutical industry due the relatively easy scale-up. Direct methods in preparative scale such as chiral chromatography based on either chiral liquid- or solid-phase need no modifications to the enantiomer prior to the fractionation. Various organic chiral selectors (reviewed by Ward, 2000) and unspecific proteins have been used as chiral phases (reviewed by Millot 2003). According to Rouhi (2003), the interest in continuous chiral chromatography is growing. The tendency is to develop the specific chiral stationary phase for preparative applications, rather than more general or broad selectivity supports (Armstrong and Zhang, 2001).

Pharmaceutical, forensic, biochemical and environmental analyses are performed for a wide variety of analytes from different matrices such as plasma, urine, tissue, saliva, hair, water, foods and soil. A common feature of all these analyses is that the sample matrices are complex, have potential interferences and the analytes are in low concentration. Tedious sample preparation with many steps is usually required. However, the use of several purification steps before analysis is not preferred because all additional clean-up steps introduce a risk of losses and decrease the reliability of the method. Sample preparation is one of the most critical steps for reliable results. Especially in the

pharmaceutical industry the trend is to develop single-step automated sample preparation methods in high-throughput format with on-line coupling to the separation and detection techniques (Koh *et al.,* 2003 (review)). Different sample preparation approaches were described by Stevenson, 2000. In general, enantioselectivity is not included in the sample preparation but rather the following separation step.

1.3.2 Antibody-based affinity chromatography

Immunoaffinity chromatography (IAC) is a chromatographic method based on immobilised antibodies which bind the target compound from the sample matrix efficiently and in a specific manner. After subsequent removal of unwanted impurities from the support, the bound compound is eluted by disrupting the antibody-antigen complex using chaotropic agents, extreme pH, organic solvents or a different combination of these. A regeneration step is added to the protocol in the case of reusable immunoaffinity supports before the next round of separation. Immunoaffinity chromatography (IAC) has been used for specific purification of antigens (enzymes, receptors, peptides) and haptens (drugs, hormones, antibiotics, carbohydrates). For recent reviews see Hennion (1999), Muronetz and Korpela (2003). IAC is one of the most powerful methods for the purification and concentration of antigens from various sample matrices. An excellent review of different types of analytical applications of immunoaffinity chromatography was presented by Hage (1998), and the challenges facing separation of enantiomers was reviewed by (Maier *et al.,* 2001). Various support materials for immunoaffinity applications were listed by Stevenson (2000).

Most of the antibodies used hitherto in immunoaffinity columns have been polyclonal or monoclonal antibodies produced by immunisation. However, the use of recombinant antibody fragments would have many advantages over poly- and monoclonal antibodies, such as lower production costs and improved binding capacity due to the smaller size and oriented immobilisation via a tag. The possibilities to use antibody libraries in the search for new specificities, and antibody engineering to introduce tags and to adjust the properties, if needed, also increase the potential of immunoaffinity supports. An important parameter of immunoaffinity chromatography is the efficiency and specificity of the hapten binding, especially for low-concentration and high-volume samples. Modifying the selection method for

the antibody libraries would offer a possibility to obtain antibodies with suitable kinetic parameters (Section 1.2.6.). In immunoaffinity-based purification or in analytical applications, antibodies with fast association kinetics are preferred. Fast dissociation of the antigen or hapten in mild elution conditions is required for low volume recoveries or in the case of reusable affinity supports. (Ohlson *et al.,* 1997, Burgess and Thompson, 2002). Reusability increases the economical feasibility of the immunoaffinity supports. Hofstetter *et al.* (2002) reported the continuous use of an immunoaffinity column for over 1000 cycles of purification of a hapten in 11 months, without significant change in column performance. Reusability of an antibody support has been improved by crosslinking immobilised antibody fragments with glutaraldehyde (Vuolanto *et al.,* 2004).

Enantioselective antibodies have been used to fractionate chiral synthesis products (Mertens *et al.,* 1982, Knox and Galfre, 1986) and other mixtures of enantiomers (Hofstetter *et al.,* 1998, 2002) hitherto in a relatively small scale, probably due to the use of poly- and monoclonal antibodies. Enantiomers from biological samples such as sera, plasma and urine have been extracted by specific antibody supports for analytical purposes. Examples of enantioselective antibodies both in preparative and analytical applications have been collected into Table 7. Other applications for enantioselective antibody fragments are presented in section 1.3.5.

Table 7. Examples of enantioselective antibodies in immunoaffinity applications.

Enantiomers	Sample matrix	Antibody origin	Reference
Abscisid acid	Buffer	Antisera	Mertens *et al*., 1982
Abscisid acid	Buffer	Monoclonal	Knox and Galfre, 1986
Loxoprofen metabolites	Plasma	Antisera	Takasaki and Tanaka, 1992
Amino acids	Buffer	Monoclonal	Hofstetter *et al*., 1998, 2002
Bufuralol and metabolites	Plasma	Antisera	Ikegawa *et al*., 1998
Warfarin	Serum	Polyclonal	Clarke *et al*., 2001
Ibuprofen-modified peptide	Buffer	Polyclonal	Ikegawa *et al*., 2001
Amphetamine	Urine	Monoclonal	Lua and Chou, 2002

1.3.3 Antibody-based solid-phase extraction as a sample preparation method

Solid-phase extraction (SPE) has become a popular method for sample preparation, and the development of new formats and sorbents has been rapid. Solid-phase extraction can be used to simultaneously isolate, concentrate, purify and change the medium of the analyte. Antibody-based solid-phase extraction methods follow the same principles as immunoaffinity chromatography except having much less theoretical plates due to the shorter length of the column. The difference between immunoaffinity chromatography and antibody-based solid-phase extraction is that the former is a separation method coupled to a detection system whereas the latter is an extraction method coupled to separation and detection systems either on- or off-line. Most commonly the SPE is combined with the chromatographic and electrophoretic separation methods. SPE-HPLC (high performance liquid chromatography)-combinations are routinely used due to the relatively easy connection of these two techniques. The coupling with capillary electrophoresis (CE) is less widely used because of the technical complexity, although some examples already exist even for immunosorbents (Guzman, 2000). SPE-CE combinations have potential for microscale analysis due to their low sample and reagent consumption, great resolution and speed.

Different sorbent types can be used in SPE. Non-specific sorbents include inorganic oxides (silica gel, alumina, diatomaceous earth), chemically bonded silicas (C8/C18), porous polymers and carbon. Compound- and class-specific sorbents are based on ion exhange, restricted access or specific affinity of proteins or molecularly imprinted polymers. Different sorbent types were compared and examples were presented by Poole, 2003. A common feature of non-specific sorbents is their lack of specificity; they are unable to distinguish between enantiomers and they co-extract interfering molecules from the sample matrix (Maurer *et al.,* 2000, Rolcik *et al.,* 2002, Hennion and Pichon, 2003), which can become a problem especially when the analyte is at a trace level and the interfering compounds are present at higher concentrations. Co-eluted interferences may cause problems and even suppress the formation of analyte ions in the electrospray process of the MS-detection. Specific antibody-based extraction prior to MS-detection has been shown to improve the sensitivity of the analysis considerably by increasing the signal-to-noise ratio (Ferguson *et al.,*

2001, Rolcik *et al.,* 2002). Figure 5 illustrates clearly the importance of specific extraction of the analyte before MS-detection.

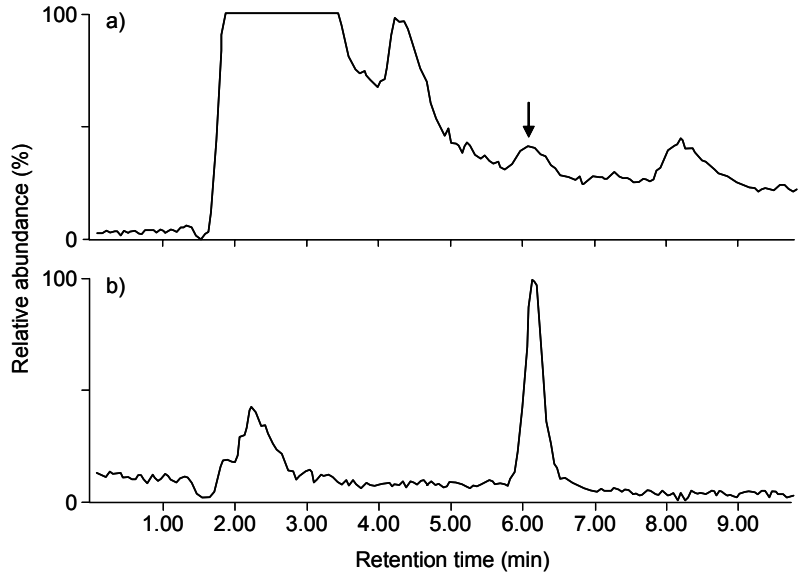


Figure 5. Comparison of LC-MS analyses. a) melatonin- spiked human plasma extracted by C_{18}-SPE (the arrow indicates the melatonin). b) melatonin-spiked plasma extracted by a specific immunosorbent (Modified from Rolcik et al., 2002).

Immuno-SPE is an antibody-based solid-phase extraction providing an interaction with high affinity and specificity for the analyte. The properties of the antibodies are the key determinants for successful extraction due to a strong correlation between recovery of the analyte and the affinity of the antibody (Hennion and Pichon, 2003). Immuno-SPE has potential to solve some of the problems of the unspecific SPE. The breakthrough volumes for immunosorbents are higher than for non-specific sorbents which makes the binding of low-concentration analytes from high volumes more efficient. Immunosorbents can also be enantioselective and therefore provide an additional advantage. For examples of different immunosorbents in SPE see the recent review by Delaunay-Bertoncini and Hennion, 2004.

Most of the present immunoaffinity sorbents are based on poly- or monoclonal antibodies. Few examples of antibody fragments, digested from monoclonals,

have emerged (Phillips and Krum, 1998, Guzman, 2000, Clarke *et al.,* 2000). Hitherto the potential of antibody libraries as a source of specific binders has not been utilised. According to Delaunay-Bertoncini and Hennion (2004) the use of antibody fragments is one of the future directions in the development of analytical systems, the others being miniaturization of analytical microsystems and chip technology.

In classical immunoassays cross-reactivity is typically a problem, whereas in solid-phase extraction it may be even desirable. Cross-reactivity is preferred if a class of compounds, such as closely related pesticides or a drug and its metabolites, are to be extracted and the following separation and detection steps are to be used for the identification. The cross-reactivity of an antibody must be determined experimentally in the format of intended use. For example, although only a little binding of related compounds (phenylureas) was observed in ELISA (Enzyme-linked immunosorbent assay) -assay of isoproturon and chlortoluron, a strong retention of these phenylureas was observed in the column format (Stevenson, 2000). Most examples reported as cross-reacting immunosorbents are made of using polyclonal antibodies and a few of immunosorbents based on actually cross-reacting or group-specific monoclonal antibodies have been reported (Houben *et al.,* 1999, Delaunay *et al.,* 2003). Mimicking in a controlled way the polyclonal approach the immunosorbents simultaneously binding different analytes could be created by immobilising more than one specific antibodies to the SPE support when the analytical method is used to identify the eluted compounds. This possibility greatly increases the versatility of the immunoaffinity sorbents.

On-line connections in general require compatibility between the extraction and the separation, e.g. the chemical composition of the buffers and the pressure resistence of the sorbents should be compatible. On-line connection is automatable and minimal sample handling and solvent consumption is necessary. On the other hand the off-line connection does not limit the properties of the buffer or the sorbent because the eluent of the first step can be evaporated to dryness and later reconstituted to the conditions preferred by the later separation or analysis. With an off-line method an enrichment factor of over 100 has been obtained for one analyte (Ferguson *et al.,* 2001).

Most frequently the final analysis of the compounds has been performed by measuring the UV-absorbance or fluorescence. Mass spectrometric (MS) detection has also been used after the SPE and the separation technique (Maurer *et al.,* 2000, Wu *et al.,* 2001, Ferguson *et al.,* 2001, Rolcik *et al*., 2002). Mass spectrometric detection is especially useful after a class-selective extraction if the components of the eluent are identified in addition to their quantitation (Wu *et al.,* 2001). The advantage of the direct coupling of SPE and MS is the increased speed when the separation step between the extraction and detection techniques is excluded.

Extraction formats differ depending on the sample matrix. Single columns of unspecific supports have been used for environmental analysis, whereas the analysis of drugs, metabolites and endogenous compounds has moved from small columns towards the automated 96-well filtered microtiter plates (Souppart *et al.,* 2001, Matthews *et al.,* 2002, Bakhtiar *et al.,* 2002, Shou *et al.,* 2002, Pommier *et al.,* 2003, Svennberg *et al.,* 2003, Yang *et al.,* 2003, Kim *et al.,* 2004) and also recently to 384-well filtered microtiter plates (Rule *et al.,* 2001) Automated solid-phase extraction is gaining popularity, especially in replicate analysis. Automation saves operation time and improves precision and safety if hazardous samples are used. Contamination between close wells may however limit the use of the plates at the moment (Majors, 2001). These aspects and future prospects of automatisation of SPE were more widely reviewed by Rossi and Zhang (2000). Hitherto, antibody-based solid-phase extractions have been performed only in the column format, although the column size has been decreasing down to microcolumns.

1.3.4 Enantioselective antibody crystals

Immunoaffinity columns separate the enantiomers efficiently and the continuous use of the affinity supports is possible (Ikegawa *et al.,* 1998, Houben *et al.,* 1999, Rejeb *et al.,* 2001). Compared to non-specific chiral columns in which the separation is based on partition leading to large elution volumes, the bound compounds from immunoaffinity columns are eluted in higher concentration. Decreased solvent consumption is one of the goals in the chemical and pharmaceutical industry. Elution volume depends on the efficiency of the eluent but the volume of the columns also plays a significant role. Typically 80–95% of

the column volume of the immunosorbents is due to the volume of the support (Vuolanto *et al.,* 2003, 2004). Crystallisation of antibody fragments is the most dense way of packing of antibodies and cross-linking the antibodies in crystals makes this type of antibody supports very stable. Separation of the enantiomers of a drug candidate was performed with crystallised and cross-linked enantioselective Fab-fragments and the miniaturised column was reusable (Vuolanto *et al.,* 2003). Cross-linked antibody crystals were insoluble even in 100% methanol (Vuolanto *et al.,* 2003). In addition to improved solvent stability increases in thermostability and protease resistence of the cross-linked protein crystals have also been reported (Govardhan, 1999, Vuolanto *et al*., 2004).

1.3.5 Examples of *in vitro* detection of enantiomers

In principle all antibody-based diagnostic methods designed for low molecular weight haptens can also be applied to enantiomer analytics. The enantiospecificity of the detection methods originates from the properties of the antibody fragments used. Natural low molecular weight chiral compounds analysed from biological matrices generally have only one enantiomer due to their asymmetric synthesis by the enzymes *in vivo*. Therefore enantiospecific detection has not played a significant role in the diagnostics of endogenous compounds. However, when the presence of exogenous compounds is to be determined, enantioselectivity may become an important factor. Drug abuse is an illustrative example. Amphetamine and its derivative metamphetamine are both chiral compounds and are widely used as illegal drugs. However they are also *in vivo* metabolites of some legal drugs and a definite knowledge of amphetamine metabolism and enantiomeric disposition is needed. (Peters *et al.,* 2002, Lua and Chou, 2002). Enantiomeric drug and metabolite analysis assists the investigation process when the source of these compounds must be confirmed in forensic analysis before legal actions. Recently an enantioselective immunoaffinity chromatography was developed to determine metamphetamine from urine samples of drug addicts (Lua and Chou, 2002).

Enantioselective antibodies have also provided a tool to investigate rare *in vivo* effects of a drug. Ito *et al.*, (2004) used enantioselective polyclonal antibodies in enzyme immunoassay and immunoblotting in order to study the enantioselective *in vivo* conjugation of ibuprofen to the proteins. They were able to identify

cellular target proteins modified by the reactive metabolite of ibuprofen. Enantioselective antibodies were used to examine the mechanism of ibuprofen-induced cytotoxity. In general, a corresponding antibody-based approach could be used to study more widely the importance of protein modifications of both chiral and non-chiral drugs or other exogenous compounds.

In addition to the various medical applications, antibodies can also be used in environmental applications (Van Emon *et al.,* 1998, Kramer and Hock, 2003). Immunoextraction has already been coupled to ELISA or enzyme assays in order to increase the sensitivity in environmental monitoring of cyanobacterial toxins (Rivasseau and Hennion,1999). Immunoaffinity purification of soil extracts prior to ELISA increased the sensitivity of a herbicide assay 100-fold (Sheedy and Hall 2001). Enantioselective antibodies have the potential to solve specific analytical tasks. For example polychlorinated biphenyls (PCBs) are stable chiral pollutants that bioaccumulate and cause potential health hazards in aquatic ecosystems. Some macrozooplanktons are able to metabolise these pollutants stereoselectively (Wong *et al.,* 2004). It is likely that the biotransformation is the major long-term removal process for these compounds. Enzymes perform enantioselective reactions and the possible involvement of biodegradation in the destruction of PCBs can be concluded from the enantiomeric excess of the chiral pollutants. The chiral analysis is a very important diagnostic tool to study these biodegradation processes and also to investigate the stereoselective biological and toxicological effects of the enantiomers of pollutants in different species in food webs. Many pesticides and herbicides are also chiral compounds, and the activity often resides preferentially in one of the enantiomers. The other enantiomer may be toxic to non-target organisms (Wang *et al.,* 2004) and the undesirable environmental loading may be due to the applied racemate. Examples of antibodies used in environmental monitoring were reviewed by Van Emon *et al.,* 1998.

1.4 Aims of the present study

The general aim of this work was to study and develop antibody-based methods for separation and analysis of enantiomers. A drug candidate was used as a model compound. The specific objectives were:

1. To study the applicability of immobilised antibody fragments in repeated separations of enantiomers in affinity columns.

2. To investigate the possibility to prepare enantioselective antibody-based nanomembranes in order to enhance the versatility of the method.

3. To determine the affinity, specificity and stability requirements for antibodies in applications developed in this thesis.

4. To develop in a high-throughput format a solid-phase extraction method based on immobilised antibody fragments.

2. Materials and methods

2.1 The methods used in the original papers of this thesis

Method	Used in
Linker synthesis for the hapten	I
Immunisation and screening of monoclonal antibodies	I
Cloning of Fab-fragments, ENA5His and ENA11His	I
Construction of a homology model of ENA5His	III
Design of mutations to ENA5His	III
Site-specific mutagenesis of ENA5His	III
Production and purification of the Fab-fragments	I, III
ELISA, SDS-PAGE, Immunoblotting	I, III
Immobilisation of the Fab-fragments	
Copper -IMAC	I, III
Cobalt - IMAC	IV
Aldehyde derivatised silica	II
Affinity measurements	I, III
Immunoaffinity columns	I, III
Continuous enantioseparation	I, III
Solid-phase extraction	IV
HPLC-analysis of the enantiomers	I, III, IV
MS-analysis of the enantiomers	IV
Derivatisation of silica nanotubes	II
Immobilisation of the Fab-fragments into the nanotubes	II
Facilitated diffusion in enantioseparation	II

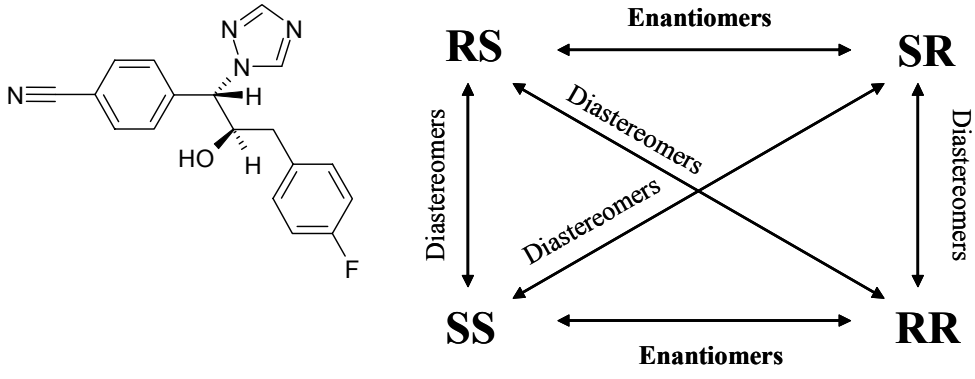
2.2 Fabrication of alumina membranes

Alumina membrane was prepared by anodisation of 0.1 mm thick (Alfa Aesar) aluminium foil. The foil was first polished manually with 94% ethanol followed by electropolishing in a mixture of 80% H_3PO_4, 10% H_2SO_4 and CrO_3 (20g/l) for 10–15 min (15 V, 70 °C). Two-step anodisation was performed in a bucket containing 5% oxalic acid/water (0 °C) at a constant voltage of 20–100 V for 24 hours. Stainless steel was used as a cathode.

The formed alumina was removed in a aqueous solution of 0.2 M CrO_3, 0.4 M H_3PO_4 at 60–70 °C for one hour. The anodisation protocol was then repeated in the same conditions, but the anodisation time was adjusted to obtain the desired thickness of the growing membrane. The voltage determined the pore size of the growing membrane. After growing the alumina membrane the residual aluminium layer from the other side of the membrane was removed in saturated $HgCl_2$ solution. Sonication for 15 min was used to open pore ends. Openess and even size of the pores was checked by atomic force microscopy (AFM). Sol-gel synthesis, protein immobilisation and diffusion experiments are described in Appendix II.

3. Results and discussion

The drug candidate 4-[3-(4-fluorophenyl)-2-hydroxy-1-[1,2,4]triazol-1-yl-propyl]-benzonitrile (Figure 6 a), called hereafter diarylalkyltriazole, has two chiral centres (Södervall and Mutikainen 2002). Therefore its chemical synthesis produces four different stereoisomers (Figure 6 b). The diastereomers can easily be fractionated from this mixture but the enantiomers are difficult to separate from each other by chemical or physical means. The spatial arrangement of the substituents of diarylalkyltriazole differs between enantiomers as illustrated in Figure 7.



a. b.

Figure 6. a) The structure of 4-[3-(4-fluorophenyl)-2-hydroxy-1-[1,2,4]triazol-1-yl-propyl]benzonitrile (diarylalkyltriazole). b) Stereoisomers of diarylalkyltriazole and their stereochemical relations.

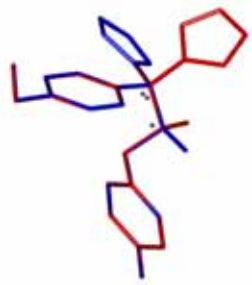


Figure 7. The superimposed conformations of the SS- (blue) and the RR-(red) enantiomers of diarylalkyltriazole. Chiral centres are marked with an asterix. (Modelled and figure provided by Nana Munck, VTT Biotechnology).

In this work, antibody-based methods for purification and analysis of racemic compounds were developed using diarylalkyltriazole, an inhibitor of the aromatase enzyme, as a model system. Efficient separation of the enantiomers is important for both the preparative fractionation of the racemic mixture and the specific analysis of the drug enantiomers. Different stereoisomers were named a-, b-, c-, and d-enantiomers according to their retention order from chiral ovomucoid column. The d-enantiomer was the most interesting enantiomer for drug development and was considered as the main target of the method development. Originally the configuration of the d-enantiomer was determined to be S,S. However, it was later found to be either R,S or S,R (Södervall and Mutikainen, 2002). The absolute configuration of the d-enantiomer has not yet been solved. However, for the experimental part of these method development studies the absolute configuration is of little importance and therefore the name d-enantiomer (and a-, b-, c-enantiomer for the others) is used throughout this thesis in order to avoid confusion.

3.1 Enantioselective Fab-fragments: PCR-cloning, production and purification (I and III)

Immunisation was used to obtain monoclonal antibodies, although at present a more convenient way would be selection from antibody libraries. A three-carbon linker was coupled to the racemic mixture containing enantiomers a and d (Appendix I, Figure 1) and this derivative was linked to the keyhole limpet hemocyanin for immunisation. Four monoclonal antibodies were obtained and screened by ELISA with racemate-BSA (Bovine serum albumin) conjugate

immobilised on microtiter plates (Appendix I). Two of them, named ENA5His and ENA11His, were cloned as Fab-fragments. A tag of six histidine residues was genetically introduced to the end of the light chains for purification and immobilisation purposes. A graphical presentation of the Fab-fragment with the hapten is shown in Figure 8. Modifications to the Fab-fragments made in this study are marked with a label.

These two clones have no apparent sequence similarity at the CDRs. Their CDR-loops vary both in length and in amino acid composition. The overall identities are 36% and 53% for the variable regions of heavy and light chain, respectively. Due to the low binding capacity of ENA11His and the too high affinity of the ENA5His to be used in continuous enantioseparation (described later in detail), a series of affinity mutants of ENA5His was designed to fine tune the affinity as described later in section 3.3. An oligonucleotide-based mutagenesis protocol was applied to introduce the mutations (Appendix III). The double mutant was cloned later by combining the most promising mutants of the heavy and light chain (Appendix III).

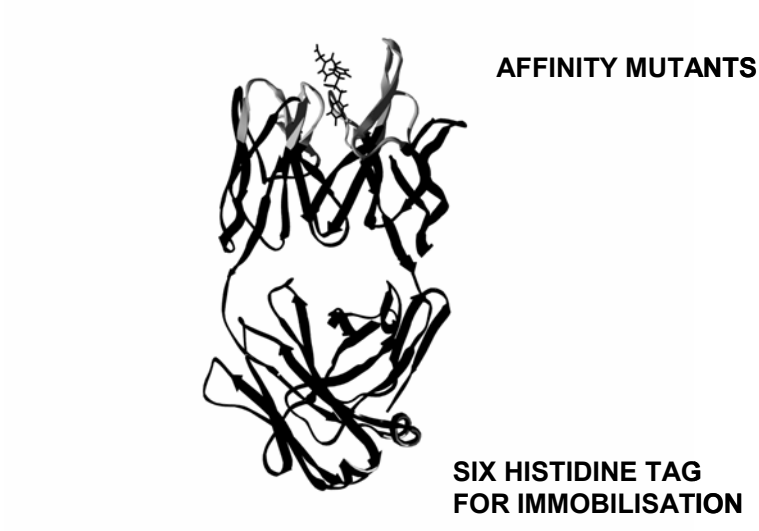


Figure 8. A graphical presentation of the Fab-fragment. Hapten is inserted to the binding site composed of six hypervariable loops (gray). Framework regions of the variable domains and the V_H1 and V_L domains are in black. A six-histidine tag was cloned to the carboxy terminal end of the light chain for immobilisation purposes. Mutants were made in order to obtain an affinity more suitable for the applications. More detailed pictures of the mutations are presented in Figure 9.

The production vector used for all constructions contained the *tac* promoter, which is controlled by the *lac*I^q repressor. The pelB signal sequence of pectate lyase of *Erwinia carotovora* was linked to both the heavy and the light chains to promote secretion. (Alfthan *et al.,* 1993)

All Fab-fragments were produced in *Escherichia coli* bacteria (RV308 strain, ATCC 31608) using the high cell density cultivation method of Pack *et al.,* 1993 (Appendix I). Production levels of different constructions varied from 20 to 150 mg purified Fab/ one litre of culture supernatant, determined by measuring the absorbance at 280 nm. The production yields are presented as purified protein because this final yield is the most relevant for the applications. Highest yields (80–150 mg/L) were obtained for the parental Fab-fragments. Cell lysis occurred during the cultivations, leading to the extensive release of the periplasmic and cytoplasmic fractions to the medium. Production levels of the Fab-fragments were reasonably high, although not nearly as high as has been reported by Carter *et al.,* 1992 (humanised Fab, 1–2 g/L, periplasm, ELISA-determination). Repeat cultivations were performed only with the parental Fabs and the mutant Tyr96Val. Due to the lack of replicate cultivations it is difficult to draw any conclusions about the effect of different mutations on the production levels.

Purifications of Fab-fragments were performed with a two-step procedure. Metal affinity chromatography was followed by ProteinG affinity chromatography. No traces of other proteins were seen in SDS-PAGE analysis after purification. However, 10–20% of the Fab-fragments, depending on the clone, had no interchain disulphide bridge and separate chains were observed in the non-reducing conditions of SDS-PAGE (data not shown). The effect of the lack of the disulphide bridge on the applications is discussed in section 3.5.1

3.2 Immobilisation of the Fab-fragments (I, II, III and IV)

Recombinant antibody fragments can be immobilised either site-specifically or randomly, either covalently or non-covalently as briefly reviewed in the Introduction (Section 1.2.7). In most antibody applications there is a need for stable and oriented immobilisation. All the separation methods for enantiomers developed in this work employed immobilised antibody fragments, and the immobilisation strategy depended on the application. Common to the

immobilisation strategies used in this study is the required stability in relatively high concentrations of organic solvents or at low pH. The widely used immobilisation protocol via a six-histidine tag to the copper-loaded metal affinity support was used in the preparative fractionation studies of the racemate of the a- and d-enantiomers (Appendices I and III). Repeatedly over 95% of the Fab-fragments in PBS (phosphate buffered saline) were immobilised to the support. Immobilisation was stable over 20 cycles of continuous enantioseparation when 40% methanol-PBS was used as eluent. No light chain was observed in dried and reconstituted eluents analysed by immunoblotting with anti-kappa chain detection. However, after the first cycle of solvent elution the maximum capacity of the immunoaffinity support decreased (see section 3.5.1), probably due to the dissociation of the heavy chain not connected via a covalent disulphide bridge to the immobilised light chain. A histidine tag could also be cloned to the carboxy terminal end of the heavy chain in order to improve the stability of the antibody support. No reduction in binding capacity of the antibody support was observed after six months of storage in PBS-0.02% sodium-azide at + 4 °C.

In analytical applications, in which the aim was to use serum samples and eluents compatible with MS-analysis, another immobilisation strategy was needed. The high concentration of methanol required to release the bound analyte is not applicable directly to MS. The common approach to elute haptens from antibodies is to use low pH, which however also releases the Fab-fragment from the copper-IMAC support. Direct MS-analysis of a mixture containing a high amount of Fab-fragment as an impurity is not possible. Stable immobilisation during the low pH elution was required to prevent leakage of the bound Fab-fragments. Therefore the protocol of Hale (1995) was used to immobilise the Fab-fragments to the cobalt-iminodiacetic acid (IDA)-Sepharose support (Appendix IV). After immobilisation of the Fab-fragment and subsequent washes the support was subjected to mild oxidation with 0.03% H_2O_2 in order to stabilize the linkage. Imidazole and EDTA washes were used to remove any unoxidized, labile attached antibodies and cobalt before equilibrating to the PBS buffer. Immunoblot analysis showed no leakage of antibodies from oxidised support in acidic elution conditions.

Histidine tag – copper-IDA interaction was also tested in the immobilisation of antibody fragments to the functionalised nanotubes on the membrane but without

success, probably due to the multistep chemical activation of the nanotube needed for copper-IDA-coating. Therefore a method based on the linkage between lysine residues of the ENA11His and trimethoxysilylpropylaldehyde in the nanotubes was developed (Appendix II). ENA11His has lysine residues in the CDRs and it is probable that some of the randomly immobilised antibody fragments are attached to the tubes via the lysine residue in the enantiomer binding region. However, sufficient antibodies were immobilised in a suitable orientation, making the antibody-assisted diffusion of the enantiomer through the pores of the alumina membrane possible. The amount of functional antibodies inside the nanotubes of the membrane probably contributed to the efficiency of the facilitated diffusion of the hapten through the membrane. The amount of functional antibody fragments inside the nanotubes is however difficult to determine.

Additional cysteines at the carboxy-terminus of the light chain of ENA5His and various cellulose-binding domain (CBD) fusions were also studied for covalent and non-covalent immobilisation of ENA5 Fab-fragments, respectively. Additional cysteines, depending their location, resulted in dimerisation or interfered with the disulphide-bridge formation of the Fab-fragment (data not shown). CBD-fusions suffered from low production levels and linker cleavage between the Fab and CBD during cultivation (data not shown).

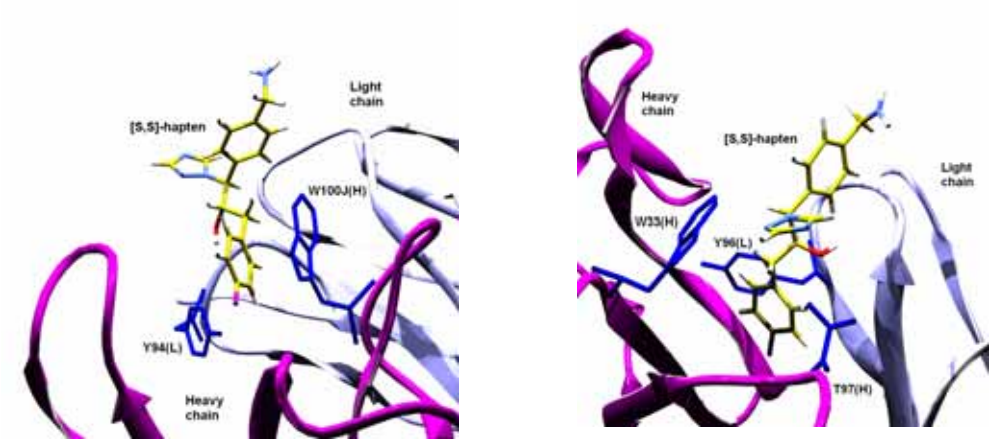
3.3 Building the homology model of the ENA5His (III)

Results obtained from the affinity chromatography studies (presented in more detail in section 3.5) showed that the reusability of the column was dependent on the affinity of the immobilised antibody. The affinity of the ENA5His was too high for the repeated use of the antibody column because the conditions needed for efficient elution of the bound enantiomer led to partial denaturation of the antibody. On the other hand ENA11His had suitable affinity for repeated use but the capacity of binding was much lower than theoretically expected. Therefore a series of affinity mutants of ENA5His with lower affinity was designed.

The aim was to reduce the too high binding affinity of ENA5His for the d-enantiomer but to retain its specificity of recognition between the a- and d-enantiomers. Two models, based on homologous antibody structures, were built for ENA5His as described in Appendix III. The amino acids most likely to have

contacts with the mutual part of the enantiomers were identified and five of them were chosen for the mutagenesis.

Two models were made, due to the general difficulties in modelling of the CDR3 loop of the heavy chain having large conformational variation. The framework regions were the same in both models but the template structures for CDRs varied. Two and three mutations were designed on the basis of the models 1 and 2, respectively (Figure 9). Their location at the sequence of ENA5His is shown in Figure 1 in Appendix III. The choice of the template structures for CDRs of the model 1 was made on the basis of the canonical class and maximal identity of the sequence. In the model 2 additional attention was paid to the structural similarity of the haptens of the template structures with respect to diarylalkyltriazole. Only the CDR2 loop of the light chain is common for both models (Table I, Appendix III). The location of the hapten is deeper in the binding pocket in model 2. All mutations (Trp33Ala, Thr97Gln and Tyr96Val) based on this second model reduced the affinity of the parental ENA5His antibody.



a. b.

Figure 9. The hapten binding sites of models 1 (9 a) and 2 (9 b). Mutated residues are indicated by the side chains. (Modelled and figure provided by Nana Munck, VTT Biotechnology)

Aromatic residues, especially tyrosines, are in general common residues at the binding sites of antibodies (Collis *et al.,* 2003). Tyrosine can participate in both

hydrophobic and hydrophilic interactions. ENA5His has 10 tyrosines and 3 tryptophans and some of them showed stacking interactions with the buried part of the hapten in the molecular dynamics simulation. Two tyrosines and two tryptophans at the binding pocket were mutated in this study. Tyr94 and Tyr96 were mutated to leucine and valine, respectively, causing loss of the aromatic ring and a possible hydrogen bond. Mutation Tyr94Leu showed a slight increase in affinity whereas the change in the other tyrosine caused up to 10-fold decrease in affinity. An exact explanation for the improved affinity of Tyr94Leu is difficult to obtain from the model. On the other hand, Tyr96 of the light chain is known from previous studies often to interact with antigen (MacCallum *et al.,* 1996, Chen *et al.,* 1999 (Pro96) Jirholt *et al.,* 2001, Kusharyoto *et al.,* 2002).

The two tryptophans of the heavy CDR-1 and CDR-3 were mutated to alanine and leucine, respectively. Trp33 has been shown to be an important residue for the binding (MacCallum *et al.,* 1996, Chen *et al.,* 1999, Jirholt *et al.,* 2001, Kusharyoto *et al.,* 2002, Kobayashi *et al.,* 1999). In ENA5His the substitution of the bulky aromatic side chain to a small methyl group of alanine decreased the affinity ca 5-fold. Trp100J, located near the surface, was subjected to more conservative change to leucine, resulting nevertheless in a 6-fold decrease in affinity. In addition to possible deletion of a direct contact to the hapten, the reduction of the side chain volume may induce differences in positioning of close residues. The fifth mutation, Thr97Gln, was designed to introduce a drastic effect with the intention of creating a control to verify the models. The substitution of threonine with glutamine side chain caused almost 10-fold decrease in affinity.

In order to understand in detail the specific effects of each mutant on the binding of the hapten the high resolution structures of the antibody with and without hapten are needed. Homology models have been used to design mutations (Ruff-Jamison and Glenney, 1993, Chames *et al.,* 1998), but more generally they provide a tool to target the mutagenesis study to the important residues or area at the binding site. Both affinity and specificity of anti-hapten antibodies have been adjusted successfully on the basis of homology models (Ruff-Jamison and Glenney, 1993, Iba *et al.,* 1998, Hemminki *et al.,* 1998a, Kusharyoto *et al.,* 2002). The chemical properties in the binding site of an antibody are much more complex than the sum of the properties of the amino acids forming the site. The characteristics of individual residues can however be used as a starting point to

understand the difference caused by mutation. The mutated amino acid may have direct contact with the hapten or it may have interactions with the antibody and thus indirectly have an impact on recognition. In some cases the binding of the hapten has been shown to induce conformational changes in antibody structure (Bhat *et al.,* 1990, Stanfield *et al.,* 1993, Arevalo *et al.,* 1993, Wedemayer *et al.,* 1997, Valjakka *et al.,* 2002,) and these changes are very difficult to predict with any current computational methods.

3.4 Binding properties of the antibody fragments (I and III)

In order to compare affinities of antibody fragments, small amounts of pure enantiomers a or d were conjugated to bovine serum albumin (BSA) via the same linker molecule that was used for racemic mixture. The conjugation with the pure a-enantiomer failed repeatedly, probably due to the steric hindrance. For the affinity measurements we therefore had only the d-BSA –conjugate, which was used for the d-enantiomer specific ENA5His and its affinity mutants. The affinity constant for ENA11His has not hitherto been determined.

Nieba *et al.,* 1996 studied how the values obtained from BIAcore measurements (based on surface plasmon resonance) corresponded to the affinity measurements in solution. They observed great discrepancies between the values. Rebinding and avidity effects influenced the values obtained by kinetic measurements in BIAcore. Furthermore identical surface coatings were difficult to obtain. Higher affinity constant values from BIAcore compared to homogeneous assays were also observed by Borrebaeck *et al.,* 1992. However, competition BIAcore can be used for affinity ranking of antibody fragments.

We used competition BIAcore to perform the affinity ranking of the ENA5His and its affinity mutants and to compare the results with the enantioseparation experiments in the small scale affinity columns. Fab-fragments are monovalent, obviating the avidity problem. The regeneration step after each run was optimised so that the same surface could be used for all constructions, thus eliminating the uncertainty caused by dissimilar binding surfaces. In general the antibody concentration should be close to the estimated K_D while the concentration of the competing hapten is varied. However, due to the lack of estimates of the affinities

of the mutants we used a 100 nM antibody concentration for all constructs, even for ENA5His with an affinity of 10–20 nM.

IC_{50}-values of a- and d-enantiomer reported for ENA5His and its affinity mutants (Table II, Appendix III) were measured with the same amount of antibody fragment, the same surface of immobilised d-BSA and the same conditions and data analysis in order to make comparison between them more reliable. Details of the measurements with the BIAcore are presented in Appendix III. Comparison of the mutants was made in order to select the most promising candidate for the affinity column experiments and for the construction of a double mutant. Surface plasmon resonance (BIAcore) was also used to visualise the association and dissociation behaviour of the mutated Fab-fragments to the parental ENA5His. In Figure 5, Appendix III, an overlay plot of the binding curves shows the differences between mutants and wild type Fab both in association and dissociation phases. The most dramatic difference was seen in Tyr96Val mutant, which reaches the equilibrium earlier than other mutants and the dissociation is much faster.

The other goal in the mutagenesis study was to maintain the specificity of the recognition between a- and d-enantiomers. The IC_{50}-values for the a-enantiomer showed up to 2-fold decrease for some mutants, so the relative affinity difference between a- and d-enantiomers was decreased for these mutants. However, in each case the affinity difference was high enough for efficient separation of the enantiomers in column format.

The efficiency of the elution of the bound d-enantiomer with 40% methanol-PBS, pH 7.4 was compared between mutants (Table III, Appendix III). Both the overlay plot and the competitive analysis made by BIAcore were in good agreement with the elution efficiency studies, except for the mutant Thr97Glu. This mutant had wider variation in all experiments, especially in column performance, and was therefore not used in later studies. The double mutant Trp33Ala/Tyr96Val showed 2-fold reduction in affinity in BIAcore measurement compared to Tyr96Val and in column experiments the bound d-enantiomer was eluted efficiently already at 35% methanol-PBS, pH 7.4.

3.5 Recombinant antibodies in affinity matrices (I, III and IV)

Due to the main interest in the d-enantiomer all the fractionation and the analytical studies in columns were performed in order to purify or analyse the d-enantiomer. Small-scale columns (300–500 µl) were used to study the specificities and the fractionation properties of the Fab-fragments. First the 1:1 racemic mixture of a- and d-enantiomers was applied to the ENA5His column. The d-enantiomer was bound to the antibody support, whereas the a-enantiomer was found in the flow-through (Figure 2 a, Appendix I). ENA11His columns behaved in an opposite manner (Figure 2 b, Appendix I). From the racemic mixture of the b- and c-enantiomers both antibodies bound only the b-enantiomer. This suggests that the chiral carbon responsible of the different configurations and the specific binding is most probably different for these antibody fragments.

3.5.1 Immunoaffinity chromatography for preparative fractionation of enantiomers (I and III)

The affinity supports of both wild-type antibody fragments, ENA5His and ENA11His, were made by immobilisation via a histidine tag to the metal affinity support. The conditions for the enantioseparation were screened first with the ELISA and small scale columns as described in Appendix I. Efficient binding was obtained at 2% DMSO-PBS, pH 7.4 and the bound enantiomer was efficiently released by increasing the pH to 11.6. However, 4–5% of the Fab-fragments was co-eluted and the total loss of binding capacity was 85–90%, possibly due to inactivation of the remaining antibodies at high pH. Reactivation of antibodies by extensive incubation in PBS, pH 7.4 might have restored at least partially the binding activity of the immunosorbent as reported by Delaunay-Bertoncini and Hennion, 2004. However, in preparative scale fractionation applications there is a need for repeated runs with rapid turnover and such a long reactivation is not a viable approach. Therefore a search was made for milder, non-denaturing elution conditions.

The antigen-antibody interaction can be disrupted by different means including pH, buffer composition, temperature, chaotropic agents and organic solvents or a combination of these elements. We studied the effect of organic solvent, due to

the fact that in high concentration the diarylalkyltriazole is poorly soluble in water. The d-enantiomer was efficiently eluted from the ENA5His-support with 55–75% methanol-PBS, pH 7.4. However, antibodies were partially denatured in these conditions. The best results were obtained with 55% methanol-PBS, which caused 30% decrease in binding capacity in the next round of separation. By contrast ENA11His efficiently released the bound a-enantiomer at 30% DMSO-PBS, pH 7.4 and 40% methanol/ethanol-PBS, pH 7.4 without dramatically losing its binding capacity in subsequent rounds of enantioseparation.

An automated and larger scale separation system was applied to study in more detail the reusability of the ENA11His columns. The maximum binding capacity was determined experimentally by loading an excess of racemate to the column and measuring the amount of eluted a-enantiomer. Reusability tests were performed by using racemate having this experimentally determined maximum amount of a- enantiomer and an equimolar amount of d-enantiomer in order to observe even slight decreases in the binding capacity. During the first cycles the binding capacity decreased ca 20%, reaching a steady state at which the ENA11His columns bound 41–45% of the theoretical maximun of the a-enantiomer. In theoretical maximum one mole of antibody binds one mole of enantiomer. In the case of ENA11His the binding ratio was 1:0.4. The capacity was reduced after the first elution cycle, most probably because some of the Fab-fragments did not have the disulphide bond between the heavy and the light chain. Chains joined only by non-covalent interactions are more likely to be sensitive to solvent elution, due to the possible dissociation of the heavy chain from the support. The dissociation of the heavy chain does not however explain the generally low binding capacity of the ENA11His in affinity columns. Preliminary results showed that ENA11His also has affinity for the d-enantiomer and the possible competition between enantiomers might explain the reduced capacity. Further studies of the binding specificity of the ENA11His will elucidate this phenomenon. In the case of ENA5His the theoretical and the experimental binding capacity were consistent with each other. Due to the decreased binding capacity in the column, the possible cross-reactivity and the difficulty of determining the affinity data for ENA11His, the mutagenesis study was focused on ENA5His. The aim was to use these low affinity mutants to determine the functional affinity range of antibodies in affinity supports. This goal was not achieved with this first set of mutants because the critical low affinity was not reached even with the double mutant. Additional mutants have

however been made and preliminary results suggest even lower affinities (data not shown). In the future cross-reactivity studies could also be used to determine the specificity-capacity correlation in affinity columns.

Two different elution modes, gradient and step-wise, were tested for the ENA11His column. Elution volumes were 25 and 10 bed volumes, respectively. The result shows that the elution volume and thus solvent consumption can be adjusted by the mode of elution. However, the rapid change in conditions caused about 10% decrease in the binding capacity of ENA11His after 20 cycles, whereas no decrease was observed in the columns eluted with the gradient.

In Appendix III the immunoaffinity columns made of the single mutant ENA5HisTyr96Val and the double mutant ENA5HisTyr96Val/Trp33Ala were studied in a similar manner as the ENA5His and ENA11His in Appendix I. The reduced affinity made it possible to use the affinity supports of immobilised mutants for twenty cycles of enantioseparation without significant loss in binding capacity. Both columns were functional after six months of storage in PBS, pH 7.4 in +4 °C. These results with the affinity mutants confirmed that affinity supports of recombinant antibody fragments can be used to fractionate enantiomers of a racemic mixture in a repeated runs. Using anti-diarylakyltriazole antibody as an example we calculated that one litre of bacterial culture supernatant provided enough antibody to make a column for enantioseparation of 10 mg of drug candidate in 20 cycles of fractionation. This amount is sufficient for the early phases of drug development (Appendix III).

Affinity columns made of antibody crystals offer an interesting benefit over traditional immunoaffinity supports. ENA5His was also used to set up a chromatographic system based on cross-linked antibody crystals (Vuolanto *et al.,* 2002, not included in this thesis). The goal was to decrease the size of the affinity columns by using antibodies in the most dense packing format. Affinity columns made of antibody crystals were able to fractionate the enantiomers. However, only 33–50% of the theoretical maximum of the binding capacity was achieved (Vuolanto *et al.,* 2003, 2004). The decrease may have been due to the crystallisation, subsequent cross-linking or restricted diffusion inside the crystals. The ENA5His used in these studies had two lysines in the binding site, which may have reacted with the cross-linking agent glutaraldehyde. Antibody engineering could be used to study the effect of lysine residues at the binding

site on the binding capacity of the cross-linked antibodies. Antibody engineering also opens interesting possibilities to design an antibody framework with improved crystallisation properties (Wingren *et al.,* 2003). Different approaches of molecular engineering can be used to promote crystallisation of proteins (Derewenda, 2004). By combining the crystallisation-prone framework to CDR-libraries, it might be possible to facilitate the present tedious experimental screening for crystallisation conditions. This approach would be especially useful in applications such as affinity chromatography, in which the quality demands of the crystals are not as strict as in protein structure determinations.

3.5.2 Antibody-based solid-phase extraction as a pretreatment method for the analysis of enantiomers (IV)

The aim was to develop a simple antibody-based sample treatment method applicable in high-throughput format. The quality of the sample treatment method is crucial to the reliability of the whole analytical procedure. In many methods used at present an internal standard is used to compensate the poor performance of the analytical systems.

ENA5His and one of its affinity mutants ENA5HisTyr96Val were used to set up the solid phase extraction experiments. They both bind efficiently the d-enantiomer from the sample but due to its faster dissociation the ENA5Tyr96Val was used in most experiments to ensure the efficient release of the enantiomer in lower volume from the antibody support. The conditions used in previous affinity columns for these antibodies were not directly applicable to the solid phase extraction method. Whereas the antibodies in the column were able to bind the d-enantiomer from the racemate in 2% DMSO–PBS, pH 7.4 in 1:1 molar ratio, the binding in SPE-format was less efficient. Two and four molar excesses of Fab-fragments were needed for quantitative binding of the d-enantiomer from the PBS-buffer and plasma, respectively. Although the SPE functions according to the same principles as liquid chromatography, the number of theoretical plates is limited. First both 96- and 384-well plates with a filter at the bottom of each well were tested. The 25 µl of the immunoaffinity support forms flat disk at the bottom of the 96-well plate, whereas the same amount of support forms a tiny column in a 384-well plate. The spurting of eluents was extensive for the 96-well plates due to the different design of the drip director

and the recoveries from the 96-well plate experiments were not as good as in the 384-well plate studies. Therefore all the statistical experiments were made with the 384-well plates.

SPE was here developed as a sample preparation method for direct mass spectrometric (MS) analysis. Throughout the whole procedure the specific demands of MS-analysis were kept in mind. Mass spectrometry is a sensitive analytical system. The analyte must be in a volatile buffer and solvent. The unspecific binding of impurities of the sample matrix to the SPE followed by their co-elution can impair the MS-analysis by increasing the background noise and thus reducing the sensitivity (Figure 5). Impurities may even suppress the signal of the target analyte (Ferguson *et al.,* 2001, Rolcik *et al.,* 2002). We used a C_{18}-column between the immuno-SPE and MS-analysis in order to avoid any interference of possibly co-eluted compounds at this early stage of method development. However, the ultimate goal is to develop the methods further towards direct MS-analyses. A number of the parameters in sample application, e.g. washing and elution, were studied (Table I, Appendix IV). In many research reports biological samples have been diluted to buffer, pH has been adjusted or some additives have been introduced before applying the sample to the SPE. We applied plasma samples directly in order to see whether the sample handling time could be minimised by omitting protein precipitation or other additional steps such as those mentioned above. For dilute samples (3–60 ng/ml) the application volume was increased five- or tenfold in order to increase the response level clearly over the lowest limit of quantitation with a signal to noise ratio >10. The results showed that diluted samples can be quantitated by increasing the sample volume, due to the ability of the antibody to enrich the analyte.

The efficiency of washes was studied by varying the number of repetitions, different volumes and buffer compositions as listed in Table I, Appendix IV. The goal of the washing step was to remove the impurities and adjust the conditions for subsequent elution. 10 mM ammonium acetate, pH 5.0 was found to be the most suitable. Methanol caused additional spurting. EDTA and imidazole did not bring any additional advantage when the C_{18} -column between SPE and MS was used at this method development stage. However, in the future when the aim is to automate the method this additional step of C_{18}-liquid

chromatography will be rejected and more attention must therefore be paid to the washing step before the direct injection of the eluate to MS.

Efficient elution was obtained with 25 mM ammonium acetate, pH 2.8. Addition of methanol again caused increased spurting and variance in elution volume and was therefore not used. Due to the low concentrations of the enantiomer in eluents and the immediate injection to the HPLC(C_{18})-MS combination, no organic solvent was required to keep the analyte soluble before analysis. With this system setup the objectives of quantitative binding of the analyte (d-enantiomer), washing away the low affinity compound (a-enantiomer) and efficient elution of the bound analyte were all achieved (Figure 1, Appendix IV).

The specificity of the binding was studied by determining the possible unspecific binding of the d-enantiomer to the plain support and to another hapten binding antibody. No binding of the d-enantiomer to cobalt-Sepharose or to anti-morphine-Fab support was observed. In a second set of experiments we studied the co-binding of the other enantiomers (unpublished data). ENA5HisTyr96Val was used in tenfold molar excess to avoid any competition between enantiomers. A fixed amount of d-enantiomer was used. To this was added an equimolar amount of a, b, c or d. The double amount of d increased the response twofold, showing that the amount of Fab-fragments was not the limiting factor for binding. Most of the b-enantiomer was also bound, which is in good agreement with the small scale column results. The addition of a- and c-stereoisomers showed no increase in total response. In the future it would be interesting to study the correlation of the cross-reactivity between enantiomers and their relative binding to antibody support. In some applications the unwanted compound, having a slight affinity for the antibody, may be present in high concentration and create problems for the purification or analysis of the target compound.

The main objective of this SPE-study was to see how the antibody-based extraction meets the challenges of bioanalytics. Six replicates of samples for each measurement were used and only complete sets of experiments were included in the statistical analysis. The buffer and plasma samples were spiked with the d-enantiomer only. Recoveries were 79–122% and 80–108% for buffer and plasma samples, respectively (Table II, Appendix IV). For the first two series (300–3000 ng/ml, Buffer A) the recoveries were unacceptably high,

possibly due to evaporation of solvent before MS-analysis. Avoiding storage of samples improved the accuracy of the system. Our results are in good compliance with the recoveries reported for C_{18} supports in 96- and 384-well extractions for small molecular weight analytes (Souppart *et al.,* 2001, Rule *et al.,* 2001, Shou *et al.,* 2002, Pommier *et al.,* 2003, Svennberg *et al.,* 2003, Kim *et al.,* 2004). However, additional value for the antibody support was gained from the enantioselectivity. Antibody support was used in this type of set-up for the first time and therefore closely related references to evaluate and compare our approach are not yet available.

The recoveries from the spiked plasma samples were in general lower than for spiked buffer samples. We used the affinity mutant ENA5HisTyr96Val in all experiments to make comparative analysis for buffer and plasma samples. However, higher affinity antibody might have retained the d-enantiomer from the complex plasma sample more efficiently because the affinity of the antibody is crucial for the extraction. In SPE supports for analytical applications the repeated use is not an issue and therefore the high affinity antibodies could also be used. According to Rule *et al.* (2001), frothing decreases the binding efficiency of plasma samples in low affinity supports. High affinity supports are likely to decrease this effect.

The precision (RSD%) value expresses how close the individual measurements are to each other. For bioanalytical methods RSD values below 15% are acceptable (FDA: Guidance for industry. Bioanalytical method validation, 2001). In our system the experiments with spiked buffer generally showed good precision values. However, two out of eight plasma experiments had too high values. Extraction volumes were checked but they were not the cause of variance. Comparison of the precision values of ENA5His and ENA5His Tyr96Val supports might indicate whether the precision could be influenced by increasing the affinity of the support. Accuracy values describe how close the measured values are to the nominal value. For bioanalytical methods, accuracies should be below 15% (FDA: Guidance for industry. Bioanalytical method validation, 2001). Most of the buffer samples showed good accuracy, whereas many of the plasma samples had too high values. Improvements in recovery would also increase the accuracy of the system in this case. The limit of quantitation was 0.01 µg/ml for MS-analysis of the standard curve with signal to noise ratio of >10. The linearity for the whole system was determined by spiked

buffer samples over the range 0.03–3 µg/ml. The correlation coefficient r = 0.999 was very good. The measured individual values deviated from the calculated ones by 0.3–7.8%. The individual measurements of the lowest concentrations had the highest deviation, as usually expected.

Our study is the first example of the use of recombinant antibodies in a 384-well solid phase extraction system. Although these primary results appear promising, much effort needs to be put into the further development of the system. Examples of other analytes, as well as a systematic study of the affinity and specificity requirements of antibody fragments, are needed to evaluate immuno-SPE in a high-throughput format. The design of the extraction device is also very important. In the 384-well format the wells are long and narrow, which makes the sorbent form a micro-scale column. The insertion of sorbent and the sample application should avoid the possibility of creating plugs caused by air bubbles. Frits could also be added to the top of the sorbent to prevent floating of the sorbent during sample application, washes and elution (Fisk *et al.,* 2003). Cross-contamination between the wells is a problem that becomes more serious as the wells get smaller and closer to each other. Careful design of drip directors and collection plates in the 384-well format is essential to prevent contamination. Drops must become very small and/or be directly guided to the bottom of the collection well in order to prevent the formation of large droplets plugging the upper part of the collection well. With the plates used in our experiments this plugging phenomenon was occasionally observed. The possibility to modify the surface properties of the plastic material in order to prevent liquid plugs should also be evaluated. In addition, further development could be targeted to optimising the support material. The cross-linked agarose used in our experiments has ion exhange properties, causing unspecific binding of e.g. proteins from the biological samples. Whether the storage of immunosorbents is preferred in wet or dry conditions is also an important issue to be examined.

3.6 Immobilised antibody fragments in nanomembranes (II)

New strategies for fractionation of the enantiomers with enantioselective antibodies using nanomembranes (Appendix II) and nanotubes (Mitchell *et al.,* 2002) have been developed in collaboration with Prof. C. Martin's group in the

University of Florida. Lakshmi and Martin (1997) reported enzyme-based nanomembranes capable of transporting the substrate of the immobilised apoenzyme across the membrane towards the lower concentration of the substrate, a phenomenon called facilitated diffusion. However the system was restricted to the particular enzyme and its substrates or related compounds. Finding a suitable specificity, success of cloning and production (or isolation), the dimensions of the protein, inactivation of the catalytic activity and the way of immobilisation may vary a great deal between different enzymes. Our idea was to use antibody fragments instead of enzyme in order to make the system more versatile. These natural binding proteins can be obtained against wide variety of molecules. Antibodies share a common structure, and rather well established production and purification approaches can be applied to speed up the construction of nanomembranes with different specificities. Antibody libraries offer a remarkable potential to obtain new specificities.

Antibody-based nanomembranes were developed to separate a- and d-enantiomers by facilitated diffusion. The system is based on transient binding of the hapten to the antibody and the principle mimics partition chromatography. We used the ENA11His affinity chromatography support described in Appendix I for the development of suitable conditions for the partition chromatography in isocratic conditions. ENA11His was chosen due to its lower affinity and it was possible to develop a fractionation method based on partition for this fragment. The ENA5His affinity mutants were not yet available at the time of system set-up. Due to the poor water solubility of the hapten the main efforts were targeted to adjusting the affinity of the antibody with the DMSO-solvent so that the ENA11His would bind the hapten specifically but in a reversible manner. Methanol and ethanol were not assayed because the facilitated diffusion takes such a long time that the possible evaporation of solvent would create unstable conditions and affect the transport across the membrane. We found out in column experiments that at 15–20% DMSO in PBS, pH 7.4 the a- and d-enantiomers could be separated according to the principles of partitioning. With higher concentration of DMSO the chromatograms of these two enantiomers overlapped and with lower concentration of DMSO the d-enantiomer was bound to the column and a change in conditions was needed to elute it quantitatively from the column.

Membrane construction, Fab-immobilisation and facilitated diffusion studies were performed at the University of Florida in Prof. C. Martin’s group. The

membranes were based on alumina, having a highly organized array of cylindrical pores (Figure 10). The properties of the membranes can be adjusted by varying the conditions of anodisation during growing the membrane. The strength of the voltage determines the diameter of the pores and the thickness of the membrane corresponds to the duration of the anodisation. In these first experiments pore sizes of 20 and 35 nm were used and the thickness of the membranes was 35–40 μm with 2.1–2.3 × 10^{10} pores/cm^2. The silica nanotubes were deposited to the pores of the alumina membrane with a sol-gel template synthesis method. Before immobilising the antibody fragment these nanotubes were derivatised with a reactive trimethoxysilylpropyl aldehyde. Aldehyde carbonyl groups at the inner surface of the nanotubes react with the surface exposed amino groups, mainly lysines, on a Fab-fragment.

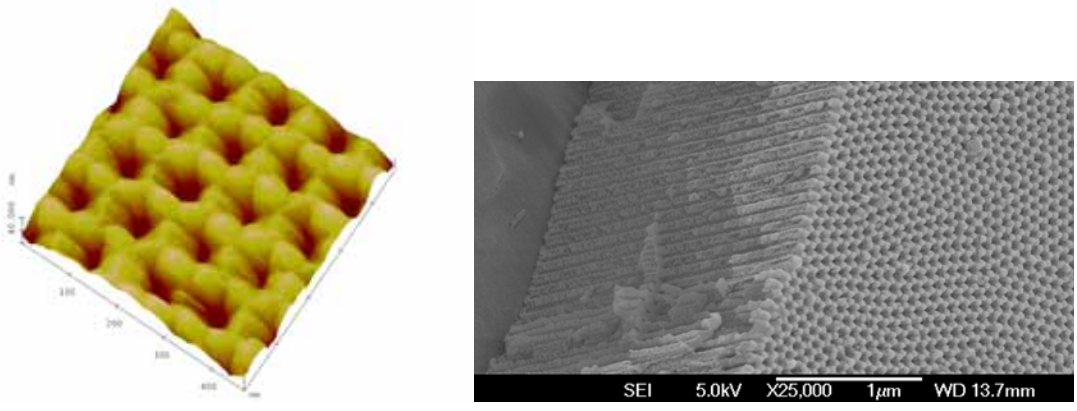


Figure 10. a) An atomic force micrograph of the surface of an alumina membrane. b) A cross-section scanning electron micrograph picture of the alumina nanomembrane. Even-sized pores are organised in arrays. Pores continue as nanotubes, which extend throughout the membrane. (Pictures provided by L. Trofin and C. Martin, University of Florida).

Nanomembranes between the chambers for feeding and receiving solutions were used to separate enantiomers of a diarylalkyltriazole. Antibody-assisted, facilitated diffusion transported the enantiomer across the membrane towards the lower concentration. The a-enantiomer, that has higher affinity for the immobilised antibody, was transported across the membrane 4.5 times faster than the other, d-enantiomer, having lower affinity. Graphical presentation of the

separation of the enantiomers by the antibody nanomembrane is presented in Figure 11.

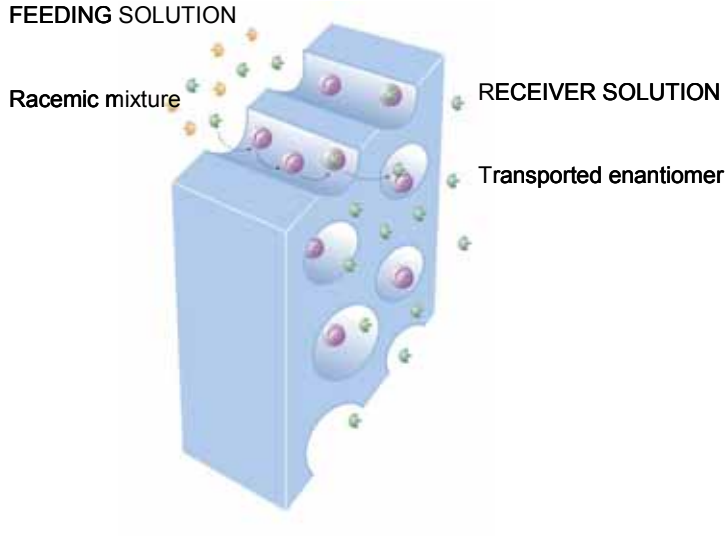


Figure 11. A graphical presentation of an antibody-based nanomembrane. The diffusion of the enantiomers from the feeding solution through the membrane to the receiver solution depends on the specificity and affinity of the immobilised antibody (magenta). The high affinity enantiomer (green) diffuses faster through the membrane than the low affinity enantiomer (yellow). (Picture provided by Janne Turunen.)

Two parameters are important for the function of the nanomembranes: the efficiency of the transport (flux) and the selectivity. Increase in the pore size increases the flux but decreases the selectivity. The higher the concentration of the feed solution, the better the flux but at the expense of selectivity. Most of the parameters influencing the diffusion have opposite effects on the flux and the selectivity. The affinity and the specificity of the antibody are important parameters. In this set of experiments the affinity was adjusted with the amount of DMSO. At lower concentration (10% DMSO) the transport of the a-enantiomer across the membrane is faster and more selective than at 30% DMSO, where the flux is decreased to the level of passive diffusion and no selectivity between the two enantiomers is observed (Appendix II, Figure 3). These results are in good compliance with the column experiments in which 30% DMSO elutes the bound enantiomer. The selectivity of the facilitated

diffusion would probably be improved by increasing the specificity of the antibody.

In Appendix II the principle of the proof-of-concept study of the facilitated diffusion in antibody-based membranes is presented for the first time. The highest selectivity coefficient (α = 4.5) and flux (17 nmol cm^{-2} h^{-1}) were obtained with 20 nm and 35 nm pore sizes, respectively. In order to utilize antibody-based membranes further, basic research is needed to determine the extent to which the properties of an antibody fragment influence the system. Antibodies with varying affinities and cross-reactivities might be used to study the range of affinity and specificity needed for optimised separation. The possibility to specifically transport the desired compound from crude mixtures would also be interesting and important to study.

By solubilising the alumina membrane the individual antibody-containing nanotubes can be collected and used to bind one enantiomer from the solution and release it in dissociating conditions (Mitchell *et al.,* 2002). Such nanotubes also have a potential in other applications such as capsulation of enzymes and drug delivery. Both surfaces of the tubes can be functionalised separately and the tubes can be constructed from various materials with desired properties such as ruggedness or biodegradability. Research in the field of nanomembranes, -particles and -tubes is very intense and different materials and fabrication methods are under development. For a recent review of biotechnological applications of nanotubes and –membranes see Martin and Kohli (2003).

4. Conclusions and further perspectives

Enantioselective antibodies can be used in various applications due to their ability to specifically bind only one enantiomer in the presence of the other. Separation and analytical needs of different enantiomers are mainly related to exogenous synthetic compounds such as chiral drugs and other organic molecules. Because of the synthetic origin of the enantiomers the separation and analytical techniques for the enantiomers have hitherto been developed mainly from the chemical point of view, using methods such as fractional crystallisation, chemical derivatisations and non-specific chiral chromatography. Some examples of enantioselective poly- and monoclonal antibodies have shown how the exceptional binding specificities and affinities of antibodies could bring additional advantage over traditional chromatographic methods.

In this thesis the applicability of recombinant antibody fragments in separation and analysis of enantiomers of a chiral drug was studied. The enantiomers in general have unique characteristics and have therefore specific needs for purification, fractionation and analysis. As shown in this work the antibody fragments can be used to construct immunoaffinity chromatography supports and antibody-based membranes for the fractionation purposes. One litre of culture supernatant provided enough antibody fragments for the fractionation of enantiomers needed at the early stages of drug development.

Antibody fragments were also used for the first time in solid-phase extraction supports as a sample preparation method prior to the analysis of the enantiomers. The 384-well plates used in this study are a potential format for high-throughput sample preparation. Miniaturisation and high-throughput approaches are one of the major trends in the pharmaceutical industry. The early selection of antibodies against drug candidates would allow both the small scale preparative purification and analysis of drug compounds already in the early phases of drug development. The methods developed here for the enantiomers are also directly applicable to other low molecular weight compounds.

The limited availability of antibodies recognising small organic molecules has restricted the use of anti-hapten antibodies in chemical and pharmaceutical studies. The present requirement of sometimes difficult and tedious linker synthesis for the hapten prior to conjugation and selection of specific antibodies

is an unfortunate disadvantage. The most demanding task in the future will be the development of selection methods for native, unmodified haptens. Another clear future need is further optimisation of the antibody frameworks to meet the requirements of hapten applications. Much research has already been done to improve expression, stability and immobilisation. Stability is a very important factor both in storage and in assay conditions, which, in the case of anti-hapten antibodies, may require the use of organic solvents. Dry storage of the immobilised antibody fragments is also important factor to be studied. Ideal frameworks could be common templates for synthetic or combinatorial libraries providing different anti-hapten specificities.

In addition to the methods developed in this thesis, interesting emerging technologies such as antibody crystals and isolated nanotubes may open new possibilities for utilisation of recombinant antibodies. Recent developments in antibody technology, including antibody libraries and advanced selection methods for obtaining new specificities and antibody engineering for improving the properties, greatly enhance the possibility to evaluate more deeply the recombinant antibodies in stereochemical applications.

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Title

Enantioselective antibody fragments

Abstract

Antibodies have a unique ability to bind to a wide variety of different molecules, ranging from large protein antigens to low molecular weight organic molecules. The specificity and affinity of the binding varies between antibodies and can be utilised in many applications. Recombinant antibody fragments have advantages over traditional poly- and monoclonal antibodies in production and immobilisation and in the optimisation of their properties.

Enantiomers of a chiral compound provide a challenge for separation methods and analytics due to their similar chemical and physical properties. Antibodies provide a highly specific way to fractionate enantiomers in both preparative and analytical applications.

In this work two different antibody-based approaches to separate enantiomers of a chiral drug candidate were developed. Antibody fragments were cloned, produced in bacteria and immobilised on a solid affinity support. Repeated affinity purification of enantiomers was achieved in optimised conditions. In the other approach antibody fragments were immobilised inside the nanotubes of an alumina membrane and the bionanomembrane was used to fractionate enantiomers of a racemic mixture.

In addition a sample preparation method, antibody-based solid-phase extraction, was developed in a way that can be applied to high-throughput format. Recoveries were comparable to those reported for non-specific sorbents, but with the advantage of the enantioselectivity. The method was used to extract an enantiomer from a spiked buffer or serum. The preparatory sample treatment protocols usually used for serum, e.g. protein precipitation, were not needed.

A homology model of one of the antibody fragments was constructed and used to design site-specific mutations in order to adjust the affinity of the antibody to be suitable for the preparative and analytical approaches developed in this work. One of the mutants, ENA5His Tyr96Val, had appropriate properties both in preparative and analytical applications.

Keywords

antibodies, recombinant antibodies, antibody fragments, enantiomers, chiral compounds, nanotubes, bionanomembranes, enantioselectivity, haptens, affinity chromatography

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