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EVOLUTION OF BIOAFFINITY REAGENTS BY PHAGE DISPLAY

by

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (**I-V**)

I Brockmann E.C., Lamminmäki U., Saviranta P. (2005) Engineering dihydropteroate synthase (DHPS) for efficient expression on M13 phage. *Biochim. Biophys. Acta*. 1724(1-2):146-154.

II Korpimäki T., Brockmann E.C., Kuronen O., Saraste M., Lamminmäki U., Tuomola M. (2004) Engineering of a broad specificity antibody for simultaneous detection of 13 sulfonamides at the maximum residue level. *J. Agric. Food. Chem.* 52(1):40-47.

III Brockmann E.C., Cooper M., Strömsten N., Vehniäinen M., Saviranta P. (2005) Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions. *J. Immunol. Methods*. 296(1-2):159-170.

IV Brockmann E.C., Vehniäinen. M., Pettersson K. (2010) Use of high-capacity surface with oriented recombinant antibody fragments in a 5-min immunoassay for thyroid-stimulating hormone. *Anal. Biochem.* 396:242-249.

V Brockmann E.C., Akter S., Savukoski T., Lehmusvuori A., Leivo J., Saavalainen O., Azhayev A., Lövgren T., Hellman J., Lamminmäki U. (manuscript) Synthetic single-framework antibody library integrated with rapid affinity maturation.

In addition, some unpublished data are included.

ABBREVIATIONS

BBP	bilin-binding protein
BITC	biotin isothiocyanate
CBD	cellulose-binding domain
CDR	complementary determining region
CEA	carcinoembryonic antigen
CGTase	cyclomaltodextrin glucanotransferase
C_H	constant heavy domains of antibody
C_{H1}	constant heavy domain 1 of antibody
CI	confidentiality index
C_L	constant light domain of antibody
CLTA-4	cytotoxic T lymphocyte associated protein 4
CTLD	C-type lectin domain
CV	coefficient of variation
2D	2-dimensional
D	antibody diversity segment
dAb	domain antibody
DARPin	designed ancyrin repeat protein
DHPS	dihydropteroate synthase
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiotreitol
dUssDNA	uridylated single-stranded DNA
ELISA	enzyme linked immunosorbent assay
EYFP	enhanced yellow fluorescent protein
Fab	fragment of antibody
FGFR	fibroblast growth factor receptor
FLISA	fluorescence linked immunosorbent assay
Fn3	fibronectin type III domain
FRET	fluorescence resonance energy transfer
Fv	fragment of antibody variable domains
GA-SAv	glutaraldehyde cross-linked streptavidin
GdmCl	guanidinium chloride
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage stimulating factor
h	human-
H	heavy chain of antibody
hCG	human chorionic gonadotropin
hFSH	human follicle-stimulating hormone
hGH	human growth hormone
hLH	human lutenising hormone
HuCAL	human combinatorial antibody library
IC_{50}	50 % inhibitory concentration
ICAM-1	intracellular adhesion molecule-1
Ig	immunoglobulin
IgNAR	immunoglobulin isotype novel antigen receptor
IPTG	isopropyl β-D-1-thiogalactopyranoside
J	antibody joining segment
Kd	dissociation constant
k_{on}	association rate constant
k_{off}	dissociation rate constant
L	light chain of antibody
LRET	luminescence resonance energy transfer
m	mouse-
Mab	monoclonal antibody

MRL	maximum residue limit
NTA	nitrilotriacetic acid
p3	phage coat protein 3
$p3^{CT}$	C-terminal domain of phage coat protein 3
p9	phage coat protein 9
PAPP-A	pregnanct-assocaited plasma protein A
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEO	polyethylene oxide
PhoA	alkaline phosphatase
PhOx	2-phenyloxazol-5-one
PVDF	polyvinylidene fluoride
RAM	rabbit anti-mouse antibody
RT-PCR	reverse transcription PCR
S/B	signal/background
scFv	single-chain fragment of antibody variable domains
SD	standard deviation
SHZ	sulfamethazine
SIP	selectively infective phage display
SOE-PCR	splicing by overlap extencion PCR
SPR	surface plasmon resonance
TIM	triosephosphate isomerase
TNF	tumor necrosis factor
TSH	thyroid stimulating hormone
UDG	uracil-DNA glycosylase
V	variable domain of antibody
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
V_H	variable heavy domain of antibody
V_L	variable light domain of antibody
WT	wild type

ABSTRACT

Antibodies are natural binding proteins produced in vertebrates as a response to invading pathogens and foreign substances. Because of their capability for tight and specific binding, antibodies have found use as binding reagents in research and diagnostics. Properties of cloned recombinant antibodies can be further improved by means of *in vitro* evolution, combining mutagenesis with subsequent phage display selection. It is also possible to isolate entirely new antibodies from vast naïve or synthetic antibody libraries by phage display.

In this study, library techniques and phage display selection were applied in order to optimise binding scaffolds and antigen recognition of antibodies, and to evolve new and improved bioaffinity reagents. Antibody libraries were generated by random and targeted mutagenesis. Expression and stability were mainly optimised by the random methods whereas targeted randomisation of the binding site residues was used for optimising the binding properties. Trinucleotide mutagenesis allowed design of defined randomisation patterns for a synthetic antibody library. Improved clones were selected by phage display. Capture by a specific anti-DHPS antibody was exploited in the selection of improved phage display of DHPS. Efficient selection for stability was established by combining phage display selection with denaturation under reducing conditions. Broad-specific binding of a generic anti-sulfonamide antibody was improved by selection with one of the weakest binding sulfonamides. In addition, p9 based phage display was studied in affinity selection from the synthetic library.

A TIM barrel protein DHPS was engineered for efficient phage display by combining cysteine-replacement with random mutagenesis. The resulting clone allows use of phage display in further engineering of DHPS and possibly use as an alternative-binding scaffold. An anti-TSH scFv fragment, cloned from a monoclonal antibody, was engineered for improved stability to better suite an immunoassay. The improved scFv tolerates 8 – 9 °C higher temperature than the parental scFv and should have sufficient stability to be used in an immunoanalyser with incubation at 36 °C. The anti-TSH scFv fragment was compared with the corresponding Fab fragment and the parental monoclonal antibody as a capturing reagent in a rapid 5-min immunoassay for TSH. The scFv fragment provided some benefits over the conventionally used Mab in anayte-binding capacity and assay kinetics. However, the recombinant Fab fragment, which had similar kinetics to the scFv, provided a more sensitive and reliable assay than the scFv. Another cloned scFv fragment was engineered in order to improve broad-specific recognition of sulfonamides. The improved antibody detects different sulfonamides at concentrations below the maximum residue limit (100 µg/kg in EU and USA) and allows simultaneous screening of different sulfonamide drug residues. Finally, a synthetic antibody library was constructed and new antibodies were generated and affinity matured entirely *in vitro*. These results illuminate the possibilities of phage display and antibody engineering for generation and optimisation of binding reagents *in vitro* and indicate the potential of recombinant antibodies as affinity reagents in immunoassays.

1 INTRODUCTION

In a living cell biomolecules mediate a number of functions through specific interactions. Protein-DNA interactions regulate gene expression. Receptor – ligand interactions induce or inhibit signalling pathways that adjust cellular functions. Protein complexes are formed through protein-protein interactation. Enzymes catalyse chemical reactions of defined substrates that they first bound.

Antibodies (also called immunoglobulins) are biological macromolecules that are found in blood and lymphoid tissues of vertebrates. They are produced by the immune system to recognise and neutralise foreign agents such as bacteria and viruses, but also play a role in allergic reactions and autoimmune diseases. The immune system has an enormous capability to develop antibodies against various targets antigens. Because of the capability for tight and specific binding, antibodies have proven valuable tools as affinity reagents in research and diagnostics. In bioaffinity assays, such as immunoassays, specific antibodies are used to bind target molecules in the sample, and quantification of the binding reaction reveals the amount of the target molecules. An increasing amount of antibodies are also used as therapeutic agents.

The first antibody preparations that were exploited as binding reagents were polyclonal and were obtained from serum of immunised animals. Isolated antisera contain multiple antibodies of varying affinity against different epitopes of the target antigen, but also unspecific antibodies. Discovery of hybridoma technology in 1975 (Kohler & Milstein, 1975) established the isolation and production of monoclonal antibodies and provided a nonlimited supply of specific antibodies. Monoclonal antibodies have become the golden standard in antibody technology, but also polyclonal antibodies are still used, mostly because of the ease of production on a short time-scale. However, each batch of polyclonal sera is unique and limited in amount.

PCR technology made it possible to clone antibody genes without prior knowledge of the coding sequence of each individual clone (Orlandi *et al.*, 1989). Initially, cloned Fv antibody fragments, consisting of only the variable domains, were expressed in bacterial cytoplasm in inactive form and needed to be purified from inclusion bodies by refolding (Cabilly *et al.*, 1984). Transport into periplasmic space established production of soluble antibody (Better *et al.*, 1988; Skerra & Pluckthun, 1988) and efficient purification was achieved by affinity tags, which could be introduced by PCR (Skerra *et al.*, 1991; Essen & Skerra, 1993). Joining the variable fragments with a flexible polypeptide linker gave rise to single chain Fv (scFv) fragments (Huston *et al.*, 1988; Stemmer *et al.*, 1993), which is one of the most popular forms of antibody fragments. Cloning also the C_L and C_{H1} domains produced recombinant Fab fragment (Better *et al.*, 1988).

In the early 90s, it was shown that antibody fragments can be displayed on the surface of filamentous phage encoding the antibody, and the binding phage could be enriched by affinity capture (McCafferty *et al.*, 1990; Barbas *et al.*, 1991; Breitling *et al.*, 1991; Garrard *et al.*, 1991; Hoogenboom *et al.*, 1991; Kang *et al.*, 1991a). This revolutionised the recombinant antibody technology as efficient antibody engineering and generation of novel antibodies *in vitro* was established. Since then, specific binders have been enriched from V gene libraries cloned from immunised animals, from naïve libraries made from non-immunised donors and also from libraries where synthetic diversity has been introduced by PCR (Benhar, 2007). Numerous vast, universal phage displayed antibody libraries, with up to 10^{11} different antibody clones, have

been constructed and used as a source of high-affinity antibodies (Hust & Dubel, 2004; Benhar, 2007). In addition, alternative, non-antibody, frameworks have been utilised for generation of novel synthetic binders by phage display and are becoming increasingly popular (Nygren & Skerra, 2004; Nuttall & Walsh, 2008; Hosse *et al.*, 2006).

Antibodies produced *in vivo* undergo affinity maturation through somatic mutagenesis and clonal selection. Similarly, cloned antibodies or antibodies isolated from naïve or synthetic antibody libraries can be affinity matured *in vitro* through targeted or random mutagenesis followed by selection with phage display. In addition to affinity, recombinant antibodies have also been optimised for specificity, stability and folding (Wark & Hudson, 2006); (Honegger, 2008; Sheedy *et al.*, 2007). There are also various display methods other than phage display that have been used in the selection of antibodies such as bacterial display (Fuchs *et al.*, 1992); (Francisco *et al.*, 1993), yeast display (Boder & Wittrup, 1997), ribosome display (He & Taussig, 1997; Hanes *et al.*, 1998; Schaffitzel *et al.*, 1999) and mRNA display (Fukuda *et al.*, 2006). However, phage display was the first and is still the most popular tool to select antibody libraries because of its robustness and simplicity (Hoogenboom, 2005; Sergeeva *et al.*, 2006).

In this report, the generation of high-affinity antibodies by phage display is reviewed. Different types of binding libraries, both natural and synthetic, are presented. Also the methods for affinity maturation *in vitro* and phage display selection strategies are described. In the experimental part, phage display was applied in order to optimise binding scaffolds and antigen recognition of antibodies, and to evolve new and improved bioaffinity reagents.

2 REVIEW OF THE LITERATURE

2.1 Structure and generation of antibodies *in vivo*

The immune system, which protects the body against diseases, consists of a complex network of specialised cells that have evolved to defend the body against attacks by foreign invaders such as bacteria, viruses, fungi and parasites; it can also fight cancers. The non-specific part of the immune system which can adapt to fight against almost any components recognised as foreign is comprised by T and B lymphocytes, which constitute the cell-mediated and humoral immunity, respectively. A molecule recognised by the immune system is referred as an antigen. A small molecule that is not immunogenic itself, but can elicit an immune response when attached to a large carrier protein is called a hapten.

Immunoglobulins (Ig), also known as antibodies, are a central part of the humoral immune system and are produced by the B lymphocytes as a response to foreign antigens. Immunoglobulins are Y-shaped protein molecules consisting of two identical light (L) chains and two identical heavy (H) chains (Fig.1). The chains are connected through disulfide bridges at the hinge region. The light chain consists of a variable (V_L) domain and a single constant (C_L) domain. There are two types of light chains: kappa and lambda. The heavy chain consists of a variable (V_H) domain and three constant (C_H) domains. Depending on the type of the constant region of the heavy chain, antibodies are classified into five different isotypes: IgM, IgG, IgE, IgD and IgA. All the antibody domains adopt an immunoglobulin fold, which is composed of two-layers of an antiparallel β-sheet. (Janeway *et al.*, 2001).

Proteolytic cleavage of immunoglobulins *in vitro* at the hinge region results in an Fc fragment and either two Fab fragments or a single Fab_2 fragment. Fc-portion (Fig. 1A) mediates the effector functions of antibodies and Fab fragment (Fig.1A) contains the antigen-binding site. More precisely, the antigen-binding site is located in the variable domains, at the tip of the two arms of the Y-shaped immunoglobulin molecule (Fig. 1A). Each of the V_L and V_H domains has three hypervariable complementarity-determining regions (CDR) and a framework sequence surrounding the CDR regions (Fig. 1B). The CDR regions are in loops of the anti-parallel β-sheet, at one end of the variable domain and form the antigen-binding surface (Fig. 1C). The sequence diversity of the binding site and the variable domains is generated by recombination of germline variable (V), diversity (D) and joining (J) gene segments. When B cells mature in the bone marrow, the heavy chain is generated first by recombination of a V_H, D_H and J_H gene segment with an IgM type constant region. The heavy chain is then combined with a subsequently rearranged light chain, made from a V_L and J_L segment in conjunction with a C_L gene (Janeway *et al.*, 2001).

Mature B cells circulate through blood and secondary lymphoid organs. On the surface of the cell membrane they express an immunoglobulin, also known as a B cell receptor. From the millions of B cells in the periphery only a small fraction expresses an Ig with the ability to bind any one antigenic determinant. When a B cell encounters an antigen and binds it through the B cell receptor, it is activated. As a result, the B cell starts proliferating and differentiates into antibody producing plasma cells. The antibodies raised in a primary immune response, in the first exposure to the antigen, have low affinity and are of IgM type. The selected antibody clones then acquire mutations in a process called somatic hypermutagenesis and are affinity-matured towards higher affinity. The selected antibodies also undergo class-switching from IgM to IgG isotype. Thus, in a second immune response typically high-affinity IgG antibodies are produced (Janeway *et al.*, 2001).

The most popular recombinant antibody formats are single-chain Fv (scFv) and Fab antibody fragments (Fig. 1A). ScFv molecule contains only the antigen-binding V_L and V_H domains, which are connected through a flexible polypeptide linker into a single polypeptide. Fab fragment contains additionally the C_L and C_{H1} constant domains.

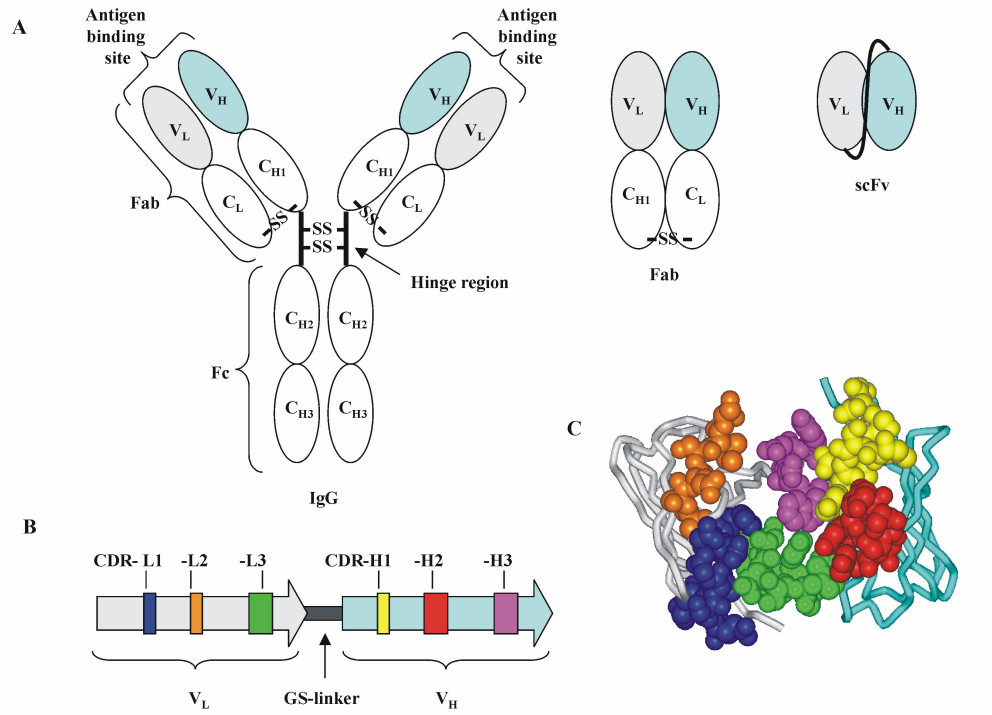

Figure 1. Schematic antibody structure. **A**) Schematic structure of an immunoglobllulin molecule and Fab and scFv antibody fragments. Immunoglobulin consists of two identical heavy (H) and light (L) chains composed of variable (V) and constant (C) domains. The antigen-binding site is located at the tip of the variable domains. The variable light (V_L) domain is shown in grey and the variable heavy (V_H) domain in blue. **B**) The schematic structure of a single-chain Fv (scFv) fragment gene. A scFv fragment consists of a V_L and V_H domain which are linked through a flexible glysine-serine linker (**A**). Hypervariable CDR regions that form the antigen-binding surface in a folded protein (**C**) are indicated with colours: CDR-L1 blue, CDR-L2 orange, CDR-L3 green, CDR-H1 yellow, CDR-H2 red, CDR-H3 purple.

2.2 Diversity and design of primary antibody libraries

New antibodies can be isolated *in vitro* from phage displayed natural and synthetic antibody libraries. These libraries are called primary antibody libraries in contrast to secondary antibody libraries that are generated for affinity maturation or further optimisation of isolated antibodies (Griffiths & Duncan, 1998). While *in vivo* the B cells present an antibody, *in vitro* the antibody is displayed on the surface of a filamentous phage. Otherwise the process of antibody generation *in vitro* follows the same principle as the antibody generation *in vivo*: clonal selection, clonal expansion and affinity maturation.

2.2.1 Natural antibody libraries

Natural antibody libraries are assembled from rearranged variable domain genes cloned from source B cells. Such B cells may be isolated from diverse lymphoid sources, such as peripheral blood, spleen, lymph nodes, bone marrow and tonsils (Hoogenboom, 2005). Depending on the donors, natural antibody libraries are either immune or naïve.

Immune libraries

Immune libraries are created to obtain antibodies specific for a certain antigen. They are assembled from rearranged variable domain genes isolated from immunised donors. Because the antibodies are raised as a response to immunisation, immune libraries have a strong bias towards the antigen and a modest library size ($<10^8$) is sufficient for isolating specific antibodies. The antibodies have been affinity-matured *in vivo* and thus immune libraries provide high-affinity antibodies (Clackson *et al.*, 1991).

In the first cloned immune libraries, binders were isolated by screening (Ward *et al.*, 1989); (Huse *et al.*, 1989). Later filamentous phage display provided an efficient route in order to enrich the specific antibodies (Clackson *et al.*, 1991; Burton *et al.*, 1991). Construction of a separate immune library is needed for each antigen, but simultaneous immunisation with several antigens is also possible and reduces working efforts (Li *et al.*, 2000a). Usually, immunised libraries are constructed by separate cloning of the V_L and V_H genes isolated from lymphocytes. During cloning, the V_L and V_H are recombined, which also produces new non-functional V_L-V_H combinations and reduces the number of specific binders. If specific B cells are separated from the non-specific cells before cloning, the proportion of specific antibodies in the final library increases and improves the outcome of the selection (Kramer, 2002a).

Most often immune libraries are produced from mouse (Clackson *et al.*, 1991; Orum *et al.*, 1993; Ames *et al.*, 1994; Shen *et al.*, 2007), but sheep (Li *et al.*, 2000b; White *et al.*, 2001), rabbit (Hawlisch *et al.*, 2000; Li *et al.*, 2000a) cow (O'Brien *et al.*, 1999), monkey (Men *et al.*, 2004), chicken (Yamanaka *et al.*, 1996), camel (Arbabi Ghahroudi *et al.*, 1997) and shark (Dooley *et al.*, 2003) have also been used as donors. Because it is not ethical to immunise humans, human antibodies can be obtained from immune libraries made from patients naturally infected with viruses (Burton *et al.*, 1991; Bowley *et al.*, 2007) or parasites (Cheng *et al.*, 2007). Special disease-related libraries have also been generated from cancer patients (Clark *et al.*, 1997; Lee *et al.*, 2002; Rothe *et al.*, 2004), allergic patients (Laukkanen *et al.*, 2003) and patients with autoimmune diseases, such as rheumatoid arthritis (Raats *et al.*, 2003b), systemic lupus erythematosus (Guchhait *et al.*, 2004) or coeliac disease (Rhyner *et al.*, 2003). These libraries can provide specific antibodies for autoantigens and disease markers. Autoantibodies isolated from autoimmune patients can be special and differ from the antibodies in healthy individuals.

Naïve/non-immune antibody libraries

Naive (non-immune) antibody libraries (Table 1) are constructed from *in vivo* rearranged antibody genes, isolated from B cells of healthy individuals that were not intentionally immunised.

IgM B cells are the primary source of naïve antibody genes. They are formed in bone marrow and non-functional or self-reacting B cell clones have been eliminated or inactivated during the

B cell development. From bone marrow B lymphocytes enter the blood and migrate to the spleen. When they encounter antigen, usually in germinal centres of lymphoid organs (lymph nodes, spleen and gut associated lymphoid tissue) the V regions acquire somatic mutations which result in affinity maturation and the antibodies undergo class-switching to IgG. Activated, antigen-stimulated B lymphocytes specialise either to plasma or memory B cells. Plasma cells are found in secondary lymphoid organs (especially spleen) and bone marrow, whereas memory cells are found in circulation. About 75 % of the blood lymphocytes are naïve and produce unmutated IgM, but 10 % have somatic mutations and belongs to the secondary antibody pool raised after antigen response. A majority of the secondary pool, 15 % of blood lymphocytes, have undergone both affinity-maturation and class-switching and presents IgG lymphocytes. Thus, IgG lymphocytes represent an immunised, affinity-matured antibody pool. Because mutated B cells contain 7 –11 times more mRNA than unmutated cells, most V genes isolated from peripheral lymphocytes are mutated (Janeway *et al.*, 2001; Bradbury & Marks, 2004; Monroe & Dorshkind, 2007).

Marks (Marks *et al.*, 1991) was the first to show that it is possible to make antibodies without immunisation from a single, naïve, universal antibody library. Marks constructed two phage displayed libraries by amplifying the rearranged V genes either from peripheral IgM or IgG lymphocytes (Marks *et al.*, 1991). Most binders were obtained from the IgM library and the only binder isolated from the IgG library had the poorest binding affinity and was barely detectable by ELISA. This suggested that naïve IgM lymphocyte pool is a better and more universal source for antibodies than the secondary lymphocyte pool generated after antigen response. However, the affinities of the binders isolated from the library were moderate, at the micromolar range, and needed affinity-maturation (Marks *et al.*, 1992). Antibodies against human anti-self antigens could also be derived from the same library (Griffiths *et al.*, 1993).

Genetic diversity is an essential determinant for the functionality of an antibody library, and there is a linear correlation between the library size and the highest affinity that has been isolated (Ling, 2003). In the Marks library, scFv was assembled directly from reverse transcription polymerase chain reaction (RT-PCR) amplified V_L and V_H, and the library was limited in size (10^7 – 10^8) compared to currently available antibody libraries. Larger libraries have been made by constructing separate V_H and V_L libraries first, using more efficient cloning strategies for scFv assembly (Sheets *et al.*, 1998; de Haard *et al.*, 1999) doing hundreds of transformations (Vaughan *et al.*, 1996) and by utilising *in vivo* recombination (Sblattero & Bradbury, 2000). Library diversity has also been increased by using optimised primer sets (Okamoto *et al.*, 2004); (Rojas *et al.*, 2005), several donors, up to 140 healthy human individuals (Pansri *et al.*, 2009), and by using B cells from several lymphoid sources (blood, spleen, tonsils, bone marrow) (Vaughan *et al.*, 1996; Rojas *et al.*, 2005). Large and diverse libraries have established isolation of high-affinity antibodies (Table 1). One of the most functional naive libraries is the Vaughan library (Vaughan *et al.*, 1996). With diversity of $1.4x10^{10}$, it is among the largest naive libraries and has provided high-affinity antibodies with 0.3 – 8 nM Kd for all the seven tested antigens, including haptens, proteins and anti-self antigens. For the library, V genes were amplified from bone marrow, peripheral blood and tonsil B cells of 43 donors. All known V_H, Vκ and Vλ families were exploited and random hexamers were used in amplification so that all the known five antibody classes (IgM, D, G, A and E) could potentially be represented. Most of the selected V genes contained mutations, suggesting that they actually were from the secondary antibody pool (Vaughan *et al.*, 1996).

Table 1. Naïve human phage displayed antibody libraries.

Reference	Source of diversity		N of donors	Library size	Antigens	Antibody/ antigen (average)	Best affinity (Kd)
Marks *et al.*, 1991	Peripheral blood lymhocytes	IgM/ IgG, Vκ,Vλ	2	IgM: $2.9x10^7$ IgG: $1.6x10^8$	Lysozyme, phOx	2.5	0.1 µM, 0.5 µM
Vaughan *et al.*, 1996	Bone marrow, blood, tonsils	All known V_H, Vκ,Vλ	43	$1.4x10^{10}$	Proteins, haptens, self-antigens	7.0	0.3 - 8 nM
Sheets *et al.*, 1998	Spleen, blood	IgM, Vκ,Vλ	5	$6.7x10^9$	14 protein antigens	8.7	0.2 - 72 nM
de Haard *et al.*, 1999	Spleen, blood	IgM, Vκ,Vλ	5	$3.7x10^{10}$	Tetanus toxoid, phOx, MUC1, hCG, hLH, hFSH	14.4	2.7 - 38 nM (hormones)
Sblattero & Bradbury, 2000	Peripheral blood lymphocytes	IgM, IgG, Vκ,Vλ	40	$3x10^{11}$ (calculated, *in vivo* recombination)	15 antigens	6.1	15 – 83 nM
Rojas *et al.*, 2005	Blood, tonsils, bone marrow, spleen	IgM, IgG, Vκ,Vλ	32	$5x10^8$	Peptide, six proteins	5.6	0.2 - 14 nM
Schofield *et al.*, 2007	Peripheral blood	V_H, Vκ,Vλ	43	$1.1x10^{10}$	292 antigens	25	n.d.
Pansri *et al.*, 2009	Peripheral blood lymphocythes	V_H, Vκ,Vλ	140	$1.5x10^8$	Proteins, hapten, snake venom, rabies viruses, cancer cells	2	n.d.

2.2.2 Semi-synthetic / combinatorial antibody libraries

Semi-synthetic and combinatiorial antibody libraries combine natural and synthetic sequences (Hoogenboom, 2005). Such libraries can be constructed e.g. by PCR assembly of germline genes and introduction of additional synthetic diversity (Table 2), introducing synthetic diversity into a naïve library by PCR (Table 3) or recombination of *in vivo* formed CDRs (Table 4).

Combining germline V genes with synthetic CDR-H3 diversity

In naïve antibody libraries the diversity is limited, not only by the number of transformants, but also by the number of isolated B cells (~$5x10^8$ in mice and ~10^{12} in humans) (Griffiths *et al.*, 1994). Moreover, naïve antibody libraries are rarely truly non-immunised because they contain antibodies also from the secondary, antigen-stimulated pool. Also self-reacting clones have been deleted during clonal anergy. This has resulted in attempts to reproduce antibody diversity from germline genes. Such antibody library would not have been selected for self-tolerance and thus would be completely naïve and provide antibodies also against self-antigens.

The genetic diversity of antibody repertoire is generated by *in vivo* recombination of V, D and J gene segments. Human antibodies are assembled from 65 different germline V_H genes and 70 different V_L genes (30 Vλ and 40 Vκ). CDR1 and CDR2 loops are encoded by the V genes, whereas the diversity of CDR3 is generated by recombination of the V_L genes with four (λ) or five (κ) J_L gene segments (for CDR-L3) or recombination of the V_H genes with 27 D_H and 6 J_H gene segments (for CDR-H3). Additional diversity can be created by small deletions or insertions at the junctional site (Tomlinson *et al.*, 1994; Frippiat *et al.*, 1995; Janeway *et al.*,2001). CDR-H3 is very complex and varies in both length and sequence diversity. The length of the CDR-H3 loop found in human antibodies varies from one to over 35 (Knappik *et al.*, 2000; Hoet *et al.*, 2005; Zemlin *et al.*, 2003). Reproducing the natural diversity of CDR-H3 for libraries is challenging, because the theoretical diversity exceeds the possible size of phage libraries (<10^{11}). Several groups have, therefore, selected to generate synthetic diversity for the CDR-H3 using fully randomised loops of variable lengths.

In the first library, where germline V_H genes were rearranged *in vitro*, 49 human germline V_H genes were recombined with a synthetic fully randomised CDR-H3 loop of five or eight residues (Hoogenboom & Winter, 1992). In this library only a single V_L chain was used. The size of the library was limited (10^7 clones) and as in the first naïve antibody library (Marks *et al.*, 1991), the affinities of the isolated binders were moderate (in the µM range). Thus, affinity maturation was needed. Nissim (Nissim *et al.*, 1994) constructed a similar but slightly more diverse library (library size > 10^8). From this library, functional monospecific antibodies were isolated for a wide range of antigens, including haptens, foreign and self-antigens as well as intracellular antigens, several of which are difficult targets for hybridoma technology. Nissim (Nissim *et al.*, 1994) also introduced the term “single-pot” library, which means a highly-diverse, universal, antigen-unbiased library. Once constructed, specificities for a wide range of targets can be isolated from the library.

The repertoire of *in vitro* assembled germline genes was widened to the light chain by Griffiths (Griffiths *et al.*, 1994). In the library, 49 human germline V_H and 47 V_L (26 Vκ and 21 Vλ) genes were used, which covers the vast majority of the *in vivo* used V gene segments. Part or all of the CDR3 loop was encoded by random sequence. A highly diverse library (estimated size

Table 2. Antibody libraries combining germline V genes with synthetic diversity.

Reference (Library)	Framework	Diversity	Display vector	Library size	Antigens	Antibody/ antigen (average)	Best affinity (Kd)
Hoogenboom & Winter, 1992	Human scFv	Single unmutated Vλ, 49 germline V_H, synthetic CDR-H3 (length 5 or 8 aa: random 15 base D + JH4)	pHEN1, phagemid	10^7	PhOx, NIP	20 10	0.7 µM, 3.1 µM
Griffith *et al.*, 1994	Human Fab	26 Vκ, 21 Vλ, 49 V_H, synthetic CDR-H3 (4-12 aa); recombination of V_L and V_H *in vivo*	pUC19-2lox, fdDOG-2lox	$6.5x10^{10}$	NIP-CAP, fluorescein, Mab, HGF/SF, plasmin	2 - 58	4 - 217 nM
Nissim *et al.*, 1994	Human scFv	single Vλ3, 50 V_H + random CDR-H3 (lenght 4 -12 aa)	pHEN1 phagemid	$> 10^8$	18 antigens (haptens, foreign and self-antigens; secreted, from cell-surface, intracellular, endoplasmic reticulum, nucleus)	n.d.	n.d.
de Kruif *et al.*, 1995	Human scFv	Vκ1-4, Vλ1-3; 49 V_H, synthetic CDR-H3 (length 6-15; short full randomisation flanked by regions with limited variability (bias towards natural sequences)	pHEN1 phagemid	$3.6x10^8$	13 different antigens	n.d.	0.1 – 2.5 µM
Knappik *et al.*, 2000 (HuCAL1)	Fully synthetic human, scFv	49 framework combinations (7 consensus V_H, 4 Vκ and 3 Vλ), fully randomised CDR-L3 and CDR-H3 cassettes	pMORPH7 phagemid	$2.1x10^9$ (61% correct)	Proteins (ICAM-1, CD11b, hEGFR), peptides (Mac1. Hag, NFκB)	n.d.	1 – 1600 nM
Rauchenberger *et al.*, 2003 (HuCAL-Fab1)	Human, fully synthetic Fab	Entire V_H and V_L from HuCAL1 library transferred into Fab library	pMORPH18 phagemid	$2.1x10^{10}$ (67 % correct)	Human FGFR-3	49	1.5 – 37 nM
Rothe *et al.*, 2008 (HuCAL GOLD)	Human, fully synthetic Fab	7 consenstus V_H, 3 Vλ and 4 Vκ. All six CDRs diversified.	pMORPH23 Cys display	$1.6x10^{10}$	IL18R-Fc, b-Galactosidase, β-estradiol	17 - 25	0.1 – 10 nM
Shi *et al.*, 2010	Human Fab	4 V_H, 3 Vκ. Synthetic diversity at CDR-H1, H2 and H3	p9 display phagemid	$12x \sim 10^{10}$	4 proteins (mouse tissue factor, mCP-5, hTNFa, hIgE)	10 - 28	0.2 – 5 nM

$6.5x10^{10}$) was estabiliseshed by recombination of separate V_L and V_H libraries in bacteria. From this library, binders with relatively high affinity (Kd = 4 – 217 nM) were isolated for proteins and haptens. Even though the library was not somatically hypermutated, affinities were similar to those of mouse monoclonal antibodies from the secondary response (Griffiths *et al.*, 1994).

In the Hoogenboom & Winter (1992), Nissim *et al.* (1994) and Griffiths *et al.* (1994) libraries, CDR-H3 was diversified with fully random loops of variable lengths. However, in natural antibodies the sequence is not fully random. Particular residues are more hypervariable than others and certain amino acid residues occur more frequently at the binding site. Furthermore, the loop conformation is determined by the sequence. Extending the randomisation at the key residues beyond what is used in nature might reduce the number of functional loops. Therefore, de Kruif *et al.* (1995) constructed a semi-synthetic library using several V_L and V_H genes and a partly randomised CDR-H3 loop, but diversity at the CDR-H3 directed towards natural sequence. This was practical because of the limits in library size.

Combining naïve and synthetic diversity

Somatic hypermutation is critical in the generation of high-affinity antibodies and naive libraries, from the basic, rarely produce affinity-matured binders. CDR-H1 and H2 are the sites where somatic hypermutagenesis often occurs. Hoet *et al.* (2005), therefore, constructed an antibody library where CDR-H3 and light chain diversity was collected from natural antibodies, but the key contact residues of CDR-H1 and H2 loops were diversified synthetically (Hoet *et al.*, 2005). In contrast to the other semi-synthetic antibody libraries, where various CDR-H3 designs have been exploited, natural diversity at CDR-H3 was justified by the enormous sequence and length diversity of natural CDR-H3 repertoires, which is impossible to be covered using DNA synthesis. Moreover, diversification of CDR-H1 and –H2 provided characteristics of affinity-matured clones. The library was highly functional and provided < 10 nM binders for several antigens (Hoet *et al.*, 2005). Details of this and other libraries that combine naïve and synthetic diversity are summerised in Table 3.

Combining single framework with in vivo formed diversity

Another approach to generating a semisynthetic antibody library is to recombine *in vivo* rearranged CDRs into a single master framework for a so called n-CoDeR library (Soderlind *et al.*, 2000). *In vivo* formed CDRs are conceptually proofread and guarantee high functional diversity at the binding site. The design is based on the presumption that CDRs derived from other germline gene sequences have the potential to be included in a specific single framework. The approach proved to be functional since a number of different specificities could be generated with 0.9 – 420 nM affinity. (Soderlind *et al.*, 2000). In another library using the n-CoDeR principle, only the CDRs that belong to the framework subgroup were amplified (Azriel-Rosenfeld *et al.*, 2004). In this Ronit1-library (Azriel-Rosenfeld *et al.*, 2004), the same V_H (DP47) and V_L (DPL3) were used as in the original n-CoDeR library (Soderlind *et al.*, 2000), but additionally, also a V_L kappa (DPK-22) was included to the framework. The best affinities of the scFvs derived from the Ronit1 library were similar to the original n-CoDeR library and varied from 0.9 to 200 nM (Table 4).

Table 3. Antibody libraries combining naïve and synthetic diversity.

Reference (Library)	Framework	Diversity	Display vector	Library size	Antigens	Antibody/ antigen (average)	Best affinity (Kd)
van Wyngaardt *et al.*, 2004 (Nkuku)	Chicken scFv	*In vivo* formed V genes from chicken bursae. Synthetic CDR-H3 (length 6 - 14 aa).	pHEN1, phagemid	$2x10^9$	Several haptens, proteins, viruses	n.d.	0.4 – 2.8 nM
Baek *et al.*, 2004	Human scFv	V_H and Vκ from peripheral blood lymphocytes. Bone marrow, fetal liver, random CDR-H3 (4 – 12 aa).	Ex-phage	$2x10^8$	Malonyl CoA carboxylase	n.d.	n.d.
Hoet *et al.*, 2005	Human scFv	Natural Vκ and Vλ and CDR-H3 (IgM cells from 35 autoimmune and 10 healthy donors). Single V_H (VH3-23), synthetic CDR-H1 and -H2.	pMID9 (phage) / pMID21 (phagemid)	$3.5x10^{10}$, $1.0x10^{10}$	4 human proteins (TIE-1, endotheliase-1, endotheliase-2, human tissue kallikrein)	35 - 293	0.22 – 1.5 nM

Table 4. Antibody libraries combining single framework with *in vivo* formed CDRs.

Söderlind *et al.*, 1995 (n-CoDeR)	Human scFv	Single V_L (DPL3) and V_H (DP47) framework. *In vivo* formed CDRs.	pAfb5c-His	$2x10^9$	11 antigens (haptens, peptides, proteins, carbohydrates)	3.8	0.9 – 67 nM
Azriel-Rosenfeld *et al.*, 2004 (Ronit1)	Human scFv	Single V_H (DP47), Vλ (DPL-3), Vκ (DPK-22). All possible *in vivo* formed CDRs that belong to the master framework families.	pCC_{16}, Ex-phage	10^9	Peptide, protein, peptide-protein complex	4.7	0.9– 200 nM

2.2.3 Synthetic antibody libraries

Synthetic antibody libraries are constructed entirely *in vitro*, using oligonucleotides that introduce diversity into the CDRs of one or more V genes. In fully synthetic libraries the framework sequence is also synthesised *in vitro* and the framework can be selected to be optimal in expression, display and stability. The use of synthetic formats bypasses the need to isolate antibody genes and allows for the generation of sequences with predefined properties, e.g. optimised cocon usage, unique cloning sites, or defined CDR lengths and compositions (Knappik *et al.*, 2000). Moreover, sequence diversity can be extended beyond that found in nature, which may allow for the generation of antibodies to antigens that do not raise immune response in animals (Borrebaeck & Ohlin, 2002).

Use of multiple framework genes in synthetic antibody libraries

In natural antibodies, diversity comes from both the framework and binding site sequence. An ideal way of obtaining a fully synthetic "naïve" library would be to use several frameworks and randomisation of all six CDR regions. However, because of limits in the achievable library size, it is not possible to cover the full diversity and approximations have to be done. One approximation is to limit the number of framework sequences by using consensus frameworks (Knappik *et al.*, 2000).

Based on sequence homology, human germline genes can be grouped into six V_H, seven V_L kappa and ten V_L lambda subfamilies. The number of members in each group varies and many of the germline genes are never or only very rarely used during immune response.In point of fact, more than 95 % of human antibody repertoire is covered by five V_H and seven V_L (four Vκ and three Vλ) germline families (Knappik *et al.*, 2000). In the HuCAL library (Knappik *et al.*, 2000) each of the frequently used subfamilies were represented by one synthetic consensus sequence. This limited the number of framework genes from > 3,500 to 49, while preserving the conformational diversity determined by the framework (Knappik *et al.*, 2000). In another synthetic library the different subfamilies were represented by three selected V_H and four V_L frameworks encoded by frequently used IGV and IGJ germline genes (Shi *et al.*, 2010).

The framework genes used in the HuCAL library were synthesised *de novo*, using optimal codons for expression in *E.coli*. Additionally, unique restriction sites flanking each CDR region were introduced into the framework. The presence of unique restriction sites across the whole library made it possible to introduce CDR cassettes into the frameworks by cloning (Knappik *et al.*, 2000).

Single framework antibody libraries

Natural antibodies utilise several frameworks, but their usage, expression and stability are not equal (Griffiths *et al.*, 1994; Ignatovich *et al.*, 1997; Knappik *et al.*, 2000; de Wildt *et al.*, 2000; Ewert *et al.*, 2003). The most frequently used germline segments in human repertoires are often also selected from synthetic repertoires (Griffiths *et al.*, 1994). Because the antigen-binding site is formed by six CDRs, one approach to the construction of antibody libraries is to use a single framework of V genes often found in natural antibodies. The framework can be selected to be stable (Desiderio *et al.*, 2001) and have optimal expression, display and immunogenecity, producing favourable characteristics for downstream applications. Using only a single framework makes library construction easier and the isolated binders can readily be recombined and shuffled for affinity maturation (Soderlind *et al.*, 2000).

Table 5. Antibody libraries combining single framework with synthetic diversity.

Reference (Library)	Framework	Diversity	Display vector	Library size	Antigens	Antibody/ Antigen	Best affinity (Kd)
Barbas *et al.*, 1992	Human Fab (anti-tetanus toxoid)	Randomised CDR-H3	pComb3, phagemid	$5x10^7$	Fluorescein	17	140 nM
Braunagel & Little, 1997	Human scFv	CDR-H3: random eight residue loop, trinucleotide codons	pSEX81	$8x10^8$	Dinitrophenol, fluorescein isothiocyanate and 3-nitro-4-hydroxy-5-iodophenylacetic acid	n.d.	n.d.
Pini *et al.*, 1998	Human scFv (V_H DP-47, Vκ DPK22)	CDR-L3 (4 residues), CDR-H3 (4 residues)	pDN332, phagemid	$> 3x10^8$	Several antigens	n.d.	18 nM
Desiderio *et al.*, 2001	Mouse scFv	CDR-L3 (4 residues), CDR-H3 (4 residues)	pDN332, phagemid	$5x10^7$	Several protein antigens	n.d.	10.5 µM
Sidhu *et al.*, 2004	Humanised scFv (anti-Erb2 4D5)	CDR-H1 (5 residues), CDR-H2 (6 residues), CDRH3 (randomised with DVK, 12 different aa)	Bivalent display Monovalent display	$1.4x10^{10}$ $2.1x10^{10}$	Six protein antigens (mVEGF, hIGF, hIgE, hIgG, hGH, hGHbp)	117 32	VEGF: IC50 = 130 nM VEGF:
Lee *et al.*, 2004	Humanised Fab (anti-Erb2 4D5)	CDR-H1, CDR-H2 (limited natural mimicking diversity); CDR-H3 (various designs were explored (DVK-> NVT-> NNS -> XYZ), multiple lengths)	Bivalent Fab.zip p3 display	$4x10^{10}$	13 protein antigens	5.4	<10 – 130 nM
Völkel *et al.*, 2004	Synthetic scFv diabody (V_H DP-47, V_L DPK9)	CDR-H3 (9aa + JH4), CDR-L3 (9aa + Jκ2): 16 positions randomized with either KMT, NHK or NNK.	pHEN3, phagemid	$1.2x10^9$	Endoglin, endothelial cells	2	0.7 µM
Silacci *et al.*, 2005 (ETH-2-Gold)	Human scFv (V_H DP-47, V_L DPL16, DPK22)	CDR-L3 (6 residues), CDR-H3 (4 - 6 residues)	pHEN1 phagemid p3	$3x10^9$	16 antigens (serum proteins, recombinant extracellular matrix protein domains, viral proteins, toxins)	TNC: 8	670 nM
Yang *et al.*, 2009	Fully synthetic scFv (V_H DP47, V_L DPL3)	All six CDRs (synthetic diversity)	pComb3, phagemid	$7.6x10^9$	8 human and nonhuman proteins, fluorescein	4.9	9 – 670 nM

Table 6. Single-framework antibody and other binding libraries with minimalist diversification.

Reference	Framework	Diversification		Library size	Display	Antigens	Best affinity (Kd)
Fellouse *et al.*, 2004	Fab	All CDRs	Y, A, S, D	10^{10}	Bivalent Fab'-zip, phagemid	hVEGF, mIgG, hIGF-1	2 – 10 nM
Fellouse *et al.*, 2005	Fab	CDR-L3,-H1,-H2-H3 (solvent accessible residues)	Y, S	10^{10}	Phage display	Maltose-binding protein,, neutravidin, Erbin PDZ domain, insulin, hVEGF, human death receptor 5.	60 - 220 nM
Fellouse *et al.*, 2007	Fab	CDR-L3, -H1, -H2, -H3 (solvent accessible residues)	Y, S + 2-4 amino acids at paratope residues	$3x10^{10}$	Bivalent phagemid display	hVEGF	9.7 nM
Fellouse *et al.*, 2007	Fab	CDR-L3,-H1,-H2-H3 (solvent accessible residues): YS +2-4 aa at nonparatope residues. CDR-H3: All (excluding C), biased towards Y, S and G.		$3x10^{10}$	Bivalent phagemid display	14 protein antigens	0.5 – 4.3 nM
Koide *et al.*, 2007b	Monobody (fibronectin type III domain)	Y, S		10^{10}	3x phage -> 1x yeast display	Maltose-binding protein, human and yeast small ubiquitin-like modifier.	5 – 89 nM
Birtalan *et al.*, 2008	Fab	CDR-L3, -H1, -H2 (solvent accessible residues): YS. CDR-H3: Y, S, G / Y, S, R / Y, S, G, R.		$> 10^{10}$	Phage display	Insulin, VEGF and HER	0.3 – 188 nM
Gilbreth *et al.*, 2008	Monobody	Y, S, X (40% Y, 20% S, 10% G and 5% of each R, L, H, D, N, A)		n.d.	Phage -> yeast display	Maltose-binding protein	5.7 nM

In synthetic (Table 5) and semi-synthetic (Table 3) single-framework antibody libraries, the DP-47 [VH3-23] germline gene that belongs to the V_H3 subgroup is almost exclusively used as the master V_H sequence (Soderlind *et al.*, 1995; Pini *et al.*, 1998; Azriel-Rosenfeld *et al.*, 2004; Lee *et al.*, 2004a; Volkel *et al.*, 2004; Silacci *et al.*, 2005; Yang *et al.*, 2009; Hoet *et al.*, 2005). V_H3 genes are stable, well expressed, common in natural antibodies and pair with almost all light chains (Griffiths *et al.*, 1994; Knappik *et al.*, 2000; Ewert *et al.*, 2003; Schofield *et al.*, 2007). DP-47 represents 12 % of the human antibody repertoire (Griffiths *et al.*, 1994). In addition to V_H3, V_H1 genes are also often used in binding, as was shown by an analysis of a more than 4,400 antibodies selected from a naive library of 292 different antigens. More than 80 % of the isolated binders used either V_H3 (51 %) or V_H1 (34 %), and these germline genes were found in combination with several different V_L genes (Schofield *et al.*, 2007).

The number of different V_Ls used as master genes in single-framework libraries is greater than the V_Hs. Most libraries use DPK22 [Vκ3] (Pini *et al.*, 1998; Azriel-Rosenfeld *et al.*, 2004; Silacci *et al.*, 2005) or DPL3 [Vλ1] (Soderlind *et al.*, 1995; Azriel-Rosenfeld *et al.*, 2004; Yang *et al.*, 2009), but also DPK9 [Vκ1] (Volkel *et al.*, 2004), and DPL16 [Vλ3] (Silacci *et al.*, 2005) have been used. DPK9 (12 % of Vκ), DPK22 (25 % of Vκ) and DPL16 (16% of Vλ) are frequent in natural antibodies and DPL3 (50 % of Vλ) was common in a library constructed by recombination of germline gene *in vitro* (Griffiths *et al.*, 1994).

Diversity design in synthetic antibody libraries

In the simplest synthetic libraries only the CDR-H3 loop in a single framework is randomised (Barbas *et al.*, 1992). Introducing a fully random loop of 16 residues can generate > 10^{20} different sequences, which is already beyond the achievable library size. Because CDR-H3 region contributes most to the antigen binding (Xu & Davis, 2000), it has been reasonable to try whether randomisation of only the CDR-H3 loop would be sufficient for isolation of specific binders. This was the first step towards generation of totally synthetic antibody libraries (Barbas *et al.*, 1992).

In nature, sequence diversity in the primary antibody repertoire is focused at the centre of the antigen binding site, which is formed by the CDR3 loops. By contrast, somatic hypermutation spreads diversity to the periphery. The pattern was mimicked in several libraries, where diversity was limited to the CDR-L3 and CDR-H3 loops (Pini *et al.*, 1998; Volkel *et al.*, 2004; Silacci *et al.*, 2005), including the HuCAL library (Knappik *et al.*, 2000). The isolated binders could then be affinity-matured by optimising the peripheral residues (Pini *et al.*, 1998; Knappik *et al.*, 2000). In the HuCAL library, separate CDR cassettes were constructed for each CDR for affinity maturation (Knappik *et al.*, 2000).

Affinities of the binders isolated from the HuCAL library were 1 – 1600 nM prior to any affinity maturation. The library was in scFv format and displayed on phage (Knappik *et al.*, 2000). When the same library was selected by ribosome display, an anti-insulin antibody with 82 pM affinity could be isolated (Hanes *et al.*, 2000). In ribosome display, random mutations are produced in each cycle and the exceptionally high affinity resulted from accumulation of mutations during the selection cycles. Compared to the parental consensus antibody sequence, the produced mutations resulted in 40-fold improvement in affinity. Later the entire HuCAL library was cloned also in Fab phage format (Rauchenberger *et al.*, 2003).

In the next generation, in the HuCAL GOLD library (Rothe *et al.*, 2008) diversity was added to all six CDRs and the phage display format was changed to Cys display. In Cys display, the

antibody is coupled to the phage coat protein 3 through a disulfide bridge, which establishes affinity-independent elution in panning by reduction of the disulfide bond. The library proved to be a reliable source for high-affinity antibodies. Antibodies were obtained for several different antigens, with best affinities in the picomolar range (Rothe *et al.*, 2008).

Diversification of all six CDR loops was also applied to a single-framework antibody library (Yang *et al.*, 2009). In the library, the CDR-H3 loop was fully randomised using three different loop lengths: 7, 12 and 18 amino acid residues. In one sub-library with an 18 residues long CDR-H3 loop, there was an intraloop disulfide bond, encoded by the human D_H2 gene family, relatively common in human antibodies (Yang *et al.*, 2009). This feature has not been exploited in other synthetic antibody libraries.

In the Genentech library (Lee *et al.*, 2004a), only the three heavy chain CDRs were diversified, and the light chain CDRs were optimised subsequently. The first Genentech library used scFv framework, both in monovalent and bivalent formats (Sidhu *et al.*, 2004). While the scFv libraries provided binders with IC50-values of 0.1 nM, in Fab format the same library did not perform well and resulted in only > 5 µM binders. Therefore, various CDR-H3 designs were explored to improve the functionality of the Fab library (Lee *et al.*, 2004a). It was found that diversification with NNS codon (the commonly used nomenclature for incomplete nucleotide bases (Cornish-Bowden, 1985) is used here and later) resulted in higher affinities than diversification with DVK codon. In the final library, performance was improved further by introducing multiple CDR-H3 lengths (from 7 to 19) and the sequence was biased towards amino acid usage in natural antibodies by using a modified NNS codon, referred to as "XYZ" (Lee *et al.*, 2004a). In the "XYZ" codon, different proportions of nucleotides are exploited in different positions. (Lee *et al.*, 2004a).

Compared to the "XYZ" codon, a simpler way to bias the codons towards natural sequences is to use trinucleotide building blocks for diversification, as in the HuCAL library (Knappik *et al.*, 2000). The benefit of using trinucleotides is that stop codons can be fully avoided from the randomisation and exactly defined codon mixtures can be designed. In addition, the codon usage can be selected to be optimal for the host (Braunagel & Little, 1997).

2.2.4 Minimalist libraries

Sequence analysis of natural antibodies has revealed that certain amino acids occur more frequently in the CDR regions than others. It is also known that certain types of amino acids more frequently make contacts with ligand/antigen than others. Using such amino acid for diversification could allow for the making of antibody libraries where a single antibody framework is diversified at the binding site only with a limited set of amino acids. Such minimalist libraries are summarised in Table 6. In the first minimalist libraries, eleven different four amino acids mixtures were compared in randomisation of solvent accessible heavy chain CDR residues (Fellouse *et al.*, 2004). Among the libraries, randomisation by codon KMT (creating tyrosine, alanine, aspartate and serine) was most successful in generating binders for protein antigens. The highest affinities for anti-hVEGF binders isolated from the minimalist library were 2 – 10 nM. Structural analysis of the binders indicated, that antigen binding was mediated mostly through tyrosine. Alanine and serine did not make direct contacts with the antigen, but allowed space and conformational flexibility (Fellouse *et al.*, 2004). Soon afterwards, it was shown that it is possible to generate functional antibodies by diversification of solvent accessible heavy chain residues only with tyrosine and serine (Fellouse *et al.*, 2005).

A recent study demonstrated the effectiveness of tyrosine/serine binary library also on a non-antibody framework: a single-domain fibronectin type III scaffold, known as monoboy (Koide *et al.*, 2007). The affinities of the antibodies isolated from the binary tyrosine/serine library were moderate (Kd > 300 nM). However, when glycine was added to the randomisation mixture, specific and high-affinity, subnanomolar recognition of the antigen was established. (Birtalan *et al.*, 2008). The effect of arginine, often involved in protein-protein interactions, was also studied. However, addition of arginine to the randomisation mixture mainly increased binding to nonspecific proteins (Birtalan *et al.*, 2008).

The performance of a binary library diversified by tyrosine/serine only on the heavy chain CDR regions was improved by addition of diversity to also CDR-L3 loop and by allowing variations at non-paratope residues that determine the conformation of CDR loops (Fellouse *et al.*, 2007). Further diversification of the CDR-H3 with all (except cysteine) residues, biased towards tyrosine, serine and glysine, led to a highly functional library yielding diverse binders with subnanomolar affinity to numerous antigens (Fellouse *et al.*, 2007). The expansion of the amino acid diversity over tyrosine/serine also improved the functionality of a synthetic monobody library (Gilbreth *et al.*, 2008).

2.2.5 Focused antibody libraries for difficult targets

There are distinct differences in antibodies binding to different types of antigens. These differences have resulted in construction of specific libraries focused on certain types of antigen groups. In these libraries, the sequence is designed to form the canonical structures that are often used with the antigen type. Such focused libraries have been constructed not only for haptens (Persson *et al.*, 2006) and peptides (Cobaugh *et al.*, 2008) but also for proteins (Almagro, 2004). In addition, a carbohydrate-oriented library has been constructed by taking into account the length and amino acid composition of CDR-H3 loop (Schoonbroodt *et al.*, 2008).

Canonical classes and sequence characteristics

All CDR loops, except for the highly variable CDR-H3, adopt only a limited number of backbone conformations, called canonical structures. The canonical classes are determined by the loop size and certain key residues in both the loop and framework sequence (Chothia & Lesk, 1987; Chothia *et al.*, 1989; Chothia *et al.*, 1992). CDR loops L1, L2, L3, H1 and H2 can form 300 possible combinations of canonical structures, but only a small number of the combinations are preferentially used. Ten classes describe 87 % of canonical classes of these five loops, and mostly it is the H1 and H2 loops that are varied (Vargas-Madrazo *et al.*, 1995). The use of canonical classes varies between the different groups of antigens: proteins, haptens, polysaccharides, peptides and nucleic acids, and some canonical classes are specific to certain antigen groups (Vargas-Madrazo *et al.*, 1995).

Canonical structures determine the type of antigen-binding surface (Vargas-Madrazo *et al.*, 1995). There are three main types of surfaces: cavity, grooved and flat. Antibodies recognising protein antigens usually have large, flat binding surface and bind apical residues in the CDR loops. In anti-peptide antibodies smaller grooved binding-sites are preferred and contacts are established with residues more internally to the binding site than in the anti-protein antibodies. The antigen-binding site of anti-hapten antibodies is typically the smallest and deepest, and the contact making residues are located at the bottom of the cavity (Almagro, 2004). In anti-protein and anti-peptide antibodies there are typically more residues (on average 7) making contacts

with the antigen than in the anti-hapten antibodies (on average 5 redsidues). Most of the contacts are mediated through the CDR-H3 loop, which usually contributes 40 % (proteins) and 60 % (haptens) to the contacts with the antigen (Almagro, 2004).

Sequence composition and loop length characteristics of the binding site also vary depending on the antigen type, but loop lengths are different in mouse and human antibodies (Collis *et al.*, 2003). In human antibodies, the longest CDR-H3 loops are observed particularly in antibodies that bind proteins, viruses and sugars (Collis *et al.*, 2003). However, the average length of human carbohydrate-binding antibodies is shorter than in antibodies in general (Schoonbroodt *et al.*, 2008). The sequence compositions of the antigen-binding site also differ between the groups of antigens. For example, arginine occurs more frequently at the binding site of nucleotide binding antibodies than in other gropus, possibly because of the negative charge of phosphate backbone, whereas histidine is overrepresented in hapten binders (Collis *et al.*, 2003). Basic residues are commoc in CDR-H3 loop of anti-carbohydrate antibodies (Schoonbroodt *et al.*, 2008).

Hapten oriented libraries

Small molecules, haptens, have been difficult targets for antibody generation because they are not immunogenic themselves. To raise immunoglobulins against haptens they need to be conjugated to a carrier protein, which usually results in high background to the carrier (Kramer, 2002a). Initially, hapten-specific antibodies have been obtained from immunised libraries by phage display selection. These antibodies have gone through affinity maturation *in vivo* and are of relatively high-affinity (Clackson *et al.*, 1991). The highest affinities measured for binders isolated from immune libraries vary from 0.2 nM to 13 nM (Charlton *et al.*, 2001; Kramer, 2002b).

Even though immune libraries provide an efficient route for isolation of high-affinity antibodies, constructing a separate library for each individual antigen is labour-intensive. Single-pot libraries would provide a more general approach for specific antibodies, but the first anti-hapten antibodies isolated from naïve libraries had low affinity, in the micromolar range (Gram *et al.*, 1992). However, some highly diverse combinatorial libraries (Griffiths *et al.*, 1994) have yielded 4 nM high-affinity binders even for haptens.

Because hapten-binding antibodies use canonical loop structures that form a cavity where the hapten binds, there have been trials to construct hapten-oriented libraries that exploit the canocical structures that form a cavity typical for hapten binding antibodies (Persson *et al.*, 2006). For example, a stable and well-expressing anti-fluorescein antibody has been used as a scaffold for a hapten-oriented library. The library was created introducing diversity into residues lining the proposed cavity. In addition to diversification of 14 selected residues at CDR-H1,-H2,-H3 and L1, CDR-L3 loop was fully randomised with natural loops and small insertions were optionally allowed at CDR-H2. The library was more efficient in generating binders for haptens than a conventional library, but affinities of the isolated binders were rather low (0.3 – 3 µM), which is similar to the anti-hapten antibodies isolated from naive libraries. Theoretical diversity of the library was only $7x10^5$, which may limit the library performance. However, several high-affinity (0.8 – 4 nM) binders were obtained for fluorescein, the original antigen for the template, and thus the library clearly affinity matured the anti-fluorescein antibody (Persson *et al.*, 2006).

Anti-hapten antibodies have also been used as a framework in other semi-synthetic libraries. In one study, a semi-synthetic library was constructed using a hapten phenyloxazolone specific antibody as a framework. The library proved to be efficient in the generation of antibodies to hapten aflatoxin-B1, unlike a naive library. The highest affinity obtained for soluble aflatoxin was as high as 6 nM (Moghaddam *et al.*, 2001). Another semi-synthetic library was constructed by Barbas *et al.* (1992; 1993a) by using an anti-tetanus toxoid Fab as a framework. In the first library, CDR-H3 was fully randomised with a 16 amino acid loop (Barbas *et al.*, 1992). In the second library (Barbas *et al.*, 1993a), diversity was introduced also to CDR-L3 and different loop lengths were allowed both at CDR-L3 and –H3 loops. From this library, 29 – 140 nM binders were isolated for fluorescein by phage display.

Anti-carobohydrate libraries

While bacterial polysaccharides can elicit strong immune response, carbohydrates usually have low immunogenecity and hybridoma technologies have been ineffective in producing monoclonal antibodies against a variety of carbohydrate antigens. Immunisation with carbohydrates often leads to primary IgM response and the isolated antibodies have low affinity. Immune phage libraries from cancer patients have provided some antibodies against cancer-related carbohydrate antigens (Mao *et al.*, 1999). Additionally, some anti-carbohydrate antibodies have been isolated from semi-synthetic phage libraries (de Kruif *et al.*, 1995; Soderlind *et al.*, 2000).

Because of the difficult generation of anti-carbohydrate antibodies, Schoonboordt (Schoonbroodt *et al.*, 2008) constructed a semi-synthetic anti-carbohydrate library. The library was modified from the library of Hoet (Hoet *et al.*, 2005). Antibodies binding charged carbohydrates have specific features, such as short CDR-H3 loops with high content of basic residues. Therefore, in the library, the CDR-H3 loop was replaced with a short, synthetic, randomised, 6-residue cassette, where basic lysine and arginine residues were introduced in positions often found to be basic in anti-carbohydrate antibodies (Schoonbroodt *et al.*, 2008). After converting an isolated antibody to bivalent, 54 nM Kd was measured, which is relatively high compared to other described anti-carbohydrate antibodies.

2.2.6 Improving quality of antibody libraries

In naïve antibody libraries the amplified antibody sequences have been proofread *in vivo*. In contrast, in synthetic antibody libraries errors during oligonucleotide synthesis accumulate in the library. Single base deletions cause frameshifts and result in production of truncated protein. Furthermore, stop codons are not excluded in the randomisation pattern, unless full oligos are produced using trinucleotide blocks (Rothe *et al.*, 2008). In the n-CoDeR library, where *in vivo* formed CDRs were recombined to a single master framework, PCR amplified primers were used to eliminate errors during oligonucleotide synthesis. However, 20 – 25 % of the sequences were still not in frame (Soderlind *et al.*, 1995).

For the Ronit1 library, also composed using the n-CoDeR principle, intermediate V_H and V_L libraries were expressed on phage as fused to a cellulose-binding domain (CBD), which can be bound to cellulose membranes. Frameshift mutations result in production of truncated V domains, which also lack the CBD. Functional clones were then isolated based on binding to cellulose membranes. As a result, the library composed of > 90 % intact clones (Azriel-Rosenfeld *et al.*, 2004).

For several other libraries, scFv have been expressed as a fusion with beta-lactamase and in-frame clones can be selected based on antibiotic resistance (Seehaus *et al.*, 1992). To avoid reducing diversity of the final library, purification has been targeted to all CDRs except for the most diverse CDR-H3, which was randomised last. In most libraries, where beta-lactamase fusion and selection with carbenicillin has been used, 61-70 % of the library clones have been functional or fully correct (Knappik *et al.*, 2000); (Rauchenberger *et al.*, 2003; Yang *et al.*, 2009). However, in the final HuCAL Gold library 9 % of the clones had frameshifts, but as the majority of the frameshifts were in the unpurified CDR-H3, efficiency of the purification was shown (Rothe *et al.*, 2008). Additionally, hyperphage display has been reported to result in 10-fold increase in open reading frames (Hust *et al.*, 2006). In the hyperphage display all the p3, the phage coat protein responsible for phage infecticity, arises from the fusion protein. Thus phage produced from out-of frame clones lack the p3 and cannot be rescued by infection.

Other methods to purifiy the in-frame clones in antibody libraries are based on recognition of the folded scFv instead of a fusion partner. For example, Vκ antibodies can be purified by binding the correctly folded Vκ to protein L (Loset *et al.*, 2005), and V_H3 family antibodies have been purified based on binding to protein A (Nissim *et al.*, 1994).

In addition to errors in the oligo synthesis and gene assembly, the quality of the library is also affected by the efficiency of diversification. The display of such non-randomised and "premature" partially randomised clones has been prevented by inserting stop codons into the template CDRs (Lee *et al.*, 2004b; Rothe *et al.*, 2008).

2.3 Libraries using alternative binding scaffolds

Antibodies are natural binding proteins with enormous diversity and recognition potential, but they also have limitations. For example, monoclonal antibodies are difficult and expensive to manufacture. Smaller recombinant antibody fragments are easier to produce, but scFv fragments can have low stability and be prone to aggregation. Fab fragments are generally stable, but they are structurally more complex and they can be more difficult to display on phage than scFv fragments. Because of the intradomain disulfide bonds, neither scFv nor Fab is compatible with intracellular expression and only very stable scFv fragments have been expressed in the cytoplasm of *E.coli* (Martineau & Betton, 1999); (Ohage & Steipe, 1999). Moreover, the patent situation of antibodies is also complex and there has been an increasing trend towards developing alternative binding scaffolds.

An ideal binding scaffold is a small, monomeric, single-chain polypeptide and contains no post-translational modifications such as glycosylations. For therapeutic use, human origin is ideal. The scaffold should be stable without disulfide-bonds, expressed in high amounts and compatible with display techniques. On the surface of the protein, there should be loops or other structures that can be modified to form the paratope. The paratope can be a natural binding site or created *de novo*. Randomisations made to the paratope region should be able to generate binders with high specificity and affinity (Binz *et al.*, 2005; Gronwall & Stahl, 2009). Based on the structure of the recognition site, binding scaffolds are categorised to antibody related, such as the single-domain antibodies, and to non-antibody structures (Hey *et al.*, 2005; Skerra, 2007). At the present, approximately 50 different non-antibody protein scaffolds have been reported for generation of affinity binders. Several of them are commercially exploited and designed especially for therapeutic use. These scaffolds have been reviewed in several recent papers

(Nygren & Skerra, 2004; Binz *et al.*, 2005; Hey *et al.*, 2005; Hosse *et al.*, 2006; Skerra, 2007; Gronwall & Stahl, 2009).

2.3.1 Antibody-related scaffolds

Camelids and catrilaginous fish (shark) have antibodies composed only of H chains (Hamers-Casterman *et al.*, 1993; Roux *et al.*, 1998). The antigen-binding variable domains in the camelid antibodies are called V_HHs. Unlike mammalian V domains, the V_HHs, are, in general, soluble and stable (Arbabi Ghahroudi *et al.*, 1997). Compared to mammalian V_H domains, camelid single-domain antibodies have an extra hypervariable region exclusively in the CDR-H1, which compensates for the lack of V_L. Furthermore, camelid CDR-H3 loops are on average longer than in humans (Nguyen *et al.*, 2000) and carry a non-canonical cysteine residue that froms a stabilising intradomain disulfide bond with a CDR1 cysteine (Muyldermans *et al.*, 1994). Like scFv and Fab fragments, single domain antibodies have been used as binding scaffolds in both immune (Arbabi Ghahroudi *et al.*, 1997; Dooley *et al.*, 2003), naïve (Liu *et al.*, 2007) and synthetic libraries (Shao *et al.*, 2007). Binders isolated from camelid are called nanobodies and shark antibodies are called immunoglobulin isotype novel antigen receptors (IgNAR). In addition to these natural single-domain antibodies, mammalian single V_H and V_L (van den Beucken *et al.*, 2001) variable domains have been used as a framework in libraries for so called domain antibodies (dAbs) (Ward *et al.*, 1989; Holt *et al.*, 2003; Chen *et al.*, 2008; Chen *et al.*, 2009). Stability of the isolated mammalian single domain antibodies can be improved by camelisation (Davies & Riechmann, 1996) and such stable domain antibodies are excellent for intracellular use as intrabodies (Tanaka *et al.*, 2003).

2.3.2 Non-antibody scaffolds

The non-immunoglobulin scaffolds used in alternative binders are structurally variable. The paratope can be formed by a single loop, by several loops, or it can be a flat surface formed by secondary structure elements. Some alternative binding proteins have oligomeric domain structure (Hey *et al.*, 2005; Skerra, 2007). The binding site can be modified from a pre-existing specificity or generated *de novo* (Hey *et al.*, 2005).

Single-loop

The display of single peptide loop on a structurally robust protein scaffold is a proven strategy for modifying pre-exsisting binding specificity. This approach is used by several different disulfide-bond containing small proteins with new specificites. Examples include, kunitz domains (Dennis & Lazarus, 1994), knottins (Christmann *et al.*, 1999; Kimura *et al.*, 2009), cellulose-binding domain (Smith *et al.*, 1998), transbodies (Ali *et al.*, 1999; Weaver & Laske, 2003) and peptide aptamers (Borghouts *et al.*, 2005). Kunitz domains are engineered from a Kunitz-type protease inhibitor. Randomisation of a single loop in them has generated pharmacaeutically interesting protease inhibitors with modified specificities (Nygren & Skerra, 2004; Hey *et al.*, 2005; Hosse *et al.*, 2006; Gronwall & Stahl, 2009). Knottins, from e.g. Squash-type protease inhibitor, contain a cysteine knot motif where peptides loops can be inserted (Hosse *et al.*, 2006; Skerra, 2007). Transbodies are engineered from human serum transferring, which contains one or two possible loops for insertion of polypeptides (Hey *et al.*, 2005). Peptide aptamers are short peptides derived from random peptide libraries. Usually, they are presented in a platform or scaffold protein for example thioredoxin, which provides for a constraint conformation (Borghouts *et al.*, 2005). Kunitz domains (Dennis & Lazarus, 1994) and some knottins (Smith *et al.*, 1998) have been selected by phage display, whereas cell surface display, mRNA display and yeast display, have often been exploited in selection of new

specificites of several of the single-loop scaffolds (Christmann *et al.*, 1999; Baggio *et al.*, 2002; Kimura *et al.*, 2009).

Multiple loops

Some proteins engineered for novel specificities use multiple hypervariable loops in a rigid scaffolds, an approach similar to immunoglobulins. AdNectins (also called monobodies, engineered from fibronectin type III domain) and anticalins (from lipocalins) are the most popular alternative binding scaffolds using multiple loops, but also several other scaffolds have been used, such as cytotoxic T lymphocyte associated protein 4 (CTLA-4) (Hufton *et al.*, 2000) and C-type lectin domain (CTLD) (Etzerodt *et al.* 2002) and T cell receptors (Chlewicki *et al.*, 2005).

Fibronctin type III domain (Fn3), which naturally mediates protein-protein interactions with human integrins, has immunoglobulin like structure with three CDR-like loops, but is monomeric and contains no disulfide bonds (Bloom & Calabro, 2009). In Fn3 based binding libraries, two (Koide *et al.*, 1998) or all three (Xu *et al.*, 2002) loops have been modified. Binders have been isolated by phage display (Koide *et al.*, 1998), mRNA display (Xu *et al.*, 2002) yeast display (Lipovsek *et al.*, 2007) and a yeast two hybrid system (Koide *et al.*, 2002). The highest affinity using Fn3 scaffiold has been 1 pM for lysozyme (Hackel *et al.*, 2008). Adnectin binding to vascular growth factor receptor (VEGFR)-2 have found potential in cancer therapy and has proceeded to clinical trials (Bloom & Calabro, 2009).

Lipocalins are a family of small, stable and soluble beta barrel proteins involved in transport and storage of hydrophobic substances. There are four loops at one end of the cup-like barrel that form entrance to the ligand-binding cavity. These loops vary between different lipocalins, while the barrel itself is structurally conserved (Skerra, 2008). Anticalins, binding proteins based on a lipocalin scaffold, have been evolved for both small ligands and proteins. The randomised area is dependent on the type of target: For low-molecular weigth targets the randomised areas are in the ligand-binding cavity, while for protein targets loops are randomised. Bilin binding protein (BBP), a prototype of anticalins, has been evolved and selected by phage display to bind fluorescein (Beste *et al.*, 1999; Vopel *et al.*, 2005) and digoxigenin (Schlehuber *et al.*, 2000; Schlehuber & Skerra, 2002) with 1 and 12 nM affinities, respectively. Due to the high affinity, and natural binding of small moleclules, anticalins have been called as true hapten binders. Anticalins have also been developed for protein antigens e.g. haemoglobin (Vogt & Skerra, 2004) and CTLA-4 (Schonfeld *et al.*, 2009). For therapeutic use anticalins have potential as drug carriers and receptor/ligand antagonists (Schlehuber & Skerra, 2005; Schonfeld *et al.*, 2009).

Other secondary structures

In several binding scaffolds, the binding site is a surface formed by secondary structure elements, such as α-helix or β-sheets. Examples of this group include designed ancyrin repeat proteins (DARPins), affibodies (based on Z-domain which is modified from the Staphylococcal IgG binding protein A) and affilins (evolved from γ-crystallin and human ubiquitin).

Affibodies were one of the first described non-immunogobulin scaffolds engineered for new binding specificities (Nord *et al.*, 1995; Nord *et al.*, 1997). As a scaffold they use domain Z, a small (6 kDa) three-helix bundle, derived from the IgG binding staphylococcal protein A. The domain is stable, soluble, contains no cysteines and can be expressed in high amounts. Affibody

libraries have been constructed by engineering surface formed by two of the α-helices. Selections of the libraries, mostly by phage display, have raised binders for a large number of antigens. Affinities from initial libraries have been similar to antibodies isolated from naïve libraries (Nygren, 2008). The highest affinity obtained after affinity-maturation (22 pM) has been reported for the breast cancer marked ErbB2 /Her2 (Orlova *et al.*, 2006). Affibodies have found use in various applications from bioseparations to *in vivo* imaging and tumour targeting (Tolmachev *et al.*, 2007; Nygren, 2008).

Designed ankyrin repeat proteins (DARPins) are another novel class of artificial, non-immunoglobulin binding-proteins that use secondary structure elements for recognition (Forrer *et al.*, 2003; Binz *et al.*, 2003). DARPins are constructed by attachment of repeated structural units, ancyrin motifs, which form the binding surface. A single motif is a 33 residue domain, consisting of β-turn followed by two α- helices. The size of the binding surface in DARPins is dependent on the number of repeat units, which can be varied. DARPins exploit a designed consensus sequence of natural ancyrin repeat motifs (Stumpp *et al.*, 2008). In nature, ancyrin repeat motif is present in numerous proteins with a wide range of activities, including protein-protein interactions (Bork, 1993; Sennhauser & Grutter, 2008). DARPins are stable, well-expressed, monomeric in solution and fold fast (Binz *et al.*, 2003; Merz *et al.*, 2008), but also have potential as multispecific binders (Eggel *et al.*, 2009).

High-affinity binders were initially selected from DARPin libraries by ribosome display (Binz *et al.*, 2004; Schweizer *et al.*, 2008; Zahnd *et al.*, 2007). Phage display of DARPins was inefficient because of premature folding of the protein in cytoplasm, which prevented translocation to periplasm. However, efficient phage display was established later by using another (signal recognition particle) translocation pathway, and subnanomolar binders have then also been selected by phage display (Steiner *et al.*, 2008). DARPins have found use and potential both in therapeutics (Zahnd *et al.*, 2007; Schweizer *et al.*, 2008; Stumpp *et al.*, 2008; Eggel *et al.*, 2009; Winkler *et al.*, 2009) and as crystallisation aids (Sennhauser & Grutter, 2008).

2.4 Improving binding-affinity by mutagenesis

2.4.1 Evolution of high-affinity antibodies

In nature, antibodies selected from the primary repertoires usually have low affinity, at the μM range and affinity is subsequently improved, usually to nM range, through somatic hypermutagensis of the antibody genes and selection of B cells with improved phenotypes (Foote & Eisen, 1995). Antibodies with affinities < 100 pM are rarely obtained by repeated immunisation, because slower dissociation rates cannot be discriminated from the intrinsic B cell receptor internalisation rates. Thus, higher affinities are not actively selected *in vivo* (Foote & Eisen, 1995; Foote & Eisen, 2000; Batista & Neuberger, 1998). Such constraints do not limit affinity maturation *in vitro*.

Because affinity is an essential determinant for antibody function, several antibodies, either cloned from monoclonal antibodies or isolated from universal antibody libraries, have been targeted to affinity maturation *in vitro*. High affinity is required for both therapeutic and diagnostics use. Improved affinity has provided e.g. improved neutralisation potential of viruses and toxins (Barbas *et al.*, 1994; Laffly *et al.*, 2008), improved inhibition of ligand-induced

activity (Lu *et al.*, 2003; Yoshinaga *et al.*, 2008), higher cytotoxic activity (Chowdhury & Pastan, 1999; Ho *et al.*, 2005) and stronger agglutination (Proulx *et al.*, 2002). The principle of affinity maturation *in vitro* is similar to affinity maturation *in vivo*, including generation of amino acid changes and selection for improved antibodies by display techniques (Neuberger *et al.*, 1998; Dufner *et al.*, 2006). The lower the affinity of the starting antibody the higher the potency for improved affinity (Dufner *et al.*, 2006).

The highest-affinity antibody evolved *in vitro* has been made by introduction of a covalent disulfide bridge between the antibody and ligand, which produces infinite affinity. However, this example is exceptional, because the ligand was also modified (Chmura *et al.*, 2001). The strongest non-covalent antibody-antigen interactions evolved *in vitro* have been femtomolar and selected by yeast display (Boder *et al.*, 2000; Midelfort & Wittrup, 2006). The highest antibody affinity evolved *in vitro* (Kd = 48 fM) was for a fluorescein-binding antibody (Boder *et al.*, 2000). This is close to the affinity between bacterial streptavidin and biotin (Kd 40 fM) (Green, 1990), which is known as one of the strongest non-covalent biomolecular interaction in nature. Actually, with a half-time of > 5 days, fluorescein dissociation from the antibody was even slower than dissociation of the biotin-streptavidin complex. Several high-affinity antibodies with picomolar Kd have also been evolved by ribosome-display (Knappik *et al.*, 2000; Zahnd *et al.*, 2004; Luginbuhl *et al.*, 2006) and phage display (Yang *et al.*, 1995; Lu *et al.*, 2003; Brocks *et al.*, 2006; Steidl *et al.*, 2008).

Antibody affinity can be improved by amino acid changes in either the CDR region or framework. Changes targeted to CDR regions optimise binding interactions through introduction of electrostatic charges or new hydrophobic interactions. A decrease in the size of a amino acid side chain can make room for bulky residues or allows conformational changes, which improve the exposure of charged residues and thereby optimize the contacts with an antigen (Laffly *et al.*, 2008). Affinity optimisation of very high affinity binders is often achieved by modulating existing interactions via subtle changes in the framework rather than introducing new contacts (Zahnd *et al.*, 2004). Such changes in the framework residues can be found by random mutagenesis targeted to the whole variable region of an antibody.

2.4.2 Methods for random mutagenesis

Error prone PCR

The most common method for generating random mutations into antibody variable domain genes *in vitro* is amplification of the DNA in PCR by error-prone polymerase, such as Taq DNA polymerase. The natural error rate of Taq polymerase is 0.001 – 0.02 % per nucleotide per pass of the polymerase, but the error rate can be increased to 2 % by increasing the concentration of $MgCl_2$, adding $MnCl_2$ to the reaction mixture and unbalancing the dNTP concentrations (Leung *et al.*, 1989; Cadwell & Joyce, 1992). The final mutation frequency in error-prone PCR is dependent on the number of duplications. Thus, increasing the number of PCR cycles results in accumulation of mutations (Fromant *et al.*, 1995). The mutation rate in PCR can also be enhanced using nucleotide analogues, which introduce mismatch mutations (Lin & Brown, 1989; Zaccolo *et al.*, 1996; Zaccolo & Gherardi, 1999; Luginbuhl *et al.*, 2006).

When mutation rate increases, the fraction of active clones produced by error-prone PCR decreases exponentially. However, in highly mutated libraries the number of active clones can be larger than expected. With high mutation rates most of the sequences are unique, which

Table 7. Examples of antibodies affinity-matured by random mutagenesis.

Reference	Diversification	Frame-work	Library size	Selection	Target antigen	Best affinity (Kd)	Affinity gain
Error-prone PCR							
Hawkins *et al.*, 1992	Taq polymerase, Leung *et al.*, 1989	ScFv	n.d.	Multivalent phage display, off-rate selection (competing free antigen)	Lysozyme	9.4 nM	4x
Gram *et al.*, 1992	Taq polymerase, Leung *et al.*, 1989	ScFv	~10^6	Phage display (1st round multivalent pVIII -> monovalent p3 display)	Progesterone	~3 nM	13x and 30x
Deng *et al.*, 1994	Chemical mutagenesis (nitrous acid) and error-prone PCR (Taq polymerase)	ScFv	5x10^5	Phage display (selection on mi-crotiter plate, extensive washing)	Serogroup B lipopolysaccha-ride	25 nM	8.3x
Daugherty *et al.*, 2000	Taq polymerase, Fromant *et al.*, 1995	scFv	6x10^6	Bacterial cell surface display, labelled digoxigenin, flow cy-tometric selection (FACS), off-rate selection with competing antigen.	Digoxin	0.5 nM	1.9x
Graff *et al.*, 2004	Error-prone PCR (nucleotide analogues, Taq polymerase)	ScFv	10^5	Yeast display (binding in solution, cell sorting by flow cytometry)	Carcinoembryon ic antigen (CEA)	80 pM	100x
Persson *et al.*, 2008	AmpliTaq polymerase	Fab	3-4x10^7	Phage display, decreasing antigen concentration	Testosterone	2 nM	200x
Mutator strains							
Irving *et al.*, 1996	*E. coli* mutator strain mutD5	ScFv	10^{12-14}	Phage display (immunotube selection + cell panning)	Erythocyte surface antigen glycophorin A	100 nM	480x
Low *et al.*, 1996	*E. coli* mutator strain mutD5 (multiple rounds of growth and selection)	cFv	6x10^{11}	Phage display (increasingly stringent selection)	Hapten 2-phenyl-5-oxazolone	3.2 nM	100x slower off-rate

compensates for the loss of function and despite the low fraction of active mutants, moderate (mutation rate 0.5 %, active clones 7 %) and high (mutation rate 3 %, active clones 0.2 %) error rate libraries have given rise to clones with the greatest affinity improvements (Daugherty *et al.*, 2000). The optimal error rate also depends on the number of transformants as well as from the mutated protein and mutagenesis protocol (Drummond *et al.*, 2005). In the study of Daugherty (Daugherty *et al.*, 2000), affinity was only improved by error-prone PCR by 1.9-fold (to 0.5 nM). However, in another study, where 0.2 – 5 % error rate was produced by error-prone PCR with nucleotide analogues and Taq polymerase, 100-fold affinity improvement to 80 pM binding was achieved (Graff *et al.*, 2004). This is one of the highest increases in affinity obtained during one round of antibody optimisation by error-prone PCR. Other examples of antibodies affinity-matured by error-prone PCR are shown in Table 7.

Not only the error rate, but also the mutation types produced by Taq-polymerase can be adjusted by unbalanced deoxyribonucleotide triphosphate (dNTP) concentrations. However, the mutations produced by Taq DNA polymerase are in any case biased and still 85 % of the mutations are covered by three out of the six possible base changes (Fromant *et al.*, 1995; Rasila *et al.*, 2009). Another error-prone polymerase has been made lowering the fidelity of *Pyrococcus furiosus* (Pfu) DNA polymerase by mutations (Biles & Connolly, 2004). Mutation pattern of the low-fidelity Pfu DNA polymerase is less biased than with Taq polymerase, and all six single-base changes are observed at reasonable frequency (Biles & Connolly, 2004). Commercial Mutazyme (Biles & Connolly, 2004) and Mutazyme II (Rasila *et al.*, 2009) DNA polymerases also produce less biased mutation distribution than Taq polymerase. However, the error-rate of these polymerases is lower than with Taq polymerase (Biles & Connolly, 2004; Rasila *et al.*, 2009). An interesting approach for randomising antibody sequences for affinity maturation is to use low-fidelity human DNA polymerases, mutases, which are involved in the somatic hypermutagenesis process of immunoglobulin genes (Mondon *et al.*, 2007).

Mutator strains

Random mutations can also be produced in mutator strains. *E.coli* mutD5 is a strain defective in proofreading and post-replicative mismatch repair. It is a conditional mutant, which produces single base substitutions at high frequency, 10^5 over the normal cells (Fowler *et al.*, 1986; Schaaper, 1988). The strain has been used to generate random mutations in recombinant antibody fragments in order to improve affinity. Mutations can be produced in the initial library or between selection cycles (Irving *et al.*, 1996; Low *et al.*, 1996). A benefit of mutator strains as a means of random mutagenesis is that mutations are produced after the transformation step, which usually limits the size of the library. Therefore, randomly mutated libraries of even 10^{12-14} clones can be produced easily. However, mutations are also produced for the vector part and the mutation rate needs to be carefully controlled in order to avoid deleterious effects. If a mutator strain is used to rescue phage after phage display selection, additional mutations are produced between selection cycles and establish further affinity maturation in the course of panning. This strategy resulted in a 100-fold improved off-rate of a hapten 2-phenyl-5-oxazolone by accumulation of four sequentially produced mutations (Low *et al.*, 1996). Randomisation in a mutator strain between selection cycles resembles a ribosome display where mutations are produced in each cycle especially in the reverse transcription step.

Table 8. Random recombination by DNA shuffling.

Reference	Diversification	Frame-work	Library size	Selection	Target antigen	Best affinity (Kd)	Affinity gain
Boder *et al.*, 2000	DNA shuffling + further error-prone PCR (Taq polymerase) (repeated between selection cycles)	ScFv	n.d.	Yeast display (off-rate selection)	Fluorescein	48 fM	210x
Jermutus *et al.*, 2001	Error-prone PCR (initial library), DNA shuffling (after each selection)	ScFv	n.d.	Ribosome display (selection in solution with biotinylated antigen, off-rate selection)	Fluorescein	40 nM	30x
Fermer *et al.*, 2004	DNA shuffling (two rounds between selections)	ScFv	10^6	Phage display (selection on microtiter plate, stringent washings)	Progastrin releasing peptide	n.d.	800-900x in-crease in ELISA signal
Graff *et al.*, 2004	DNA shuffling + error-prone PCR (nucleotide analogues, Taq polymerase)	ScFv	$2x10^5$	Yeast display (binding in solution, off-rate selection, cell sorting by flow cytometry)	Carcinoembry onic antigen (CEA)	n.d.	5x improved off-rate
Zahnd *et al.*, 2004	DNA shuffling + Error-prone PCR (dNTP analogues) (repeated between selections)	ScFv	n.d.	Ribosome display (off-rate selection)	Short peptide	5 pM	500x
Luginbühl *et al.*, 2006	DNA shuffling + further Error-prone PCR (dNTP analogues / Taq polymerase), (epPCR repeated between selections)	ScFv	n.d.	Ribosome display (off-rate selection)	Peptide from prion PrP	1 pM	13x
Chodorge *et al.*, 2008	error-prone PCR (initial library), DNA recombination with L shuf-fling (between selections)	ScFv	n.d.	Ribosome display (selection in solution, decreasing antigen concentration)	Human death receptor Fas	0.6 nM	22x

Random recombination by DNA shuffling

Homologous DNA sequences can be recombined *in vitro* by DNA shuffling (Stemmer, 1994a; Stemmer, 1994b). In DNA shuffling, DNA is first randomly fragmented by deoxyribonuclease (DNAse) I to < 50 bp fragments and the fragments are then recombined in subsequent PCR assembly step to a full-sized gene. DNA shuffling has been almost exclusively used to randomly recombine antibody sequences for affinity maturation, but there are also other methods for random *in vitro* recombination that could be exploited, such as ligation-based L-shuffling (Chodorge *et al.*, 2008), random priming recombination (Shao *et al.*, 1998), staggered extension process (Zhao *et al.*, 1998) and degenerate homoduplex recombination (Coco *et al.*, 2002). In addition, shuffled antibody libraries have been generated by homologous *in vivo* recombination in yeast (Swers *et al.*, 2004).

In the original DNA shuffling method, DNA digestion in the presence of Mg^{2+} created single-strand nicks, which made it difficult to control the size of the generated DNA fragments. In an improved method, DNase I digestion is carried out in the presence of Mn^{2+} to generate double-strand breaks (Lorimer & Pastan, 1995). Moreover, additional mutations are produced in DNA shuffling. The number of new mutations depends on the polymerase used in assembly and amplification of the reassembled product (Zhao & Arnold, 1997). The highest mutation rate in DNA shuffling has been obtained using Taq polymerase (Zhao & Arnold, 1997).

DNA shuffling has been used to randomly recombine mutations in affinity-matured antibody clones for further improvement in affinity (Table 8). Typically, the recombined sequences are a pool of mutants enriched from a randomly (e.g. by error-prone PCR) mutated library (Boder *et al.*, 2000; Jermutus *et al.*, 2001; Chodorge *et al.*, 2008). Often DNA shuffling is combined with error-prone PCR amplification of the recombined library to generate additional mutations (Boder *et al.*, 2000; Graff *et al.*, 2004; Zahnd *et al.*, 2004; Luginbuhl *et al.*, 2006), and either error-prone PCR or DNA shuffling can be repeated between selection cycles (Boder *et al.*, 2000; Jermutus *et al.*, 2001; Zahnd *et al.*, 2004; Luginbuhl *et al.*, 2006; Chodorge *et al.*, 2008).

Because new combinations of mutations are formed in DNA shuffling, accumulation of beneficial and elimination of deleterious mutation is allowed, while new potential mutations are produced by error-prone PCR (Boder *et al.*, 2000; Luginbuhl *et al.*, 2006). Therefore, it is rational that recombination of clones enriched from a randomly mutated library has resulted in enrichment of larger proportion of high-affinity variants than selection directly from the random library (Chodorge *et al.*, 2008). A too high mutational load can destroy the functionality of the selected library pool, but repeating homologous recombination can allow additive recombination of positive mutations and lead to functional improvement of such a non-functional pool (Luginbuhl *et al.*, 2006). The largest improvement in affinity achieved by repeated recombination-randomisation cycles have been over four orders of magnitude (Boder *et al.*, 2000).

2.4.3 Targeted mutagenesis for affinity maturation

CDR mutagenesis

In the targeted approach for affinity maturation, mutations are introduced to residues at CDR regions. Mutations are often targeted at the residues that directly contact the antigen or are solvent exposed (Yang *et al.*, 1995). Such residues can be selected by combining previous mutational data (Schier *et al.*, 1996b) with analysis of a crystal structure or a molecular model (Chames *et al.*, 1998; Chen *et al.*, 1999; Barderas *et al.*, 2008) or randomisation can be targeted

to the positions in CDRs that generally interact with antigens (Padlan *et al.*, 1995; MacCallum *et al.*, 1996). Mutations can also be targeted to sites identified as mutational hot spots in affinity maturation *in vivo* (Chowdhury & Pastan, 1999; Ho *et al.*, 2005; Ho & Pastan, 2009). Such germline hot spots are often located nearby or at RGYW DNA sequences (Neuberger *et al.*, 1998). However, in addition to CDR residues, mutations that do not directly contact antigen can also result in enhanced affinity (Hawkins *et al.*, 1993; Chen *et al.*, 1995; Finlay *et al.*, 2009).

In various experiment (Table 9 and 10), randomisation of an individual CDR loop has resulted in 2 – 30 fold improvement in affinity (Yang *et al.*, 1995; Hemminki *et al.*, 1998; Nagy *et al.*, 2002; Brocks *et al.*, 2006; Steidl *et al.*, 2008), but in a single case optimisation of CDR-H2 loop resulted in nearly 300-fold improved affinity (Steidl *et al.*, 2008). In addition to point mutations, an increase in the length of CDR-H2 loop has improved the binding-affinity of hapten-binding antibodies (Lamminmaki *et al.*, 1999; Parhami-Seren *et al.*, 2002).

Scanning saturation mutagenesis, where single residues are substituted with all possible amino acids, provides information about the residues that modulate specificity or are essential for retaining activity and helps to identify substitutions that result in higher affinity (Burks *et al.*, 1997; Chen *et al.*, 1999). Larger sequence space can be studied if several positions are randomised at a time. However, randomisation of only six positions with the 20 different amino acids already produces $6.4x10^7$ variants. Thus, if all the introduced diversity is going to be presented, randomisation should be limited to maximally six residues. Even with such a limited randomisation, degeneration of as few as two key residues results in an inactivation of 99.75 % of the antibody clones. Biasing diversity towards the wild type codon, therefore, helps to avoid the deletorious conditions and preserves the functionality of the library. Such a biased library can be constructed using oligos doped with e.g. 50 % of the wild type codon (Balint & Larrick, 1993; Chames *et al.*, 1998). Diversity can as well be limited by using low-redundancy codons in doping oligos. Such codons present only a limited set of residues e.g. hydrophilic or aromatic amino acids (Balint & Larrick, 1993).

Because randomisation of all possible contact residues in all six CDR loops is not applicable, other semi-random methods have been developed. Affinity on an anthrax toxin neutralising antibody was improved almost 20-fold (to 18 nM) by Massive mutagenesis® (Saboulard *et al.*, 2005). For the library, altogether 73 positions at the six CDRs were randomised with NNS nucleotide bases so that on average 3.5 mutations were produced to each scFv clone. The mutagenesis was based on the synthesis of several 33-mer oligos, each of which contained one randomised position in the middle.

In a more generalised approach entire CDR regions are randomised. For example, in the HuCAL library (Knappik *et al.*, 2000) there are unique restrictions sites flanking each CDR so that separate randomised CDR cassettes can be introduced by simple cloning (Nagy *et al.*, 2002; Brocks *et al.*, 2006).

Additivity effects

Mutations in separately optimised CDR-loops can be additive and combining them can result in further improved affinity (Table 9) (Yang *et al.*, 1995; Schier *et al.*, 1996a; Steidl *et al.*, 2008). However, the additivity effects are unpredictable and an increase in affinity can be less than expected from the individual mutations (Yang *et al.*, 1995) or affinity can even be lowered

Table 9. Examples of antibodies affinity-matured by CDR mutagenesis: Parallel optimisation of CDRs (combining mutations that improve affinity).

Reference	Diversification	Frame-work	Selection of initial muta-tions	Target antigen	Best affinity (Kd)	Affinity gain (expected)
Yang *et al.*, 1995	CDR-H1 + H3 (Parallel optimisation by saturation mutagenesis. Mutations that improve affinity were combined.)	Fab	Phage display (off-rate selection compet-ing with wt Fab)	HIV-1 envelope glycoprotein gp120	22 pM	286x (246x)
	CDR-H1&H3 + L1&L3 (Parallel opti-misation by saturation mutagenesis. Mutations that improve affinity were combined.)				15 pM	1.5x (3.5 x 5.6 = 19.6x)
Ousbourn *et al.*, 1996	V_L + CDR-H3 (V_L from chain shuffling. CDR-H3 optimised by parsimonious mutagenesis. Mutations that improve affinity were combined)	ScFv	Phage display (binding in solution, off-rate selection with competing anitgen)	human carcinoembry-onic antigen (CEA)	0.6 nM	12.8x (2.1 x 5.5 = 11.6x)
Schier *et al.*, 1996b	CDR-H3 (full randomisation of 100A-D) + CDR-H3 (100K-102)	ScFv	Phage display (binding in solution, decreasing anti-gen)	Tumor antigen c-erbB-2	0.13 nM	77x (8.3 x 3.7 = 31)
Chen *et al.*, 1999	CDR-H1 + CDR-H3 + 2 framework mutations	Fab	Monovalent phage display (selection on plate, off-rate incubation with increased time)	VEGF	0.14 nM	22x
Rajpal *et al.*, 2005	Look-through mutagenesis	ScFv	Yeast display (selection in solution)	TNF-α	1 pM	500x and 870x
Steidl *et al.*, 2008	CDR-L3 + CDR-H2 (Parallel optimisa-tion with HuCAL randomisation cas-settes. Mutations that improve affinity were combined.)	Fab	Phage display (binding in solution, increased washing stringency)	granulocyte-macro-phage stimulating factor (GM-CSF)	0.4 pM	5000x (300 x 30 = 9000x)

Table 10. Examples of antibodies affinity-matured by CDR mutagenesis: Sequential optimisation of CDRs (CDR walking).

Reference	Diversification	Framework	Library size	Selection	Target antigen	Best affinity (Kd)	Affinity gain
Barbas *et al.*, 1994	CDR-H1 -> CDR-H3(96-99), Saturation mutagenesis of CDRs (NNK or NNS doping), two-step CDR walk.	Fab	n.d.	Phage display (panning on wells, immobilised antigen)	HIV-1 envelope glycoprotein gp120	0.8 nM	8x
Yang *et al.*, 1995	CDR-H1+ CDR-H3(97-100) -> CDR-L1 -> CDR-L3. Saturation mutagenesis, four-step CDR walk.	Fab	$2\text{-}5x10^8$	Phage display (off-rate selection, competition with the parental Fab)	HIV-1 envelope glycoprotein gp120	66 pM	96x (= 8.2 x 3.5 x 3.3)
	CDR-H1+ CDR-H3(97-100) -> CDR-L3. Saturation mutagenesis, four-step CDR walk.	Fab	$4x10^8$			1.4 nM	46x
	CDR-H1 + H3(H97-100) -> CDR-H3(100A-E).Saturation mutagenesis, three-step CDR walk.	Fab	n.d.			0.10 nM	63x (= 8.2 x 7.7)
Schier *et al.*, 1996b	CDR-L3 (partial randomisation of nine residues) -> CDR-H3 (full randomisation of 100A-D).	ScFv	$0.3\text{-}1x10^7$	Phage display (solution panning, decreasing antigen, stringent washing)	Tumor antigen c-erbB-2	1.1 nM	145x (= 16 x 9.1)
Nagy *et al.*, 2002	CDR-L1-> CDR-L3. Randomisation with HuCAL gold consensus cassettes.	Fab	n.d.	Phage display (solid phase or cell panning, low antigen, ammonium isothiocyanate, increasing number and length of washes, competing antigen)	human leukocyte antigen-DR (HLA-DR)	2.7 nM	130x (= 6 x 20)
Brocks *et al.*, 2006	CDR-H2-> CDR-H3. Randomisation with HuCAL consensus cassettes.	Fab	$1x10^8$	Phage display (solution panning, limiting antigen, stringent washing with competing antigen)	tissue inhibitor of metalloproteinase 1 (TIMP-1)	0.2 nM	10x (= 4 x 2.5)
	CDR-H1+CDR-H2 -> CDR-L3. Randomisation with HuCAL consensus cassettes.	Fab	$1x10^8$			0.15 nM	13x (= 3.3 x 4)

(Schier *et al.*, 1996a; Laffly *et al.*, 2008). Even though significant improvement in affinity can be obtained by combining mutations (Yang *et al.*, 1995; Steidl *et al.*, 2008), generally, a more reliable strategy to improve affinity is obtained by sequential or iterative approaches (Table 10); (Yang *et al.*, 1995). In CDR walking mutagenesis, individual CDR loops are optimised one after the other (Yang *et al.*, 1995). Depending on the number of residues targeted for randomisation in each CDR, one or typically two CDR loops are optimised at a time (Yang *et al.*, 1995; Nagy *et al.*, 2002; Brocks *et al.*, 2006).

Look-through mutagenesis (Rajpal *et al.*, 2005) systematically searches for additive effects. In this method, initially each six CDRs were randomised separately with residues representing the nine major side-chain chemistries. Single mutations were presented in each individual CDR and the libraries were enriched to identify individual mutations improving affinity. These individual mutations were then combined in separate V_H and V_L libraries and the beneficial mutations were finally screened for further additivity in V_H/V_L combinations. The method effectively looked-through the entire binding site and finally resulted in 500 – 870-fold improvement in affinity, generated by 1 – 4 mutations per CDR in four or five CDRs (Rajpal *et al.*, 2005).

Chain shuffling

In chain shuffling, a variable heavy or light chain of a specific antibody is recombined with a complementary variable domain library. Depending on the antibody, chain shuffling can result in loss of binding activity (Kang *et al.*, 1991b; Liu *et al.*, 1999) or generation of several new active combinations (Barbas *et al.*, 1993b), some of which have improved affinity (Marks *et al.*, 1992). Chain shuffling establishes new combinations of the variable domains, and improved clones have been found even when a specific heavy chain has been recombined with the same naïve library where the antibody was originally isolated (Marks *et al.*, 1992; Lu *et al.*, 2003).

Usually heavy chain shuffling is limited to CDR-H1 and H2 (Table 12) (Marks *et al.*, 1992; Schier *et al.*, 1996a), because CDR-H3 is crucial for the antigen-binding. However, there are also exceptions to this paradigm and for a preS1 of hepatitis B virus specific antibody, a light chain was shown to be the most important determinant of affinity and shuffling the light chain with an entire naïve V_H library resulted in an antibody with 6.5-fold improved affinity (Park *et al.*, 2000).

In various experiments, where a specific heavy chain has been recombined with a naïve V_L domain library (light chain shuffling, Table 11), the affinity improvement has been 2 – 30 fold (Marks *et al.*, 1992; Osbourn *et al.*, 1996; Schier *et al.*, 1996a; Lu *et al.*, 2003; Kim & Hong, 2007; Yoshinaga *et al.*, 2008). Often the new, selected chain has been homologous to the original light chain, had identical canonical structures or loop lengths and belonged to the same germline family the parental V gene, even if a specific heavy chain has been recombined with a library containing all the natural sequence and length diversity (Marks *et al.*, 1992; Ohlin *et al.*, 1996; Osbourn *et al.*, 1996; Schier *et al.*, 1996a; Lu *et al.*, 2003). As with the light chain shuffling, in the libraries where the heavy chain has been replaced with naïve V_H segments, the enriched binders have also been derivatives of the same germline gene as the parental V_H (Schier *et al.*, 1996a; Thompson *et al.*, 1996). This indicates co-operativity between the two domains in high-affinity binding (Ohlin *et al.*, 1996).

Table 11. Examples of antibodies affinity-matured by light chain shuffling.

Reference	Diversification	Frame-work	Library size	Selection	Target antigen	Best affinity (Kd)	Affinity gain
Marks *et al.*, 1992	Naïve V_L (κ and λ)	ScFv	$2x10^6$	Phage display (phagemid), (panning on coated tube)	hapten 2-phenoxazol-5-one (phOx)	15 nM	20x
Ousbourn *et al.*, 1996	Naïve V_L	ScFv	$3x10^7$	Phage display (binding in solution, off-rate selection)	human carcinoem-bryonic antigen (CEA)	3.6 nM	2.1x
Schier *et al.*, 1996	Naïve V_L (κ and λ)	ScFv	$2x10^6$	Phage display (binding in solution, de-creasing antigen concentration)	tumor antigen c-erbB-2	2.5 nM	6x
Kim & Hong 2002	Naïve V_L (κ)	Fab	$2x10^7$	Phage display (panning on microtiter plate, increasing washes)	tumor associated gly-coprotein	0.4 µM	2.7x
Kramer *et al.*, 2002b	Group-specific immune V_L library	ScFv	$9.1x10^6$	Phage display (immunoaffinity chroma-tography)	IPR-triazine (herbi-cide)	0.7 nM	12.3x
Lu *et al.*, 2003	Naïve V_L	Fab	$1.5x10^8$	Phage display (panning on immunotube)	vascular endothelial growth factor (VEGF)	0.10 nM	31x
Lee *et al.*, 2004	Synthetic, single-frame-work V_L, covers > 70 % of natural CDR-L1, L2 & L3 diversity	Fab	n.d.	Phage display (binding in solution, de-creasing antigen concentration, off-rate selection with competing antigen)	VEGF	16 pM	60x
Yoshinaga *et al.*, 2008	Naïve V_L	ScFv	$4.3x10^7$	Phage display (panning on plate, exten-sive washing (off-rate); low input & short binding (on-rate)	monocyte chemotac-tic protein-1 (MCP-1)	1.4 nM	~15x
Shi *et al.*, 2010	Synthetic V_L	Fab	10^9	p9 phage display (binding in solution, decrasing antigen concentration)	IgG	45 pM	92x
					Mouse tissue factor	3.6 nM	5.7x

Most of the affinity maturation experiments with chain shuffling, exploit naïve V domain repertoires. However, synthetic diversity has also been used for affinity maturarion (Shi *et al.*, 2010; Lee *et al.*, 2004a), using a strategy similar to light chain shuffling. For example, affinity of an anti-VEGF antibody isolated from a synthetic single framework library was improved 60-fold by randomisation of the three light chain CDRs. The library used a single synthetic framework and the designed diversity at CDR-L1,-L2 and –L3 covered at least 70 % of the natural diversity (Lee *et al.*, 2004a), thus mimicking light chain shuffling.

In addition to affinity, fine-specificity and recognition of the epitope can also be changed by chain shuffling (Lu *et al.*, 2003; Ohlin *et al.*, 1996). As a result, cross-reactivity can be reduced. For example, chain shuffling between a group of binders, specific to tumour associated antigen Lewis Y, generated a new combination of heavy and light chains with optimised affinity and high specificity (Christensen *et al.*, 2009). These chain shuffling experiments have suggested that heavy chains dominate in binding but affinity and specificity can be fine-tuned by light chains (Lu *et al.*, 2003; Christensen *et al.*, 2009). Because the entire V_L domain is changed in light chain shuffling, light chain shuffling is often used also for humanisation of mouse antibodies (Kim & Hong, 2007). Additionally, light chain shuffling has helped to overcome expression problems in bacteria (Rojas *et al.*, 2004).

Table 12. Examples of antibodies affinity-matured by heavy chain shuffling (CDR-H1 & H2).

Reference	Diversification (chain shuffling with)	Frame-work	Library size	Selection	Target antigen	Best affinity (Kd)	Affinity gain
Marks *et al.*, 1992	Naïve V_H1 library	ScFv	$2x10^5$	Phage display (pan-ning on coated tube)	hapten 2-phenoxazol-5-one (phOx)	1.1 nM	15x
Schier *et al.*, 1996a	Naïve V_H1, V_H3, V_H5 library	ScFv	$1\text{-}2x10^6$	Phage display (bind-ing in solution, decreasing antigen)	tumor anti-gen c-erbB-2	3.1 nM	5x
Thompson *et al.*, 1996	Naïve V_H library	ScFv	$1x10^6$	ELISA screening	peptide (HIV loop)	n.d.	2x (k_{off})
Park *et al.*, 2000	Naïve V_H library	ScFv	n.d.	Phage display (pan-ning on microtiter plate, increasing washes)	preS1 of hepatitis B virus	40 nM	6.5x
Kramer *et al.*, 2002b	Group-specific immune V_L library (entire V_H)	ScFV	$1.9x10^7$	Phage display (im-munoaffinity chro-matography)	IPR-triazine (herbicide)	9 nM	1.4x

2.5 Selection of binders from phage displayed libraries

Antibodies against specific antigens can be enriched from phage displayed antibody libraries in a process called biopanning (Fig. 2 and 3). The procedure includes binding the antibody phage to antigen and capture to solid surface, washing to remove unbound and unspecifically bound phage, elution to release the bound phage and amplification of the eluted phage through bacterial infection. Typically 3 – 5 enrichmnet cycles are sufficient to enrich the library (Willats, 2002; Bradbury & Marks, 2004).

2.5.1 Selection platforms

In phage display selections, binding of antibody phage to antigen can be carried out in the solution or at the solid phase. For binding in solution, biotinylated antigen is incubated with the phage, after which the antigen bound phages are captured onto a streptavidin coated surface for example on magnetic beads (Hawkins *et al.*, 1992). For solid phase panning, antigen is first immobilised through biotin onto streptavidin coated surface or antigen is coated directly onto surface of immunotubes (Marks *et al.*, 1991), microtiter plates (Barbas *et al.*, 1991) or polystyrene beads (Zhuang *et al.*, 2001). Immobilisation of protein antigen through passive adsorption can result in denaturation of significant amount of the antigen. Biotinylation is less harsh to the antigen and immobilisation with biotin-streptavidin system is much better than passive adsorption to gain effective antigen concentrations (Zhuang *et al.*, 2001). Antibody can also be blotted onto nitrocellulose (Nakamura *et al.*, 2001) or PVDF membranes (Liu & Marks, 2000), or selection can be carried out in column (Breitling *et al.*, 1991; Kretzschmar *et al.*, 1995). Small molecules such as haptens cannot be directly immobilised onto plastic and they are either conjugated to protein carriers or directly chemically biotinylated. Carbohydrate antigens, however, have been immobilised on plastic plates via lipid anchors (Sakai *et al.*, 2007). Phage display selections have also been carried out on cells (Figini *et al.*, 1998; Krebs *et al.*, 2001), on tissue sections (Sun *et al.*, 2009) and *in vivo* (Krag *et al.*, 2006).

Outcome of a selection trial can be dependent on the platform used, and different clones have been enriched on immunotubes and microtiter wells from the same library (Lou *et al.*, 2001). Immunotubes provide higher surface capacity than microtitration wells. They also accept larger volumes, but because recovery is not efficient from dilute solutions, the reaction volume should be kept small (Kretzschmar *et al.*, 1995). The highest surface/volume ratio is obtained with beads. Hence, in a comparative study, panning on streptavidin coated magnetic beads was more effective for isolating binding phages than panning on polystyrene plates (McConnell *et al.*, 1999). In addition, membranes provide large binding capacity for unmodified proteins. In one study, enrichment on nitrocellulose membrane was found to be almost equal to panning with magnetic strepatividin beads (Nakamura *et al.*, 2001). However, membrane panning has not been much exploited, possibly because membranes are not compatible with affinity selection in solution. It is more likely that membrane panning has potential in proteome-wide selection (Liu & Marks, 2000). For such selections, proteins can be separated from complex biological samples on two-dimensional (2D) gel electrophoresis and blotted onto PVDF (Liu *et al.*, 2002) or nitrocellulose (Furuta *et al.*, 2002) membranes for panning. Selections have also been carried out by using proteins eluted form 2D gel spots as a panning antigen (Pini *et al.*, 1998).

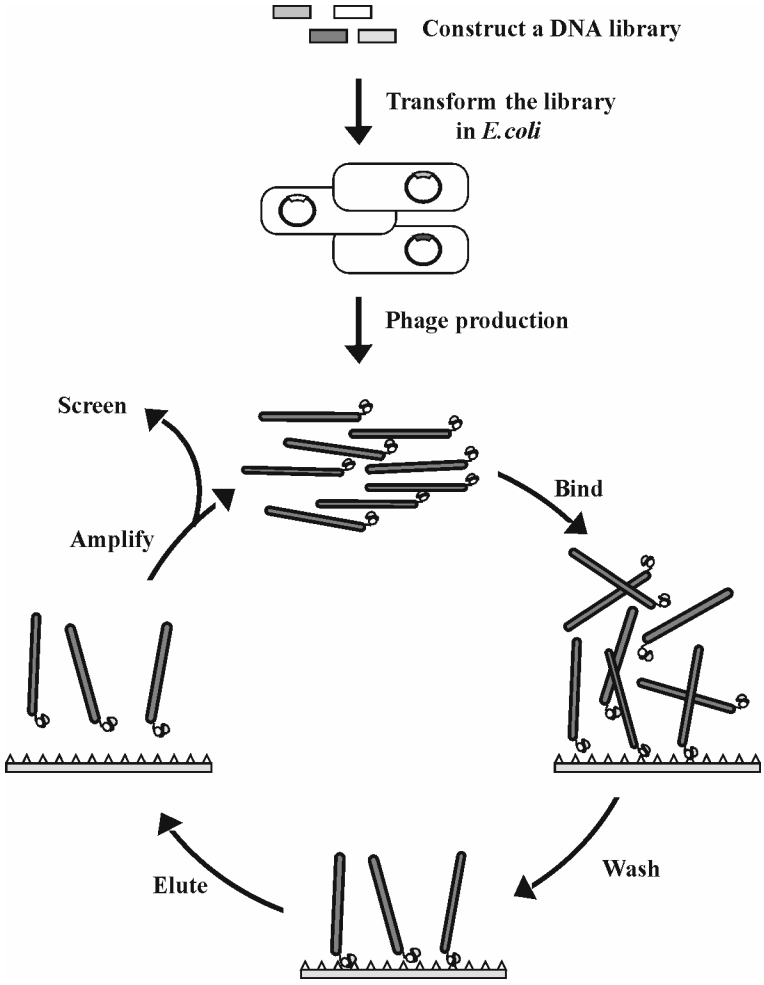

Figure 2. The principle of phage display selection of antibody libraries. A phage displayed antibody library is produced by transforming cloned antibody genes (either synthetic or naïve) in bacteria. Each bacterium receives a single type of antibody gene and produces phage that display that specific antibody. In a biopanning procedure antibodies having binding specificity against a desired antigen are selected by phage display. First, phage are incubated with the antigen in order to capture the specific antibody phage. Then unbound phage are removed by washing after which bound phage are eluted. The eluted phage are rescued by infecting bacterial cells, which then amplify the phage. The enrichment cycle is then repeated with the isolated phage. Usually 3 – 5 biopanning rounds are needed in order to enrich the antibodies that recognise the antigen. Finally, individual colonies are isolated and screened for binding activity.

2.5.2 Controlling background

The amount of background in phage display selection can be significant, especially for the first round when the proportion active clones are still low. Background is caused by unspecific binding of phage to surfaces through their capsid, but also by specific binding of an antibody to surface components other than the desired antigen. Addition of glycerol has helped to reduce

the non-specific binding of phage but it also reduces the specific binding and can slow down the selection. Glycerol can also aid in selection of unstable antibodies and more clones can be selected than without glycerol. However, the selection is biased towards clones that bind with higher affinity only in the presence of glycerol (Kjaer *et al.*, 1998). On membranes, non-specific binding of phage has been blocked by fish gelatine or reduced with 0.5 M NaCl (Liu & Marks, 2000) or drying PVDF membrane with methanol after blotting the protein (Liu *et al.*, 2002). Enrichment of anti-nitrocellulose antibodies while panning on nitrocellulose membrane was overcome by the use of a soluble anti-nitrocellulose antibody fragment (Furuta *et al.*, 2002).

Streptavidin coated surfaces provide a universal method to immobilise biotinylated antigen, but this also contains a risk for the selection of streptavidin binders. The probability of enriching unwanted binders against streptavidin is reduced by pre-selecting the library against streptavidin. However, such a pre-clearing is not 100% efficient and some contaminating binders may still be enriched from the library during subsequent selection rounds (Lu & Sloan, 1999). A more effective strategy to control and prevent the enrichment of the streptavidin binders is alternating selections between streptavidin and plastic surfaces (Lu & Sloan, 1999). Panning can also be altered between streptavidin and avidin surfaces (Hanes *et al.*, 2000). Alternatively, when biotinylated antigen is used, unspecific binders can be omitted completely using a cleavable biotin linker that releases only the antigen bound phage (Fellouse *et al.*, 2007).

2.5.3 Selection of binders from non-immune / universal libraries

In unversal binding libraries, there are only a few specific binders among billions of unspecific phage. Based on theoretical assumptions, it has been estimated that in a universal library, the frequency of antibodies with K_D values of 1 nM, 0.1 nM and 10 pM are 10^{-7}, 10^{-9} and 10^{-11} (Katakura *et al.*, 2004). Thus, one could expect that in a library with a diversity of 10^9 there are 100 binders with 1 nM affinity and one binder with 0.1 nM affinity. Experimental data has shown linear correlation between the library size and the best isolated affinity (Ling, 2003). From libraries with a size of $>10^{10}$, the best isolated antibodies have had subnanomolar affinities (Ling, 2003).

Because of the low number of high-affinity binders in non-immune libraries, it is essential to recover efficiently the rare specific binding clones, especially in the first round of selection. To also capture the low affinity phage in non-immune antibody libraries, the concentration of target molecules should be as high as possible (Kretzschmar *et al.*, 1995; Katakura *et al.*, 2004) and antigen should be tightly immobilised to a solid phase to avoid loss of positive clones (Zhuang *et al.*, 2001). Theoretical and experimental studies suggest using antigen concentration greater than 10 times the Kd (Kretzschmar *et al.*, 1995) or greater than 1 µM and long binding time, e.g. overnight, to force the binding (Katakura *et al.*, 2004). If the concentration of the target antigen is smaller than the presumed Kd, the number of recovered phage are low.

When antigen is used in a selection process in excess over the phage, the amount of bound phage is directly proportional to the input phage (Kretzschmar *et al.*, 1995). Discrimination between different affinities is not achieved under such conditions, but washing allows possibilities for fine-tuning affinity (Kretzschmar *et al.*, 1995). However, it is not only the number of input phages that count, but also the phage concentration should be sufficiently high, because recovery is not efficient from dilute solutions (Kretzschmar *et al.*, 1995).

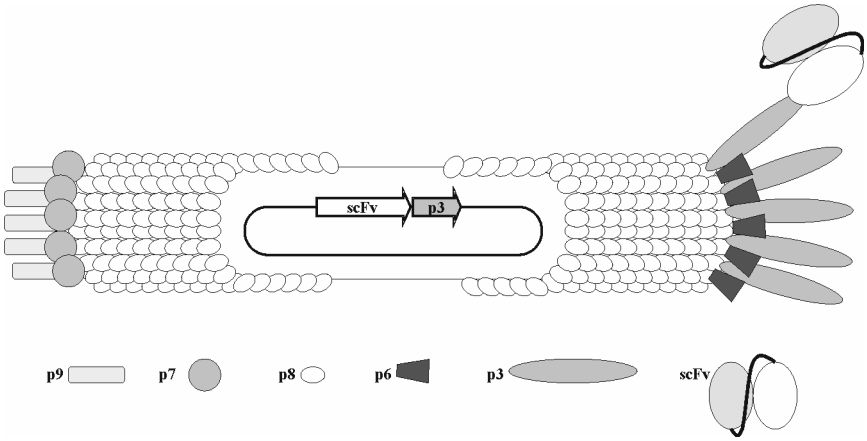

Figure 3. Schematic structure of filamentous M13 phage and the principle of phage display. Filamentous M13 phage is a long threadlike virus of *Escherichia coli*. The phage capsid contains five different surface proteins: five copies of each of the p3 (size 42.5 kDa), p6 (12.3 kDa), p7 (3.6 kDa) and p9 (3.6 kDa) and ~2700 copies of p8 (5.2 kDa). The phage genome, a single-stranded circular DNA, is packed inside the phage. In phage display, a gene of a foreign protein (for example scFv antibody fragment) is cloned into the phage genome and fused to a gene for phage coat protein (for example p3 or p9). The fusion protein encoded by the gene is expressed on the surface of a phage, creating a link between the genotype and phenotype.

Most single-pot antibody libraries use a monovalent display. However, multivalent libraries could be beneficial for the first selection round. In multivalent p3 libraries each antibody is displayed as 3 – 5 copies on the phage and the number of phage presenting antibody is increased by more than two orders of magnitude compared to monovalent phagemid display (Rondot *et al.*, 2001). Thus, the diversity of the library is better represented than by monovalent phagemid display, where only about 1 – 10% of the phage present an antibody (Bradbury & Marks, 2004). A multivalent display also effeciently selects the low affinity binders (Dinh *et al.*, 1996) because avidity slows the off-rate. As a result of using a multivalent display, more antibodies are obtained in selection and less enrichment cycles are needed to isolate binders than with a monovalent display (Rondot *et al.*, 2001; O'Connell *et al.*, 2002). Accordingly, a multivalent display has yielded binders even when selection with a monovalent display has failed (Gram *et al.*, 1992; Ravn *et al.*, 2004). However, as a drawback, the affinity of the binders enriched by multivalent phage display can be lower than those using a monovalent display (Rondot *et al.*, 2001; O'Connell *et al.*, 2002).

Multivalent antibody display can be produced by using either phage vectors which express the antibody solely as a fusion to phage coat protein p3 (O'Connell *et al.*, 2002) or phagemid hyperphage display system, where the p3 gene has been deleted from the helper phage and all p3 is expressed from the phagemid vector as antibody fusion protein (Rondot *et al.*, 2001). In addition to the multivalent vector systems, avidity on binding can be generated by multimerisation of scFv, established by a short linker. Such a multimer has been exploited in selection of anti-carbohydrate antibodies, which often have low affinity (Ravn *et al.*, 2004).

Some synthetic libraries use a bivalent display, which mimics natural immunoglobulins. Bivalent display can be generated by using a dimerisation domain consisting of for example leucine zipper (Lee *et al.*, 2004b; Sidhu *et al.*, 2004; Fellouse *et al.*, 2007). Such a display provides a controlled avidity effect making it possible to select moderate affinity antibodies from naïve repetoires with solid-phase binding. In solution, in contrast, the avidity effect is reduced because the antigen moieties are no longer linked by immobilisation. Thus, a bivalent display also allows affinity maturation of high affinity binders with stringent solution-phase selections that discriminate on the basis of intrinsic affinity (Lee *et al.*, 2004b). Even though long incubations were recommended for the first round of selection (Katakura *et al.*, 2004), binding times as short as 15 min when used in combination with 0.1 nmols of immobilised antigen were successfully used to enrich binders from a bivalently displayed universal antibody library (Fellouse *et al.*, 2007). The subsequent two to three rounds of selection were then carried out in solution to favour monovalent binding. Several antibodies with nanomolar affinities were isolated for numerous targets, proving the functionality of the library, the display and the selection strategy (Fellouse *et al.*, 2007).

2.5.4 Selecting for specificity

Antibodies isolated from binding libraries need to be specific for the desired soluble antigen. Haptens are challenging antigens because of their small size. For immunisation, haptens need to be conjugated to a carrier protein, because haptens are not immunogenic themselves. It is typical that in such immunisation, antibodies are also raised against the protein. If the same hapten conjugated protein is used for phage display selection, the addition of unconjugated carrier protein to the binding buffers can help to prevent enrichment of carrier-specific binders (Barbas *et al.*, 1993a).

For *in vitro* selections, haptens need to be either directly chemically biotinylated or conjugated to a carrier protein. As a result, the isolated antibodies often also recognise part of the biotin linker or the carrier protein and the soluble hapten is recognised less efficiently than the conjugated hapten. In such cases, isolation of antibodies more specific to the soluble hapten than the conjugate can be favoured by eluting the bound phage in the presence of the soluble antigen (Barbas *et al.*, 1992; Chames *et al.*, 1998; Moghaddam *et al.*, 2001). For example, when anti-atrazine antibodies were selected from a phage displayed immune library, binders with > 1000-fold higher specificity to the hapten were found by antigen-specifc elution than by the traditional alkaline elution (Charlton *et al.*, 2001).

In protein antigens there are several epitopes against which antibodies can be selected. Sometimes binders are wanted against a specific epitope. Several methods that direct selection for specific epitopes use monoclonal antibodies to mask the undesired epitopes (Ditzel *et al.*, 1995) or to capture and orient the antigen (Sanna *et al.*, 1995). Antibodies recognising a specific epitope can also be isolated from libraries by competitive elution with a monoclonal antibody directed against the desired epitope (Meulemans *et al.*, 1994). Anti-iditotypic antibodies, which are specific to the antigen-combining site of antibodies, have been similarly selected with competitive elution but using antigen-specific elution (Goletz *et al.*, 2002). Combining antigen elution with subsequent trypsin treatment made the selection more specific. The rationale behind the trypsin treatment is that trypsin cleaved modified p3 originating from a helper phage, rendering the phage without scFv non-infective. Whereas p3 produced from the phagemid as a fusion to scFv was stable (Goletz *et al.*, 2002). Selection can also be directed towards specific epitopes by alternating selections between protein and a peptide (Lu & Sloan, 1999).

Antibodies may also need to be specific in the sense that they do not recognise related antigens. From immune libraries, it is possible to find binders with the desired specificity by screening between the desired antigen and the related antigen that needs to be distinguished. From naïve libraries, specific clones can be isolated by phage display, if the nondesired cross-reacting specificites are selected out using the related molecules as competitors. The competing antigen is provided in solution in excess, after which antibodies are selected for the desired antigen, which is either immobilised or in a biotinylated form (Parsons *et al.*, 1996). The method, known as competitive deselection, has been used to select for example antibodies that bind preferentially foetal haemoglobin instead of adult haemoglobin (Parsons *et al.*, 1996). Addition of free antigen is useful to eliminate cross-reacting antibodies also when selecting for hapten specificity (Malone & Sullivan, 1996; Chames *et al.*, 1998).

On cells and tissues, the number of target antigens is low and there are numerous other epitopes available. When antibody libraries are selected against cell-surface antigens, the undesired specificities are first selected out in so called subtractive panning. For example, when naïve or synthetic antibody libraries are panned against tumour antigens on cancerous cells or tissues, subtractive selection is carried out on related non-tumor cell lines or normal tissue sections (Ridgway *et al.*, 1999; Jarutat *et al.*, 2007).

2.5.5 Selecting for improved affinity

Selecting for high affinity by phage display is based on the observation that high-affinity antibodies enrich more efficiently than low-affinity clones (Barbas *et al.*, 1991). However, selection is a balance between negative and positive forces and also irrelevant clones can be enriched. In addition to affinity, expression level, avidity, toxicity and effect on bacterial growth rate also contribute to the enrichment (Lou *et al.*, 2001). In libraries constructed for affinity maturation, there are typically few high-affinity binders among numerous moderate-affinity binders. Thus, searching for the highest-affinity binders is a challenge and optimal selection strategies are needed. The highest affinity binders can be favoured in selection by decreasing the antigen concentration (Duenas *et al.*, 1996; Persson *et al.*, 2008), using short incubation times (Duenas *et al.*, 1996), extensive washes (Low *et al.*, 1996) or addition of a competing antigen for off-rate selection (Hawkins *et al.*, 1992).

Antibody valency

Monovalent binding favours the selection of the highest affinity binders and is preferred in *in vitro* affinity maturation. When selections are carried out on a solid surface using immobilised antigen, dimeric or multivalent antibodies can bind with higher avidity than monomers, and variations in valency ruin the selection for affinity (Schier *et al.*, 1996a). Phage display (Fig.3.) using phagemid p3 and p9 vectors, produces a monovalent display. However, if 10 % of the phage presents an antibody on the phage capsid as estimated in the p3 system, then probably 0.1 % of the phage displays two antibodies. Unwanted multivalent display can also be caused by multimerisation of scFv fragments through domain swapping. The sensitivity to form multimers is dependent on the length of the linker peptide between V_L and V_H (Dolezal *et al.*, 2000) and the variable domain subgroups (Ewert *et al.*, 2003). Because binding in solution is based on monovalent association, panning in solution more efficiently selects the high-affinity binders than solid phase panning, unless the antigen concentration is very high. Therefore, selection in solution is preferred in affinity maturation over panning on immobilised antigen (Hawkins *et al.*, 1992; Schier *et al.*, 1996a; Thompson *et al.*, 1996).

Selection with decreased antigen concentration

Binding antigen to antibody is an equilibrium reaction, where the amount of antigen-antibody complex formed is determined by the affinity and the concentrations of antigen and antibody. A theoretical model suggests that the highest affinity antibodies in a phage antibody library should be selected if the antigen concentration is < Kd (Hawkins *et al*., 1992). Hawkings (Hawkins *et al*., 1992) used excess of antigen over the phage. They rationalised that antigen excess allows capture of a higher fraction of the phage population than limiting antigen less to phage, and decreases the risk of losing rare high affinity phage. The good yield allowes double rounds of selection between phage growth, which helps to reduce the number of rounds of selection required (Hawkins *et al*., 1992). In another study, selection was carried out using 500 times more phage than Hawkings (i..e. 10^{13} phage), which allowed limiting antigen concentration to less than the phage (Schier *et al*., 1996a). Such a low antigen concentration creates competition for binding and the high-affinity binders are favoured. However, in initial panning rounds an antigen concentration greater than the Kd of the wild type antigen is recommended to also allow capture of rare or poorly expressed antibody clones. Antigen concentration can then be reduced in the subsequent panning rounds to increase selection stringency and push selection towards the highest affinity antibodies (Schier *et al.*, 1996a). When selection with different antigen concentrations has been tested, the greatest enrichment for higher-affinity binders has been obtained by limiting the antigen concentration to less than the phage concentration (typically 10 nM) and the desired Kd (Schier *et al.*, 1996a).

Washings

Affinity is a ratio of the association rate (on-rate) and dissociation rate (off-rate), and improvement in affinity can arise from either faster on-rate or slower off-rate. Typically most of the affinity improvement is contributed by slowed off-rate (Marks *et al.*, 1992) because the washing steps used to remove non-specifically bound phage select for slow dissociation. To select for slow off-rate, the length of washing incubation is more critical than the number of washes, as the degree of dissociation depends on the incubation time. The number of washing operations is important primarily in removing the unbound and non-specific phage (Zhuang *et al*., 2001; Katakura *et al*., 2004). In addition to conventional affinity seletions using beads, plates or immunotubes, phage displayed antibodies that dissociate slowly can also be selected using Biacore (Malmborg *et al.*, 1996).

Off-rate selection

Extensive washings are hardly able to discriminate between binders with affinities at the picomolar range (Zahnd *et al*., 2004). It has also been reasoned that a capture process based on off-rate might be more powerful when selecting for affinity than limiting the antigen concentration (Hawkins *et al.*, 1992). To select for slower off-rate, the phage antibodies are first preloaded with the biotinylated antigen. Then excess of unlabelled antigen is added, and the phage that dissociate are captured by the free antigen, which makes dissociation effectively irreversible. Antibodies with slow off-rate remain bound to the biotinylated antigen and can be captured on streptavidin-coated paramagnetic beads (Hawkins *et al*., 1992). However, addition of free antigen for off-rate selection is not practical with haptens, because it results in enrichment of antibodies that recognise efficiently the biotinylated target, but not the intact hapten (Persson *et al*., 2008).

In addition to the off-rate selection in solution, described by Hawkins (Hawkins *et al*., 1992), free competing antigen has also been added to elute the surface bound phage for off-rate

selection from solid surface (Laffly *et al.*, 2008). Moreover, this method was more efficient in selecting high-affinity antibodies than selection based on limiting antigen concentration (Laffly *et al.*, 2008). However, in the study the improved affinity resulted from an improved association rate constant (k_{on}) and not from a slower off-rate. In the selection, the time of off-rate incubation was longer than the selected off-rate constant (k_{off}), and possibly fast on-rate allowed for rapid rebinding to the same or nearby adsorbed antigen molecules (Laffly *et al.*, 2008).

Because a high amount of free antigen is needed for off-rate incubation and the antigen can be costly, Yang *et al.* (1995) used an alternative off-rate biased approach where the parental antibody acts as a competitor instead of a soluble antigen. In the method, the competing antibody was added following the washing step. Improvements in affinity were dominated by the slowing of the off-rate (Yang *et al.*, 1995). Competitive off-rate selection has also been used with yeast display to select for decreased off-rates of antibodies with picomolar affinities (Zahnd *et al.*, 2004). The amount of competitor antigen needed to inhibit the binding of the scFv fragments back to the surface-immobilised antigen was found to correlate with the mean affinity of binders in the pool and decreases from round to round when binders with slower off-rate are enriched (Zahnd *et al.*, 2004).

The time the competition is allowed to continue is critical in off-rate selection. The efficiency of off-rate selection is improved by extending the time for the off-rate step (Hawkins *et al.*, 1992). If the time is too short, differences between the wild type and a slower-dissociating mutant will be indistinct. However, if the time is too long, antigen is equally lost from both the wild type and mutants (Boder & Wittrup, 1998). A mathematical model made for off-rate selection by yeast display, suggests that the optimal incubation time for off-rate selection is approximately $5/k_{off}$ (Boder & Wittrup, 1998). The model has been exploited in affinity selection of a fluorescein binding antibody with femtomolar affinity by yeast display (Boder *et al.*, 2000).

The longest off-rate incubations used in selection of picomolar affinity binders by yeast and ribosome display have been up to ten days (Boder *et al.*, 2000; Zahnd *et al.*, 2004; Luginbuhl *et al.*, 2006). Further affinity maturation of the picomolar binders would require incubation with the competitor to last for one month, which is at the upper constraint for the methodological limit in yeast and ribosome display. During this time the antibody and antigen should remain stable and resist unfolding and the display scaffold (phage, yeast, ribosome-complex) should remain viable without dividing (Foote & Eisen, 2000; Luginbuhl *et al.*, 2006).

On-rate selection

Association rate of antibody molecule to antigen depends mainly on the diffusion rate of each molecule. When antibody expressed on the surface of a huge filamentous phage binds an antigen immobilised onto a solid surface, the diffusion rate is much slower than when antigen and antibody react in solution (Katakura *et al.*, 2004). Due to the slow diffusion rate, it is difficult to select for improved on-rate. Moreover, the washes needed to remove unbound phage during the selection procedure always bias the selection towards slow off-rate. When both the binding and washing time are long, phage that display antibodies with fast on-rate are eliminated (Katakura *et al.*, 2004). However, also on-rate can be improved (Duenas *et al.*, 1996; Katakura *et al.*, 2004). Short incubation times, short washes, low antigen concentration and reducing the number of input phage favour clones with fast binding (Duenas *et al.*, 1996; Katakura *et al.*, 2004). In yeast display (Boder & Wittrup, 1997), where washes are not needed to separate bound antibodies from non-bound, it is more simple to realise true on-rate selection than with phage display.

Elution

Selection of high affinity binders by phage display is to some extent limited by the need to elute the bound antibody phage. Typically, bound phage have been eluted by the addition of an acid or a base, which are likely to work least efficiently for the highest affinity binders. If elution is not efficient enough, the high-affinity binders can be lost (de Bruin *et al.*, 1999). Because affinity-dependent elution is not needed in yeast (Boder & Wittrup, 1997) and ribosome display (Hanes & Pluckthun, 1997; Schaffitzel *et al.*, 1999), these methods are better suited, on the basis, for selection of high affinity binders. In yeast display, selection is carried out with cell sorting and in ribosome display the ribosome complexes can be dissociated by addition of EDTA. However, there are affinity-independent elution methods also available for phage display.

The affinity-independent elution methods are based on cleavage of a covalent bond either by proteases or DNase or by reduction of a disulfide bond. Some phage display vectors introduce a protease cut site between the phage and antibody, so that bound antibody phage can be released with protease digestion (Ward *et al.*, 1996); (Rondot *et al.*, 2001). In Cys display the antibody is coupled to the phage through a disulfide bridge and affinity-independent elution is achieved by reduction, which cleaves the disulfide bond and releases the bound phage (Rothe *et al.*, 2008). Selection can be also done using streptavidin beads where streptavidin is connected to the beads through a DNA linker that can be cleaved by DNase digestion (Santala & Saviranta, 2004). Moreover, it is also possible to rescue the bound phage by direct infection of bacterial cells, without eluting the bound phage (Wind *et al.*, 1997). Direct infection is most suitable for phage that display the antibody as a fusion with the p9 coat protein (Gao *et al.*, 2002), which is located at the opposite end to the p3 coat protein responsible for the infection.

All these methods elute both phage bound to the antigen and phage bound to other surfaces. More specific elution of only the antigen bound phage is achieved by specific release of the antigen. Using modified streptavidin, nitrostreptavidin, that binds biotin reversibly, antigen can be released by addition of biotin (Balass *et al.*, 1996). Biotin elution is independent of the antibody affinity, and has released antibody phage that were not eluted by acid (Balass *et al.*, 1996). Another option for antigen-specific, affinity-independent elution is available for His-tagged protein antigens (Kobayashi *et al.*, 2009). Such antigens can be immobilised to streptavidin surface through tris-NTA, a high affinity capture reagent for His tags that also contains a biotin moiety. In elution, antigen is released form the surface by imidazol (Koide *et al.*, 2009). Alternatively, antigen can be conjugated with a biotin that contains a disulfide bond in the linker. The linker is cleavable by reducing agents (Fellouse *et al.*, 2007; Koide *et al.*, 2007; Kobayashi *et al.*, 2009). Cleavage of the disulfide bond releases only the antigens, which minimises the enrichment of phage bound to other surface materials.

In addition to the numerous affinity-independent elution methods, selectively infective phage (SIP) selects directly for affinity and specificity with the antigen. In SIP, antibody is expressed on the surface of phage as fused to the C-terminal domain of p3 and there is no wild type p3 in the phage capsid. The antigen is expressed as a fusion with the N-terminal domains of phage coat protein p3, which is responsible for infectivity. Thus, the antibody phage that bind an antigen become infective and can be rescued allowing selection of high affinity clones. Because infectivity is linked to binding, the SIP method is completely free from elution and not even washes are needed to isolate bound phage (Duenas & Borrebaeck, 1994; Duenas *et al.*, 1996; Pedrazzi *et al.*, 1997).

2.5.6 Other options to improve recombinant antibodies by phage display

In addition to isolating new antibodies and optimising the binding-site of recombinant antibodies, phage display technique has been exploited in the selection of binders having improved stability, solubility or expression.

In affinity selection, it is not only the affinity that determines which clones enrich, but also the toxicity to the bacterial host, solubility, folding efficiency and stability may contribute to the outcome of a phage display selection trial (Pedrazzi *et al.*, 1997). Finally, the fittest clones will survive and enrich. The observed tendency of well expressing clones to enrich has been intentionally exploited in the selection of scFv and Fab antibody fragments which have an improved periplasmic expression level (Coia *et al.*, 1997) and/or phage display efficiency (Tuckey & Noren, 2002; Huovinen *et al.*, 2010). In these studies the well expressing clones were selected by using a specific target antigen.

Stable scFv antibody variants have been isolated from phage displayed antibody libraries based on the capability to bind an antigen after the unstable antibody variants have been inactivated by heat or chaotrope (e.g. guanidium chloride, GdmCl) induced unfolding (Jung *et al.*, 1999). In order to also prevent unfolding of the antigen during the capture step, the denaturing pressure needs to be released by lowering the temperature or in the case of chaotrope selection, by diluting the sample. However, as the chaotrope-induced unfolding is often reversible, the denatured antibodies may refold during the capture step, which reduces the stringency of stability selection. Therefore, heat inactivation, which is generally considered to be irreversible, has resulted in the selection of more stable variants than the chemical methods (Jung *et al.*, 1999). The stability of the phage itself sets the upper limits as to how stable antibody fragments can be selected by phage display. With GdmCl there are practically no limits as M13 phage tolerates even 6 M GdmCl. Against heat denaturation, the upper stability limit of M13 phage lies at 60 °C (Jung *et al.*, 1999). Amplification of the isolated antibody phage at 37 °C has also been used to favour the stable scFv variants (Jung *et al.*, 1999). Only those antibody variants that fold well *in vivo* produce functional antibody and will be displayed on the surface of a phage for the next round of selection.

Proteins other than antibody fragments have been selected for improved stability based on resistance against proteases. Protease digestion is always irreversible. If the target protein is expressed between the C-terminal and the two N-terminal domains of the phage coat protein p3, sensitivity to proteases will result in permanent loss of phage infectivity and only the stable variants remain infectious and can propagate (Sieber *et al.*, 1998). This technique is applicable also to proteins other than antibodies as no antigen-dependent capture-step is required. Anyway, the proteolytic selection has not been exploited with antibody fragments, possibly due to the proteolytic sensitivity of the scFv linker sequence and also the long CDR loops in some antibodies (Wörn & Plückthun, 2001). In ribosome display capability to fold under reducing conditions has been used as a basis of stability selection (Jermutus *et al.*, 2001).

Unlike the intrinsically very stable and highly soluble camel and llama single-domain antibodies, human antibody heavy chain variable domains (dAbs) are prone to aggregation. dAbs have been optimised for improved solubility and reduced denaturation-induced aggregation tendency by heating multivalently (on the tip of the phage coat protein p3) displayed dAb phage libraries at 80 °C. Followed by cooling, the folded antibodies were selected based on binding to protein A. Protein A, which binds V_Hs from the human V_H3 family, was expected to bind only those domains that had escaped aggregation and refolded

(Jespers *et al.*, 2004). A more generalised approach, not limited to the ligands of protein A, was designed based on the observation that aggregation of dAbs results in loss of phage infectivity. Hence the soluble clones could be selected by phage infectivity. However, sequential selection for a peptide tag was required to maintain the display level (Famm & Winter, 2006). Moreover, To *et al.* (2005) found that when displayed on the surface of phage, the naturally occurring llama single-domain antibodies produced larger plaques on bacterial lawns than fully human V_Hs with aggregation tendencies. Therefore, they used the plaque size as an identification criteria for monomeric, refoldable human V_Hs (To *et al.*, 2005). In addition to high temperatures, antibody domains also aggregate when exposed to acidic pH at physiological temperatures. Thus, exposure of phage to acidic condition has been used for selection of domains that resist acid aggregation. The isolated dAbs also showed improved thermodynamic stability (Famm *et al.*, 2008).

2.6 Possibilities of recombinant antibody fragments in immunotechnology

Recombinant antibodies can be used in all the applications where monoclonal antibodies are also used: western blotting, immunohistochemistry, immunofluorescence, fluorescence activated cell sorting, inhibition studies. For immunoassays, recombinant antibodies can provide several interesting options and they have several advantages over the monoclonal antibodies and the number of immunoassays using recombinant antibody fragments is increasing slowly but constantly. However, despite the potential of the recombinant antibody fragments, monoclonal antibodies are still the golden standard in immunodiagnostics and it is not known whether recombinant antibodies have been exploited in commercial immunoassays.

One benefit provided by antibody fragments in immunoassays is the possibility to reduce the interference caused, for example, by heterophilic and autoantibodies. Because it is often the Fc portion of antibodies that mediates the interference, use of antibody Fab fragments missing the Fc has helped to overcome some of the interference in immunoassays (Bjerner *et al.*, 2002; Vaisanen *et al.*, 2006). In addition to the proteolytic Fab' fragments, recombinant Fab (Eriksson *et al.*, 2000; Ylikotila *et al.*, 2006) and scFv fragments (Warren *et al.*, 2005) have also helped to reduce the interference. Compared to the Fab' antibody fragments produced by proteolytic digestion from Mab, recombinant antibody technology provides a simple, economical and reliable route to Fab fragments. Recombinant antibody fragments are easy to produce and the recombinant format guarantees constant source of homogeneous antibody, whereas cleavage of monoclonal antibodies by proteases can result in fragmentation at multiple sites. The proteolytic digestion of antibodies requires optimisation and some antibodies can be complicated to be digested. In addition, recombinant antibody fragments also have the option to be engineered further, for example, in order to obtain improved affinity and/or reduced cross-reactivity, which can result in more sensitive and specific immunoassays (Hemminki *et al.*, 1998; Lamminmaki *et al.*, 2003; Siegel *et al.*, 2008).

Another advantage of recombinant antibody fragments is the possibility for site-specific modifications, which do not affect the binding properties but allow oriented immobilisation. Antibody fragments have been immobilised directly onto gold surfaces through a C-terminal thiol group for so called self-assembled monolayers (Vikholm-Lundin, 2005). This method for site-specific orientation is often exploited in surface plasmon resonance (SPR) assays (Lee *et al.*, 2005). Alternatively, antibodies can be site-specifically biotinylated at the C-terminal cysteine residue by a maleimide reagent (Eriksson *et al.*, 2000) for immobilisation onto

streptavidin surfaces, which is commonly used in microtiter plate assays. In addition, it is possible to produce recombinant antibody fragments with fusion tags or other fusion partners to aid in purification, immobilisation or detection. For example, a genetically introduced hexahistidine tag often exploited for affinity puriciaction of the antibody also provides an option for oriented immobilisation of antibody fragments (Vallina-Garcia *et al.*, 2007).

The small size of the antibody fragments allows dense coating, which together with oriented immobilisation maximises the analyte binding capacity (Harma *et al.*, 2000; Peluso *et al.*, 2002; Ylikotila *et al.*, 2005; Ylikotila *et al.*, 2006). Increase in the binding capacity can promote immunoassay kinetics (Ylikotila *et al.*, 2005). In rapid point-of-care type immunoassays the equilibrium is often not achieved and in such cases fast kinetics can decisively improve the performance of an immunoassay (Tarkkinen *et al.*, 2002; von Lode *et al.*, 2003a). Dense coating also allows limiting the size of the analyte-binding surface while still providing sufficient dynamic range for an assay (Ylikotila *et al.*, 2005, Ylikotila *et al.*, 2006). Limiting the analyte-binding surface to the area where the signal is measured in spot assays has resulted in significant improvement in the sensitivity of an immunoassay (Ylikotila *et al.*, 2005). Indeed, the dense and oriented coating available for recombinant antibody fragments is especially beneficial in miniaturised and multiplexed immunoassays, where the coated surface area is very limited (Peluso *et al.*, 2003; Steinhauer *et al.*, 2006). Antibody-based microarrays are examples of such assays. They enable multiplexed protein expression profiling and are a novel and rapidly emerging technology in the proteomics field (Dexlin *et al.*, 2007; Ingvarsson *et al.*, 2007; Carlsson *et al.*, 2008).

Producing antibody arrays sets up a huge need for antibodies. Several of these arrays currently use recombinant scFv fragments (Dexlin *et al.*, 2007; Ingvarsson *et al.*, 2007; Carlsson *et al.*, 2008), which can be readily isolated from large universal antibody libraries such as the n-CoDeR (Soderlind *et al.*, 2000) or the HuCAL (Knappik *et al.*, 2000) antibody libraries. Automated panning and screening procedures developed for phage displayed antibody libraries provide an efficient route for high-throughput generation of new antibodies (Krebs *et al.*, 2001; Schofield *et al.*, 2007), and there are ongoing efforts to generate antibodies against the entire human proteome (Taussig *et al.*, 2007; Uhlen & Hober, 2009).

Universal recombinant antibody libraries are an interesting source of new antibodies particularly because of the possibility for obtaining antibodies against self-antigens, toxic and non-immunogenic antigens, which have been difficult targets with the traditional immunisation based methods (Griffiths *et al.*,1993; Griffiths *et al.*, 1994). Moreover, selection can be targeted towards the desired epitopes (Parsons *et al.*, 1996) and antibodies have even been isolated for immunocomplexes (Raats *et al.*, 2003a; Pulli *et al.*, 2005). Obtaining antibodies against immunocomplexes would be difficult by the immunisation-based methods due to the self-antigenicity of antibodies and low stability of the immunocomplex.

Antibodies against immunocomplexes have established development of non-competitive immunoassays for small molecules (Raats *et al.*, 2003a; Pulli *et al.*, 2005). Non-competitive immunoassays are usually more sensitive and provide more wide working range than competitive assays. However, haptens have typically been analysed by competitive assays because they are too small to be recognised simultaneously by two different antibodies, which is a requirement for a non-competitive sandwich-type immunoassay. Open-sandwich immunoassay is another novel type of non-competitive immunoassay, established by the use of recombinant antibody fragments. The assay uses Fv antibody fragments and the assay principle

is based on the phenomenon that antigen promotes the association of separated V_H and V_L domains (Ueda *et al.*, 1996; Suzuki *et al.*, 2000). Despite being an interesting approach, open-sandwich immunoassays may lack sensitivity due to the natural association between the variable domains, which causes high background signal. Therefore, mutagenesis of the V_L/V_H interface may be needed to weaken the intrinsic domain interaction (Ihara *et al.*, 2009).

For use as labelled reagents, recombinant antibody fragments can be chemically labelled at amino groups, a method commonly used for conjugation of monoclonal antibodies. However, random labeling at multiple sites may not be optimal for antibody fragments because due to their small size, there is an increased risk of inactivating the antibody. Inactivation can be avoided by the use of the site-specific labelling techniques available for recombinant antibody fragments. Labelling at the C-terminal cysteine residue (Eriksson *et al.*, 2000) produces antibodies with a single label and unchanged binding properties. As a drawback, low specific activity in such a single-label antibody may limit the sensitivity of an immunoassay. In addition to the chemical labelling techniques, recombinant antibody fragments also have the option to be produced as fused to fluorescent proteins (such as green fluorescent protein (GFP) and its colour variants) (Arai *et al.*, 2000; Arai *et al.*, 2001) or to enzymes that produce light (renilla luciferase; Arai *et al.*, 2001) or a visible colour change (alkaline phosphatase) (Suzuki *et al.*, 2000). These proteins can act as direct labels in enzyme-linked immunosorbent assays (ELISA) or fluorescence linked immunosorbent assays (FLISA). In addition, small peptide tags genetically introduced to recombinant antibodies can be detected by a second, chemically labelled antibody.

Expression of antibody fragments as fused to different GFP variants allows their direct use in fluorescent resonance energy transfer (FRET) assays (Arai *et al.*, 2000; Arai *et al.*, 2001; Ohiro *et al.*, 2002). In FRET assays binding an antigen brings an acceptor and donor label close to each other and creates the proximity needed for energy transfer. As an example, antibody-GFP and enhanced yellow fluorescent protein (EYFP) fusions have been used in FRET assays where simultaneous binding of two antibodies to different epitopes in a protein created the proximity needed for FRET (Ohiro *et al.*, 2002). The engineering possibilities of recombinant antibodies allowed even further advantages for the assay: Use of leucine zippers in the GFP variant tagged proteins significantly enhanced the FRET even when the antibodies bound to distant epitopes in a large protein (Ohiro *et al.*, 2002). FRET has also been exploited in open sandwich immunoassays where the V_L and V_H domains are produced as fused to different reporter proteins. Unfortunately, development of GFP based assays may be limited by the cytoplasmic expression needed for GFP. Expression of scFv fragments may be difficult in cytoplasm, because the formation of disulfide bonds is not supported there. Therefore, the protein may need to be purified by refolding from inclusion bodies (Sakamoto *et al.*, 2010). Sometimes direct chemical conjugation between GFP and antibody Fab fragment has provided better performance in FRET assays than the use of a scFv-GFP fluobody (Ohiro *et al.*, 2007).

Recombinant Fab fragments have also been used in luminescent resonance energy transfer (LRET) assays where Eu nanoparticles were coated with the antibody and binding an acceptor labelled antigen created the proximity needed for energy transfer. As the efficiency of energy transfer is dependent on the distance between the two molecules, it has been reasoned that Mabs would be less suitable than Fab fragments for the assay due to their large size and bivalency. With Mabs the distance between the antigen binding site and the surface of the particle may be too long for efficient energy transfer. Mabs also have two binding sites close to each other, so

the areas were the energy is transferred always overlap, which reduces the sensitivity of the assay compared to an assay using Fab coated beads (Kokko *et al.*, 2004).

3 AIMS OF THE STUDY

The aim of the present study was to develop new and improved binding proteins for bioaffinity assays. In the study, *in vitro* evolution techniques were exploited, including various diversification methods for the construction of libraries and selection of the libraries by phage display. The methods were used to improve expression, phage display, stability and binding properties of antibody fragments and an alternative binding scaffold. The optimised binders were used in immunoassays.

More specifically the aims were:

I To establish phage display of a TIM barrel protein dihydropteroate synthase (DHPS) for use as an alternative binding framework.

II To improve broad-specificity of a group-specific antibody for detection of sulfonamides at the level of legal maximum residue limit (MRL, 100 µg/kg in EU).

III To improve the stability of an anti-thyroid stimulating hormone (TSH) scFv antibody fragment to better suite an immunoassay.

IV To improve reaction kinetics of a point-of-care assay for TSH by use of recombinant antibody fragments and characterise the performance of the assay.

V To generate new high-affinity binders from a synthetic antibody library.

4 SUMMARY OF MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in this study can be found in the original publications (**I-V**). A summary with some additional information is presented here.

4.1 Reagents

4.1.1 Antibodies, antigens and immunoassay reagents

Monoclonal anti-DHPS antibodies 2H5, 3G4, 4G7 and an anti-M13 antibody were produced at the University of Turku, Department of Biotechnology. Monoclonal anti-TSH antibodies 5404 and 5409 were obtained from Medix Biochemica (Kauniainen, Finland). Monoclonal anti-bacterial alkaline phosphatase (phoA) antibody BAP-77 was purchased from Sigma (USA). Mabs were purified with protein G (GE Healthcare, Sweden) and biotinylated at the ε-amino groups of lysine residues with isothiocyanate (BITC, Mukkala *et al.*, 1993) or labelled with Eu(N1)-chelate (Mukkala *et al.*, 1989) or 9-dentate α-galactose Eu^{3+} chelate (von Lode *et al.*, 2003b).

Cloned dihydropteroate synthase (DHPS, EC 2.5.1.15; (Dallas *et al.*, 1992)) gene was kindly provided by Walter S. Dallas (Glaxo Wellcome, North Carolina, USA). Anti-sulfonamide scFv A.3.5 (Korpimaki *et al.*, 2002; Korpimaki *et al.*, 2003) had been previously engineered at the Univeristy of Turku (Finland) for improved affinity and broad-specificity from a cloned monoclonal antibody 27G3. The monoclonal antibody 27G3 (Haasnoot *et al.*, 2000a) was originally made by Willem Haasnoot (RIKILT-DLO, Netherlands). The cDNA for the monoclonal anti-TSH antibody 5404 was kindly provided by Medix Biochemica (Finland).

DHPS, TSH (Scripps Laboratories, USA), lysozyme (Sigma), Pregnancy associated plasma protein A (PAPP-A) (Sivanandam *et al.*, 2004), cyclomaltodextrin glucanotrasnferase (CGTase) (Hellman *et al.*, 1990) and chymotrypsinogen A (Sigma) were biotinylated at amino gropus with biotin isothio cyanate (BITC) (Mukkala *et al.*, 1993). Sulfonamides were from Sigma (USA). Biotinylated sulfamethazine was synthesised as described (**II**).

Regular and high capacity streptavidin microtitration plates and rabbit anti-mouse IgG plates were produced either at Innotrack Diagnostics (Turku, Finland) or Kaivogen (Turku, Finland). DELFIA® assay buffer, wash buffer and enhancement solution (PerkinElmer Finland, Turku, Finland) were used in immunoassays and time-resolved fluorescence from Eu^{3+} was measured with Victor plate reader (PerkinElmer Finland). The Aio assay buffer and Aio immunoanalyser were from Innotrack Diagnostics.

4.1.2 Bacterial strains, vectors, helper phage

Bacterial strains used were *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^r)]] (Stratagene, USA), SS320 (MC1061 F') [*hsdR mcrB araD139 (araABC-leu)7679 lacX74 galUgalK rpsL thi* F'[*proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^r)]] (Sidhu *et al.*, 2000) and K12 CJ236 F∆*(HindIII)::cat* (Tra^+ Pil^+ Cam^R)/ *ung-1 relA1 dut-1 thi-1 spoT1 mcrA* (New England Biolabs, USA).

The vectors used are summarised in Table 13. Phagemid vectors pAK100, pAK200, and pEB91 were used as for monovalent phage display with helper phage VCS M13 (Stratagene, USA) and pAKp3fl for multivalent phage display helper phage KO7ΔpIII (Progen, Germany) (Rondot *et al*., 2001). Amber stop codon used in vectors pAK100 and pEB91 was to reduce the display of the target protein on the phage to guarantee truly monovalent phage display.

Table 13. Summary of the vectors.

Vector	Vector properties	Origin	Reference	
pAK100	monovalent phage display with VCS M13 helper phage, fusion with phage $p3^{CT}$, myc tag, amber stop	A.Plückthun, University of Zürich, Switzerland	Krebber *et al.*, 1997	(I)
pAK200	monovalent phage display with VCS M13 helper phage, fusion with phage $p3^{CT}$	A.Plückthun, University of Zürich, Switzerland	Krebber *et al.*, 1997	(I)
pAKp3fl	multivalent phage display with KO7ΔpIII helper phage fusion with full length phage p3, trypsin cut site	University of Turku, Finland	Korpimaki *et al*., 2004a	(II)
pEB91	monovalent phage display with VCS M13 helper phage fusion with phage p9, myc tag, amber stop,	University of Turku, Finland	Manuscript Brockmann *et al.*	(V)
pAK300	periplasmic expression, His6 tag	A.Plückthun, University of Zürich, Switzerland	Krebber *et al.*, 1997	(III)
pAK3FC	periplasmic expression, CysProHis6 tag, FkpA folding modulator	University of Turku, Finland	Korpimaki *et al*., 2004b	(III)
pAK100CL	periplasmic expression, fusion with mouse C_L, His6 tag	University of Turku, Finland	Korpimaki *et al*., 2002,	(II)
pAK600	periplasmic expression, fusion with bacterial alkaline phosphatase, dimeric	A.Plückthun, University of Zürich, Switzerland	Krebber *et al.*, 1997	(V)

Vectors pAK300 and pAK3FC were used for soluble expression of intact antibody fragments. His tag from these vectors allowed affinity purification using Ni-NTA matrix (Qiagen, Germany). Vector pAK3FC introduced a C-terminal cysteine residue, which was exploited in site-specific biotinylation with maleimide PEO_2-biotin (Pierce, USA). Vector pAK3FC also expressed a folding modulator FkpA for improved soluble expression of antibody fragments (Bothmann & Pluckthun, 2000).

Vectors pAK100CL and pAK600 were used for screening antibody clones. In vector pAK100CL, scFv fragments were produced as a fusion to mouse C_L domain for binding to the rabbit anti-mouse antibody (RAM) coated plate. In vector pAK600, scFv was expressed as fused to bacterial alkaline phosphatase (phoA), which established capturing by anti-phoA antibody and direct measurement of the expressed protein concentration by phoA activity.

All vectors had IPTG inducible Lac promoter and gene for chloramphenicol resistance. Protein was directed to periplasm of *E.coli* by pelB signal sequence.

4.2 Diversification of the target genes and construction of libraries

4.2.1 DHPS (I)

In order to make a cysteine-free DHPS variant, the cloned DHPS gene was mutated at the cysteine residues (C137, C172 and C242) by using oligonucletotide directed PCR and assembly by splicing by overlap exprension polymerase chain reaction (SOE-PCR) (Horton *et al.*, 1989). The resulting libraries were cloned in vector pAK100. Each of the cysteine residues was mutated to four other residues and two libraries with a total diversity of 64 were constructed. In the first library, the cysteines were mutated to threonine, alanine, valine and isoleusine. The second library was otherwise similar, but C172 was mutated to serine, alanine, valine and methionine.

A cysteine-free DHPS was mutated further by error-prone PCR (Fromant *et al.*, 1995), which resulted, on average, in 4 amino acid mutations per DHPS subunit. The constructed libraries are summarised in Table 14.

4.2.2 Anti-sulfonamide scFv (II)

The anti-sulfonamide scFv A.3.5 was diversified by targeted oligonucleotide directed mutagenesis by using SOE-PCR (Horton *et al.*, 1989). Diversified positions were selected based on a modelled antibody structure and analysis of mutated sites in previous engineering projects (Korpimaki *et al.*, 2002; Korpimaki *et al.*, 2003). Four libraries were constructed, in each of which two CDR loops were targeted for diversification (Table 14).

4.2.3 Anti-TSH scFv (III)

The monoclonal anti-TSH antibody 5404 (Medix Biochemica, Finland) was cloned into a recombinant scFv fragment and randomly mutated by error prone-PCR with Taq DNA polymerase (Cadwell & Joyce, 1992; Fromant *et al.*, 1995). The mutated variants that were still able to bind the TSH were collected by phage display and recombined by DNA shuffling (Stemmer, 1994a). The resultant scFv library had, on average, 2.5 amino acid point mutations per scFv (Table 14).

Table 14. Summary of the constructed libraries.

Library	Vector / Helper phage	Diversification	Designed diversity	Library size	
DHPS	pAK100 / -	Targeted mutagnesis of Cys (C137T/A/V/I, C172T/A/V/I, C242T/A/V/I)	64	n.a.	(I)
DHPS	pAK100 / -	Targeted mutagnesis of Cys (C137T/A/V/I, C172S/A/V/M, C242T/A/V/I)	64	n.a.	(I)
DHPS	pAK200 / VCS M13	Error-prone PCR (4 random amino acid mutations / DHPS)	Equal to library size	4.5×10^7	(I)
Anti-sulfonamide scFv (Lib1)	pAKp3fl / KO7ΔpIII	Targeted mutagnesis of CDR-L2 + CDR-H3	1.5×10^8 (6.4×10^9)	2.0×10^7	(II)
Anti-sulfonamide scFv (Lib2)	pAKp3fl / KO7ΔpIII	Targeted mutagnesis of CDR-L1 + CDR-L3	4.5×10^8	3.2×10^7	(II)
Anti-sulfonamide scFv (Lib3)	pAKp3fl / KO7ΔpIII	Targeted mutagnesis of CDR-L1 + CDR-L2	1.5×10^{11}	7.6×10^6	(II)
Anti-sulfonamide scFv (Lib4)	pAKp3fl / KO7ΔpIII	Targeted mutagnesis of CDR-H1 + CDR-H2	1.8×10^6	1.5×10^7	(II)
Anti-TSH scFv	pAK100 / VCS M13	Error-prone PCR + DNA shuffling (2.5 aa mutations / scFv)	Equal to library size	4.0×10^6	(III)
Synthetic human scFv library (mbLib)	pEB91 / VCS M13	Targeted mutagnesis of CDR-L1, L3, H1 , H2, H3 (18 CDR-H3 loop lengths, trinucleotide oligos)	1.2×10^{22}	3.1×10^{10}	(V)
Synthetic human scFv library (amLib)	pEB91 / VCS M13	Targeted mutagnesis of CDR-L1, L2, L3	1.8×10^9	4.8×10^9	(V)

4.2.4 Synthetic human antibody library (V)

A synthetic scFv gene was designed and ordered from Entelechon (Germany). The scFv consisted of a single human V_L (kappa, subgroup III) and V_H (subgroup III) combined through a 20 amino acid long $(Gly_4Ser)_4$ linker sequence between the domains. CDR-loops L1, L3, H1, H2 and H3 were diversified for a primary binding library (mbLib) and CDR-loops L1, L2 and L3 for another library (amLib) intended for affinity maturation (Table 14). CDR-loops L1, L3, H1 and H2 were diversified sequentially using randomising oligos in Kunkel mutagenesis (Kunkel, 1985; Sidhu *et al.*, 2000). Unmutated clones were eliminated after each mutagenesis step by digestion with a restriction enzyme, the site of which was lost from the template as a result of mutagenesis. Finally, CDR-H3 was mutated by SOE-PCR.

For the light chain amLib library, CDR-L2 and L3 were diversified using oligos that contained overhangs for BspQI (a IIs type restriction enzyme). Whole plasmid was amplified using two oligos. The product was digested with BspQI and selfligated to circular plasmid. CDR-L1 was diversified thereafter using Kunkel mutagenesis (Kunkel, 1985; Sidhu *et al.*, 2000).

Lysozyme binders enriched from the primary library were affinity matured by light chain shuffling. For this, V_H of the enriched clones was recombined with the amLib light chain library.

4.3 Selection of binders by phage display (I, II, III, V)

Phage display selections were carried out by using biotinylated antigen and streptavidin coated microtiter wells (**II**), magnetic M280 Dynabeads (Invitrogen, USA) (**I, V**) or CELLection beads (Dynal, Norway) (**III**) where streptavidin is connected to the bead through a DNA linker. After binding the phage, beads were washed 4-5x and bound phage were eluted.

DHPS mutants with improved phage display efficiency and periplasmic expression were selected by binding to a biotinylated monoclonal anti-DHPS antibody. Bound phage were collected using M280 streptavidin beads and eluted with 100 mM HCl, followed by neutralisation with 2M Tris pH 7.5 (**I**).

Broad-specificity of a sulfonamide binder was improved by affinity selection using one of the weakest binding sulfonamides, sulfamethazine, as a capturing antigen. Multivalent phage display, selection on microtiter wells and elution with trypsin digestion were used (**II**).

ScFv fragments with improved stability were selected by collecting the phage that were able to bind biotinylated TSH after a denaturing treatment (heat or GdmCl inactiavation under reducing conditions, in the presence of 1 mM dithiotreitol, DTT). TSH bound phage were then collected by CELLection beads and eluted with DNase I digestion at a DNA linker in the beads (**III**).

Novel binders were selected from the mbLib library by using a monovalent p9 phage display and M280 beads saturated with the biotinylated antigen. For the first round of selection, phage 100-fold over the diversity of the library were incubated o/n with 50 µl (~$3x10^7$ beads) of the antigen coated streptavidin beads. For the 2^{nd} etc round, subtractive panning against streptavidin was included. The amount of phage and beads was reduced tenfold and incubation time was shortened to 1 – 2 h. In selection for improved affinity, the amount of antigen was reduced or off-rate selection was tried. In off-rate selection the excess of competing antigen was added

after unbound phage were removed by washing. Bound phage were eluted by 100 mM HCl and additionally, uneluted phage were infected by direct addition of bacterial cells. Selection by binding antigen and phage in solution was compared with binding phage to beads saturated with the antigen (**V**).

Eluted phage were propagated in *E. coli* XL1-Blue cells. After infection with helper phage VCS M13 (**I, III, V**) or KO7ΔpIII (**II**) cultures were induced with 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) and phage were produced o/n at 26 °C, 250 rpm. Phage were isolated from culture supernatant by double precipitation with 4% polyethylene glycol (PEG) 8000/3% NaCl. Enrichment in phage display selections was observed as increase in output/input and/or increase in binding activity in phage immunoassay. Selection was repeated for 3 – 4 cycles.

4.4 Characterisation of binders

4.4.1 Cultures for screening clones (I-III,V)

In order to screen individual clones for activity, phage clones were produced separately on a 96 well culture plate (á 200 – 300 µl SB, 0.05 % glucose, 10 µg/ml tetracycline, 25 µg/ml chloramphenicol). Cultures were infected with helper phage, induced with 100 µM IPTG and phage were produced o/n at 26 °C, 700 rpm. Culture supernantats were analysed for binding activity. (**V**). From larger 5 – 20 ml cultures phage where purified by precipitation with PEG/NaCl (**I, III**).

To screen individual clones for binding in soluble format, scFv was cloned in vector pAK100CL (anti-sulfonamide scFv (**II**), or pAK600 (synthetic scFv (**V**)). In order to measure the expressed protein concentration, DHPS was produced in a soluble format directly from the vector pAK100 (**I**) and anti-TSH scFv from the vector pAK300 (**III**). Following induction with 100 µM IPTG, clones were produced o/n at 26 °C, 300 rpm. Culture supernatants were analysed directly (**II, III**) or periplasmic protein was released from cells by incubation with lysozyme-containing lysis buffer (**I, III**) or by ultrasound sonication (**V**).

4.4.2 Measurement of the expressed protein concentration (I, II)

Concentration of DHPS was measured in a two-site (sandwich) immunoassay where DHPS was first captured with a biotinylated monoclonal antibody (3G4) onto a streptavidin surface, after which the bound DHPS was detected with a second monoclonal (4G7) antibody labelled with Eu(N1). Purified DHPS was used as a standard (**I**).

Concentration of eanti-TSH scFv was measured by a competitive immunoassay. Biotinylated scFv was bound into a streptavidin microtitration well, and made to compete with the sample scFv for binding Eu(N1)-labelled TSH. Purified anti-TSH scFv or Fab was used as a standard for quantitation of the scFv concentration in samples (**III**).

4.4.3 Measurement of the purification yields (unpublished)

Anti-TSH scFv antibody fragments containing a C-terminal cysteine residue for site-specific biotinylation and a heksahistidine tag for purification were expressed in 1l flask cultures as described (4.4.1). The periplasmic protein was released by extraction with cold Tris-EDTA solution and purified by cation excange chromatography (SP Sepharose FF, GE Healthcare) and

hisitidine affinity (Ni-NTA, Qiagen, Germany) chromatography. The scFv fragments were biotinylated with maleimide-PEO2 biotin (Pierce, USA) while bound to the Ni-NTA column. Unbiotinylated scFv was removed by a SoftLink avidin column (Promega, USA). The purification yield was measured as described (4.4.2)

4.4.4 Measurement of phage display efficiency (I)

Phage display efficiency of DHPS was measured by a sandwich immunoassay. In the assay, phage that displayed DHPS were captured in a streptavidin well by a biotinylated conformation specific anti-DHPS Mab 2H5 or 3G4, or a conformation-independent 4G7. Bound phage were detected by a Eu(N1) labelled monoclonal antibody (9E7) which binds to the capsid of M13 phage. Phage display efficiency was estimated as a ratio (S/B) between specifically to DHPS and unspecifcally (to well without DHPS) bound phage.

4.4.5 Measurement of stability (III)

Stability of anti-TSH scFv fragments was measured as residual binding activity of phage after heating (2h at 4 – 60 °C) or exposure to chaotrope guanidium chloride (1h, 0 – 5M GdmCl). DTT (1 mM) was added to samples to prevent refolding of the GdmCl treated scFv after removing the denaturation pressure. Residual binding activity was measured by an immunoassay where scFv is bound to biotinylated TSH in a streptavidin well. Bound phage were then detected by a Eu(N1)-labelled anti-phage antibody (9E7 Mab). The stability of the phage particles was analysed based on the capability to infect XL1-Blue cells after the various treatments.

Chemical stability of the scFv was analysed more precisely by spectrofluorometry. For this, scFv was expressed from vector pAK300 and purified to homogeneity using SP sepharose FF cation exchange chromatography (GE Healthcare, Sweden) and Ni-NTA affinity purification (QIAGEN, Germany) followed by another cation exchange using Resource S column on ÄKTA Explorer (GE Healthcare). To measure the stability, scFv was incubated o/n at 10 µg/ml in 0 – 5 M GdmCl. The fluorescence emission after excitation at 280 nm was recorded at 300 – 400 nm with Cary Eclipse spectrofluorometer (Varian Instruments, Walnut Creek, CA). Unfolding of the scFv caused shift in the peak maximum of the emission spectra.

4.4.6 Measurement of a cross-reactivity (II)

Cross-reaction profiles of the anti-sulfonamide scFv mutants for 13 different sulfonamides were analysed by measuring an IC50 value in a competitive immunoassay. In the assay, scFv expressed as a fusion protein into mouse C_L was bound (from culture supernantant or crude cell lysate) to rabbit anti-mouse IgG (RAM) plate. Eu-labelled sulfamethazine was then added together with a competing sulfonamide. IC50 value was the concentration of competing sulfonamide that was needed to inhibit 50 % of the binding of the labeled sulfamethazine.

4.4.7 Measurement of affinity (V)

The affinity of scFv fragments was measured by Scatchard analysis. For this, scFv was expressed as scFv-phoA fusion protein and captured from a sample into microtiter well by a biotinylated anti-bacterial alkaline phosphatase Mab BAP-77 (Sigma). A series of concentrations of Eu-labelled antigen were incubated for 2h, after which the signal from the bound label was measured. The amount of bound and free antigen at the binding equilibrium

was calculated from the measured signal and added reagent concentrations. Kd was the (-1/ slope) in a plot of Bound/Free-ratio vs Bound (M) antigen.

4.4.8 Evaluating immunoassay performance (IV)

The coating capacity of streptavidin wells for the biotinylated anti-TSH scFv, Fab and Mab was measured by adding a series of antibody concentrations to the wells. Saturation of the wells was followed measuring the amount of unbound antibody on another plate. Bound antibody was detected with a Eu-labelled antigen. Binding capacity of the saturated SA wells to TSH was calculated from the maximum signal obtained for a mixture of labelled and free TSH saturating the wells.

Antigen binding kinetics in the saturated antibody wells at 36 °C was measured by following the signal accumulation for Eu-TSH, in a two-step immunoassay and in a one-step immunoassay. In two-step assay the antigen binding step was followed by a separate incubation with labelled second antibody (5409 Mab). In the one-step assay the analyte and the labelled antibody are incubated simultaneously.

High-capacity all-in-one dry assay wells were produced by coating low fluorescent (yellow) glutaraldehyde cross-linked streptavidin (GA-SAv) wells with a saturating amount of the biotinylated capture antibody (scFv, Fab or Mab). ScFv and Fab were biotinylated site-specifically from a C-terminal Cys-residue and Mab randomly at NH_2 group. An insulation layer consisting of sugars and proteins was dried over the coated surface and tracer antibody (Eu-9dGal-labelled 5409 Mab) was dried as a 1 µl drop on top of the insulation layer. The wells were used in a 5-min one-step, two-site TSH immunoassay run on Aio immunoanalyser (Innotrack Diagnostics, Turku, Finland). Aio assay buffer (25 µl) and sample (50 µl of calibrator or patient serum) was applied to the well by the instrument, incubated 5 min at 36 °C, washed six times and dried. Time-resolved fluorescence was measured from the bottom of the well surface. The analytical sensitivity, the high-dose hook effect, the within-run and between-run precision, recovery, cross-reactivity and correlation to a commercial AutoDELFIA hTSH Ultra Assay (sensitivity <0.005 mIU/L, PerkinElmer, Finland) were all investigated.

5 SUMMARY OF RESULTS

5.1 Phage display of DHPS (I)

Dihydropteroate synthase (DHPS) from *E. coli* was cloned in phagemid vectors pAK100 and pAK200 for monovalent expression on the surface of filamentous phage M13. In these vectors, DHPS is expressed as a fusion to the C-terminal domain of phage coat protein p3 ($p3^{CT}$). Vector pAK100 additionally contains an amber stop codon between DHPS and the $p3^{CT}$, so that both soluble DHPS and DHPS-$p3^{CT}$ fusion protein is produced in a non-suppressing strain. Phage display of DHPS was not successful and practically no DHPS was observed on the phage surface using the vectors pAK100 (S/B = 1.5) and pAK200 (S/B = 1.3).

Each DHPS subunit had three unpaired cysteine residues. Because free cysteine residues can interfere with phage display, two libraries were constructed in which the cysteine residues were replaced with other amino acids. The libraries were screened for improved periplasmic expression, which is prerequisite for phage display. Based on screening, no mutants with improved periplasmic expression were found from the first library (mutations C137T/A/V/I, C172T/A/V/I and C242T/A/V/I). From the second library (mutations C137T/A/V/I, C172S/A/V/M and C242T/A/V/I) four clones (K.31, K.61, K.75 and K.90) were expressed in highr amounts than the WT DHPS. However, in larger cultures the K.31, K.61 and K.75 were expressed in less amounts than the WT DHPS, and neither was the phage display improved. Only K.90 had expression and viability comparable to the wild type (WT) DHPS (Fig.4.), but most notably, K.90 had 15-fold improved phage display efficiency (Fig.5A).

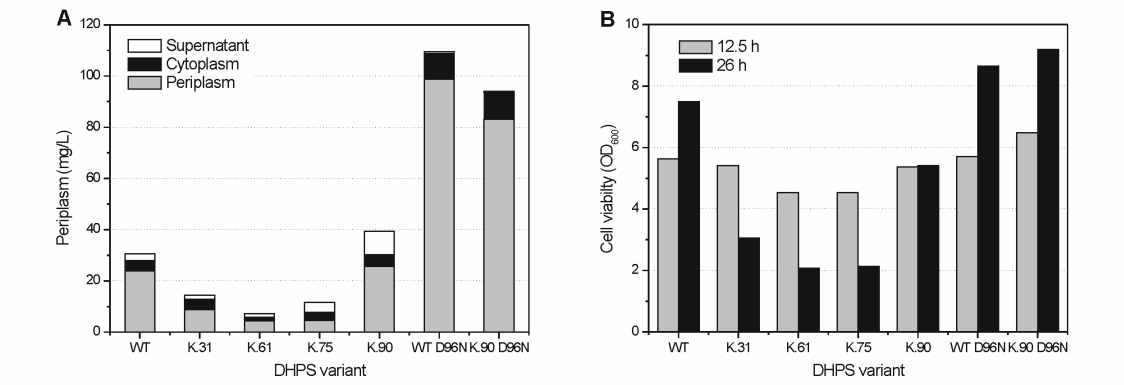

Figure 4. Periplasmic expression of DHPS. (**A**) Expression level. DHPS concentration in periplasmic extract, cytoplasm and growth medium was measured with an immunoassay after 10 hrs of expression at 30 °C from vector pAK100 in *E. coli* XL1-Blue. (**B**) Cell viability. The OD_{600} was measured after 12 hrs and 25 hrs of DHPS expression (**I**/Fig.1. and unpublished data).

To further improve the phage display of DHPS, random mutations were produced for the K.90 DHPS by error-prone PCR, and clones with improved phage display efficiency were selected from a phagemid library by a conformation-specific anti-DHPS antibody. Several clones with improved phage display efficiency were enriched. All the improved mutants had a common D96N mutation. This mutation increased periplasmic expression of both the WT and K.90 DHPS 3.5 – 4 fold (Fig.4). Phage display efficiency of the WT DHPS was improved only 4-fold

by the D96N mutation, but when D96N was combined with the K.90 cysteine mutations (C137I, C172M and C242A), phage display efficiency was improved by a total of 130-fold (Fig.5A). This DHPS mutant K.90 D96N was displayed well in both vectors pAK100 (S/B = 190) and pAK200 (S/B = 200) and was found to be in a correctly folded conformation on the surface of phage unlike the WT D96N (Fig.5B). DHPS K.90 D96N was still dimeric as is the WT DHPS.

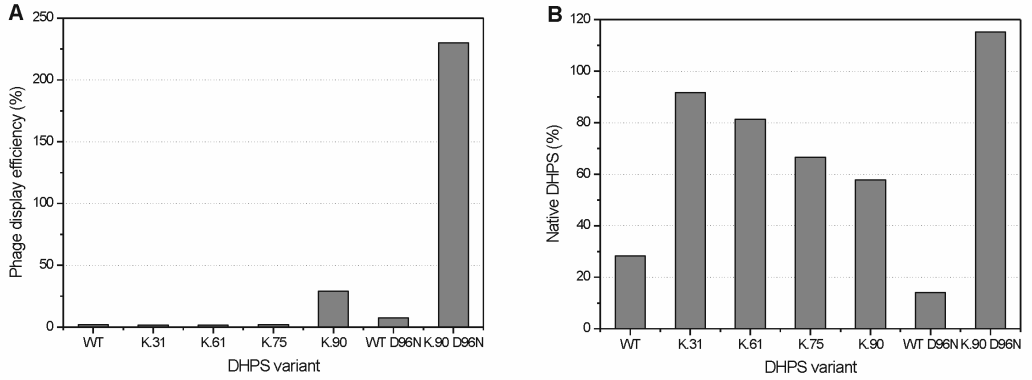

Figure 5. Phage display of DHPS in vector pAK200. (**A**) Phage display efficiency measured as phage binding (signal to background, S/B) to a conformation-specific anti-DHPS antibody 3G4. (**B**) Fraction of correctly folded DHPS on phage particles. Phage binding (S/B in phage immunoassay) to a conformation-specific 3G4 was compared with binding to a conformation-independent 4G7 anti-DHPS antibody (**I**/Fig.2.)

5.2 Broad-specific anti-sulfonamide antibody (II)

ScFv A.3.5 is a previously engineered broad-specific anti-sulfonamide antibody, which is capable of binding 10 out of 13 sulfonamides within a narrow concentration range and below the allowed maximum residue limit (MRL, 100 µg/kg in USA and EU) (Korpimaki *et al.*, 2003). The three sulfonamides, which were not detected at this concentration were sulfaquinoxaline, sulfadimethoxine and sulfamethazine (Table 15). In order to be detected by A.3.5 scFv, these sulfonamides needed to be present at 12 - 25 fold higher concentrations than sulfisoxazole and at 1000 - 2000 higher concentrations than sulfamethizole (Table 15). To also improve the binding of these three problematic sulfonamides, targeted residues at CDR loops of the A.3.5 scFv were randomised. Four libraries were constructed in each of which two CDR-loops were diversified. The designed diversities of the libraries were $2x10^6$ - $2x10^{11}$ (Table 14). The sizes of the transformed libraries were approximately 10^7 (Table 14).

ScFv fragments having improved affinity to the sulfamethazine were selected from the CDR libraries on microtiter wells using an antigen immobilised through biotin-streptavidin interaction. The antigen concentration in panning was selected based on immunoassay (Fig.6). In the assay, binding of the phage that display the parental scFv A.3.5 in a multivalent format was tested using several concentrations of the biotinylated antigen (sulfamethazine). When antigen concentration was reduced, at a certain point there was a sudden drop in the signal from bound phage. Most probably, at this concentration the antigen density was too low to support

the multivalent binding any longer. For the first and second round of panning, high antigen concentration was used, but for the third and fourth round antigen concentration was decreased to increase selection stringency (Fig.6).

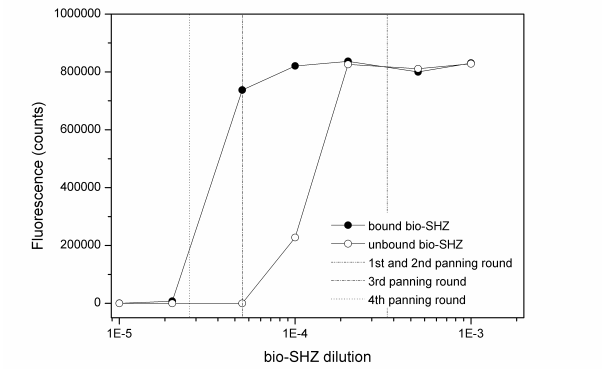

Figure 6. Capacity and binding of multivalent A.3.5 scFv phage onto streptavidin (SA) wells immobilised with biotinylated sulfamethazine (bio-SHZ). Binding capacity of the SA wells for bio-SHZ was tested using different bio-SHZ dilutions. Unbound bio-SHZ was moved to another SA well..Multivalent A.3.5 scFv phage were bound to both plates and bound phage were detected with Eu-labelled anti-phage Mab and time-resolved fluorometry. Bio-SHZ dilutions used for phage display selections are indicated (unpublished).

After three rounds of phage display selection, three out of the four libraries bound the biotinylated sulfamethazine more strongly than the parental antibody A.3.5. The fourth round of panning did not result in any further improvement. Among the individual clones isolated after three rounds of phage display selection, 7/20 (Lib1), 5/20 (Lib2), 6/20 (Lib3) and 1/20 (Lib4) bound Eu-labelled sulfamethazine with higher activity than A.3.5 scFv.

IC_{50}-values for sulfamethazine, sulfadimethoxine and sulfaquinoxaline were measured for eight of the most active clones. Binding of sulfamethazine was improved for all of them. For six clones, the IC_{50} value was below 100 µg/l for all the three sulfonamides, which means that the new binders had also detected the previously problematic sulfonamides below the concentration of the maximum residue limit. These clones had 2 – 3 mutations in CDR-L2 and clone M.3.4 had additionally one mutation in CDR-L1. Two of the less improved clones (M.2.14 and M.4.9) had only a single point mutation in CDR-L3 or CDR-H1, respectively.

The cross-reaction profile for 13 different sulfonamides was determined on four of the most promising clones (Table 15). All these clones bound the 13 different sulfonamides with higher affinity than the parental antibody A.3.5. IC_{50} for sulfamethazine was improved 7.5 – 25 fold, and for other sulfonamides 2 – 40 fold. Furthermore, M3.4 had the IC50-values within a 2-fold narrower range than the scFv A.3.5.

Table 15. Recognition of 13 sulfonamides by selected scFv clones (**II**/Table 3). The sensitivity (IC_{50} concentration) of the scFv antibodies against 13 different sulfonamides was measured in a competitive immunoassay. 27G3 is the original scFv cloned from monoclonal antibody. A.3.5 is the previously improved scFv antibody used as a template for the mutagenesis. M.1.12, M.1.17, M.3.1 and M.3.4 are new, improved scFv clones.

	IC_{50} (µg/l)					
Sulfa	27G3	A.3.5	M.1.12	M.1.17	M.3.1	M.3.4
Sulfamethizole	1.2	0.24	0.11	0.029	0.019	0.05
Sulfathiazole	8.7	2.5	0.12	0.33	0.085	0.15
Sulfachloropyridazine	9	1.3	0.41	0.24	0.066	0.19
Sulfamethoxypyridazine	19	3.1	0.63	0.38	0.12	0.25
Sulfadoxine	22	0.83	0.44	0.23	0.11	0.11
Sulfapyridine	35	9.6	1.3	1	0.27	0.42
Sulfamethoxazole	69	6.8	0.94	0.58	0.28	0.34
Sulfadiazine	160	5.8	0.48	0.47	0.26	0.24
Sulfamerazine	310	11	0.33	0.83	0.26	0.68
Sulfisoxazole	420	19	3.8	3.4	2.21	2.75
Sulfaquinoxaline	760	230	18	31	16	14
Sulfadimethoxine	1100	360	35	60	23	48
Sulfamethazine	4200	480	29	64	47	19

5.3 Anti-TSH antibody

5.3.1 Engineering for improved stability (III)

A 5404 anti-TSH antibody, which was cloned from a mouse monoclonal antibody, appeared to be rather unstable and aggregated in concentrated solutions during purification and storage. To improve the stability, 5404 scFv fragment was mutated randomly (2.5 amino acid mutations / scFv) and stabilised scFv variants were selected by phage display. Selection was based on denaturation of the antibody by heat or chatotropic agent (GdmCl), after which the clones that retained TSH binding activity were collected. Efficient selection for stability was established performing denaturation under reducing conditions. Phage production at elevated temperature (30 °C instead of 26 °C) also favoured the stabilised clones.

When the residual binding activity of 5404 scFv displaying phage was measured after chemical denaturation, scFv seemed to be more active after denaturation in 2 – 4.5 M GdmCl than in 1 M GdmCl. Even after denaturation in 4.5 M GdmCl, > 40 % of TSH binding activity was left,

even though it is very rare that scFv fragments tolerate such a high chaotrope concentration (Fig.7A). This implied that scFv refolds during the assay, especially when denatured in high GdmCl concentrations. However, when denaturation was carried out in the presence of a reducing agent (1 mM DTT), no residual TSH binding activity was observed at ≥ 1 M GdmCl. Thus reduction altered the denaturation so that it became irreversible. Phage infectivity was lost partially during denaturation in a reducing environment, but could be largely regained by refolding (Fig.7B). To select for stability, refolding should be carried out after eluting the selected phage. However, here refolding was carried out before capturing the active antibody phage in order to see whether refolding from a partially denatured state would help to reduce the aggregation.

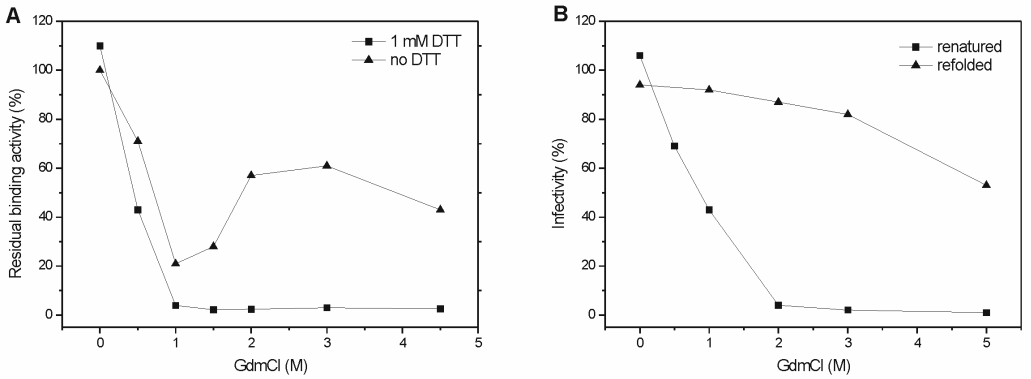

Figure 7. Chemical denturation of 5404 scFv phage. (**A**) Effect of GdmCl denaturation under reducing (1 mM DTT) and non-reducing environment (no DTT) on binding activity of 5404 scFv phage (**III**/Fig.1b). (**B**) Effect of denaturation under reducing conditions and refolding on phage infectivity (**III**/Fig. 2b).

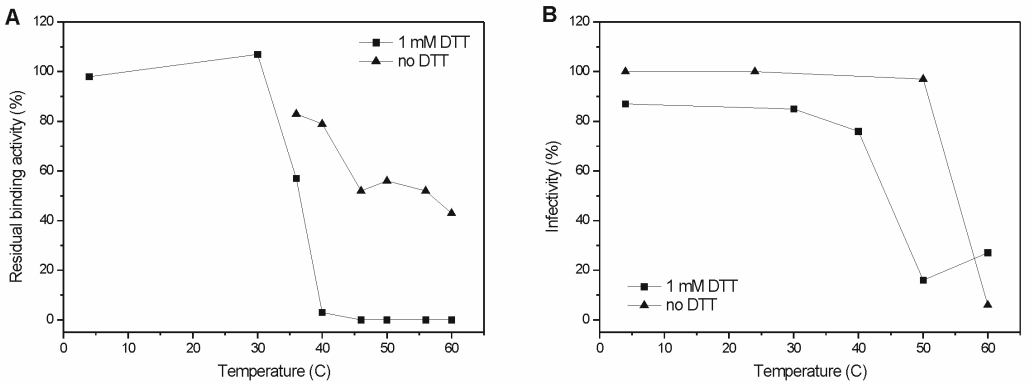

Figure 8. Thermal inactivation of 5404 scFv phage. Effect of heat on binding activity (**A**) and infectivity (**B**) of 5404 scFv phage. Denaturation was carried out under reducing (1 mM DTT) and non-reducing (no DTT) conditions. (**III**/Fig.1a and 2a).

Reducing conditions also established stringent selection when the antibodies were denatured by heat. Under non-reducing conditions, 40 % of the binding activity of 5404 scFv remained at 60

°C, where phage infectivity was lost. However, under reducing conditions 5404 scFv could be inactivated efficiently at temperatures (40 °C), which did not affect phage infectivity (Fig.8).

Stabilised antibody clones were enriched within 4 – 5 rounds of phage display selection. Under reducing conditions both methods, heat and GdmCl induced denaturation, selected for improved stability. However, the antibodies selected by thermal denaturation were on average more stable than the antibodies selected by GdmCl denaturation combined with refolding. Refolding after GdmCl denaturation apparently reduced the selection stringency for stability. The number of enriched clones that retained > 70 % activity at 40 °C was 12/20 (thermal selection) and 3/20 (GdmCl selection). Two of the most common sequences (clones T5.10/T5.11/G5.13 and T4.39/G4.31) were selected by both methods (T=thermal, G=GdmCl denaturation). These two clones had the highest periplasmic expression level among the characterized clones (Table 16).

Table 16. Stability and periplasmic expression of scFv variants (**III**/Table2 and unpublished data).

ScFv	Denaturation midpoint[a]		o/n expression at 26 °C		o/n expression at 37 °C		Purification yield [b]
	T (°C)	GdmCl (M)	Periplasm (mg/l)	Medium (mg/l)	Periplasm (mg/l)	Medium (mg/l)	µg/l
5404 (parental)	36	0.4	2.3	0.2	0.2	0.02	50
G5.14	37	0.8	3.5	0.04	0.2	0.8	16
G4.7	37	0.8	6.0	0.04	0.3	1.4	290
T4.39/G4.31	38	0.8	16	0.07	0.8	12	560
G4.38	40	0.9	3.9	0.02	0.2	0.7	320
T4.2	44	1.2	19	0.08	0.5	7.3	280
T5.10/T5.11/G5.13	45	1.3	14	0.07	1.6	23	480

[a] The denaturation midpoints are denaturing conditions in the prescence of 1 mM DTT, where the immunoreactivity to TSH is reduced to 50 %.

[b] Purification was from a 1 l flask culture by cation exchange (Sepharose FF), histidine affinity (Ni-NTA) and biotin affinity (Soft Link avidin) chormatography. The scFv was site-specifically at cysteine residue biotinylated in the Ni-NTA column.

The most stable variant isolated from the library (T5.10 scFv) had denaturation midpoints of 45 °C and 1.3 M GdmCl, which are 9 °C and 0.9 M GdmCl higher than for the parental 5404 scFv. Another stable variant, T4.2 scFv, which was found solely by thermal selection, had denaturation midpoints of 44 °C and 1.2 M GdmCl (Table 16). The denaturation midpoint was measured as the GdmCl concentration that resulted in 50 % loss of binding activity under reducing conditions. The result was very similar to the midpoint in GdmCl induced unfolding

measured by fluorescence spectroscopy, which indicates that the immunoassay gave a good estimate for stability.

In parallel with stability, expression was also improved (Table 16). The most stable scFv fragment (T5.10/T5.11/G5.13) was expressed in 5.6-fold (at 26 °C) and 100-fold (at 37 °C) higher amounts than the parental 5404 scFv. Actually, at 37 °C more of the stabilised variant (25 mg/l) was produced than at 26 °C (14 mg/l).

The stabilised 5404 scFv variants shown in Table had 3 – 7 point mutations. The effect of individual mutations was not analysed, but the most stable variants T5.10 and T4.2 had $Lys^{H}66Arg$ or $Asn^{H}52aSer$ mutations, respectively, known to stabilise other scFvs.

5.3.2 Use of anti-TSH antibodies in high-capacity assay wells (IV)

Capacity

Recombinant anti-TSH 5404 scFv and Fab antibody fragments were compared with their parental monoclonal antibody in high-capacity assay wells. The amount of biotinylated antibody needed to saturate the high-capacity glutaraldehyde-crosslinked strepatvidin (GA-Sav) wells (Fig.9A) was 2.2 pmol (Mab), 5.7 pmol (Fab) and > 11 pmol (scFv). In Mab there are two binding sites, so the number of immobilised binding sites was increases 1.3-fold by using Fab and at least 2.5-fold with scFv. TSH binding capacity (calculated from the signal of bound Eu-TSH at saturation) was increased in parallel with the number of available binding sites: 3.0-fold (Fab wells) and at least 4.1-fold (scFv wells) compared to the Mab wells.

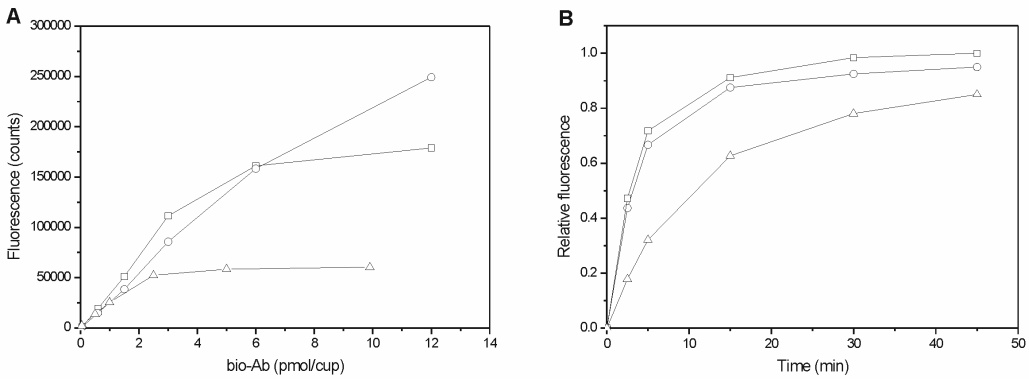

Figure 9. (**A**) Capacity of TSH all-in-one assay wells (**IV**/Fig1). (**B**) Kinetics of a one-step two-site immunofluorometric TSH assay (**IV**/Fig 2c). Capacity and kinetics were compared on high-capacity streptavidin plates using recombinant anti-TSH antibody fragments scFv T4.2 (○), Fab 5404 (□) and monoclonal antibody 5404 (Δ).

Kinetics

In high-capacity wells saturated with the biotinylated capture antibody (scFv T4.2, Fab 5404 or Mab 5404), recombinant scFv and Fab antibody fragments produced faster TSH binding kinetics than Mab. In scFv and Fab wells the maximum signal from binding Eu-labelled TSH

was reached within 5 minutes, but in Mab wells 15 minutes was needed to reach the maximum signal.

The kinetics of TSH binding in a two-step assay (where labeled antibody is incubated in a separate step after TSH is bound and washed) was similar to Eu-TSH binding. However, in the two-step assay the maximum signal with Mab was lower in relation to Fab and scFv, this differed from the Eu-TSH.

In the one-step assay (Fig.9B), where TSH and the labeled antibody were incubated simultaneously, the overall kinetics was slower than in the two-step assay. Within a 5-min incubation time, 35 – 38 % (Mab), 69 – 72 % (Fab) and 68 – 70 % (scFv) of the maximum signal compared to the 45 min incubation was achieved in the buffer and 21 – 25 % (Mab), 72-83% (Fab) and 74 – 80 % (scFv) in serum.

Storage stability

Mab 5404, Fab 5404 and a genetically stabilised variant of 5404 scFv, T4.2, were stable when stored in solution and maintained full binding activity for at least one month at 35 °C. In contrast, 66 % of the TSH binding activity of the 5404 scFv was lost and therefore, the stabilised variant T4.2 was used later in the TSH all-in-one assay.

The effect of antibody format (scFv, Fab and Mab) on the storage stability of dried all-in-one assay cups was tested. The cups contained, in a dried ready-to-use format, all the reagents needed for TSH immunoassay. Only the sample and buffer needed to be added to run the assay. The background signal in the assay cups increased gradually during storage and after two months of storage was (at 4 °C, RT and 35 °C) 2 – 28% (scFv), 14 – 36% (Fab) and 24 – 53% (Mab) higher than before storage. Despite the increase in background, the specific signal with 1, 10 and 300 mIU/L TSH was stable for at least two months.

5.3.3 Performance of an automated 5-min immunoassay for TSH (IV)

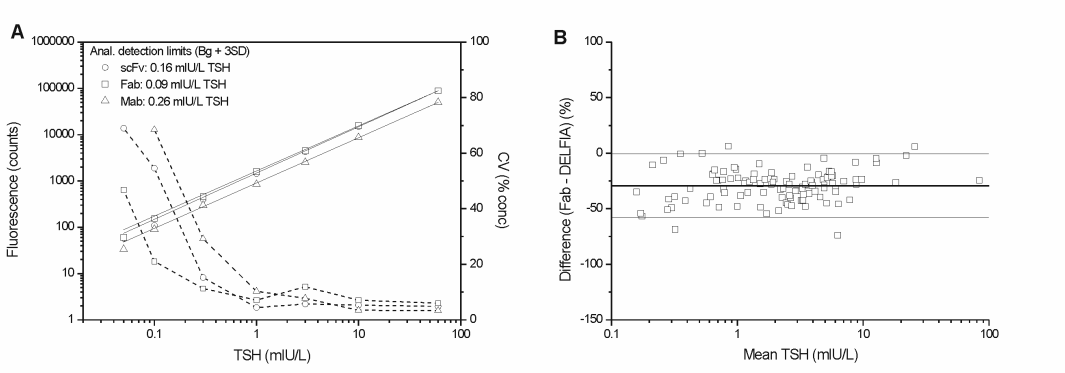

Figure 10. (**A**) Calibration curves and precision profiles for TSH all-in-one assays (**IV**/Fig.3a). Twelve calibrators in TSA, 7.5% BSA were run within assay. (**B**) Bland-Altman difference plot of TSH all-in-on assay using 5404 Fab as a capture antigen (**IV**/Fib.4b). The assay was compared with a commercial AutoDELFIA hTSH Ultra assay.

Analytical performance of an automated, two-site TSH immunoassay that used all-in-one dry reagent assay cups, 5-min incubation at 36 °C and direct surface read-out of time-resolved

fluorescence was evaluated (Table 17). Biotinylated scFv (T4.2), Fab (5404) and Mab (5404) were compared as capture antibodies in the assay. Calibration curves (Fig.10A) were linear at least up to 60 mIU/L (the highest calibrator concentration used), and there was no high-dose hook effect yet at 2145 mIU/L. The dose response in serum was linear down to 0.1 – 0.2 mIU/L/TSH and the functional sensitivities (20% concCV, calculated from three replicates of TSH diluted in TSH free serum) of the all-in-one assays were 0.30 mIU/L (scFv), 0.12 mIU/L (Fab) and 0.41 mIU/L (Mab) TSH. There was no significant cross-reactivity to human lutenising hormone (hLH), human follicle-stimulating hormone (hFSH) or human gorionic gonadotropin (hGH).

Table 17. Analytical performance of 5-min TSH all-in-one assays (**IV**).

	ScFv	Fab	Mab
Background	857 cts	441 cts	716 cts
Specific signal [1)]	88 691 cts	88 790 cts	49 632 cts
Analytical sensitivity [2)]	0.16 mIU/L	0.09 mIU/L	0.26 mIU/L
Functional sensitivity [3)]	0.30 mIU/L	0.12 mIU/L	0.41 mIU/L
Between-assay variation[4)]	4.6 – 17.7 %	4.2 – 7.9 %	5.5 – 7.2 %
Within assay variation [4)]	7.7 – 13.6 %	6.0 – 7.1 %	3.8 – 11.9 %
Mean recovery (95% CI)	101 % (73 – 130 %)	84 % (68 – 100 %)	89 % (79 to 102 %)
Mean difference to hTSH Delfia (95% CI)	-11.1 % (-64.9 to 42.7 %)	-29.4 % (-58.1 to -0.7 %)	-24.4 % (-53.2 to 4.3 %)
Correlation to hTSH Delfia	y = 1.385x – 1.325 (r=0.991)	y = 0.790x – 0.084 (r=0.989)	y = 0.860x – 0.150 (r=0.992)

[1)] 60 mIU/L TSH in buffer TSA, 7.5% BSA

[2)] Background +2SD (12 replicates)

[3)] TSH diluted in TSH-free serum (three replicates)

[4)] 0.63 – 6.3 mIU/L TSH in serum (ten replicates)

Each of the three all-in-one TSH assays correlated well with Delfia hTSH assay (r=0.99), but the differences between the Fab (Fig.10B) and Mab based assays to the Delfia assay were negatively biased. The bias was caused by the matrix effect, as the calibrators were in a buffer and the samples in serum. Recoveries were good (Table 17), but the average recoveries of the Fab- and Mab-based assays were reduced due to the calibrator matrix. The difference between the scFv and Delfia assays was dose-dependent. The Fab-based assay, with analytical sensitivity of 0.09 mIU/L TSH, was the most sensitive among these three assays. The sensitivity of the Fab-based assay was an outcome of a low background signal, high specific signal, established by fast kinetics, and low 6.0 – 7.0 % within-assay variation (Table 17). Kinetics of the scFv-

based assay was as fast as with Fab, but the relatively high background and increased assay variability (Table 17), compared to the Fab-based assay, limited the analytical sensitivity of the scFv-based assay to 0.16 mIU/L. The Mab-based assay otherwise performed well, but suffered from slow kinetics, which resulted in 45 % lower specific signal compared to the Fab and scFv-based assays. Analytical sensitivity of the Mab based assay was 0.26 mIU/L TSH.

5.4 Synthetic antibodies (V)

5.4.1 Syntehtic antibody library

A universal, synthetic antibody library was constructed using a single human scFv fragment as a framework. CDR regions were randomised using trinucleotide building blocks (Braunagel & Little, 1997), which allowed design of exactly defined diversification patterns. Randomisation was biased towards the natural sequences and Cys, Met and stop codons were omitted from the codon mixtures. CDR-H3, which is the most diverse CDR loop in nature, was randomised using twelve different loop lengths (7 –18 residues). Diversity at the CDR-H3 loop was designed according to natural antibodies (Zemlin *et al.*, 2003).

Table 18. Antibodies isolated from the synthetic mbLib binding library (**V/Table2**).

Antigen	Panning round	Active[1)] / Screened	Unique / Sequenced
Lysozyme	3	28/45	24/28
Thyroid stimulating hormone (TSH)	2	32/45	10/12
Pregnancy associated plasma protein A (PAPP-A)	3	31/45	2/11
Immunoglobulin G	2	32/45	5/6
Chymotrysinogen A	3	5/10	n.a.
Cyclodextrin glucanotransferase (CGTase)	3	10/10	n.a.
Streptavidin	3	43/45	4/9

[1)] Specific signal in phage immunoassay > 10 000, S/B > 5.

Two libraries were constructed. In the primary binding library (mbLib) diversification was targeted mostly to the heavy chain CDRs. The total designed diversity of the mbLib was 1.16×10^{22}, but transformation limitied the library size to 3.1×10^{10}. However, 55 % of the library clones were non-functional because of frameshift mutants originating from errors during oligonucleotide synthesis. Another library (amLib) was randomised only on the light chain CDRs and it was designed for affinity maturation of the binders selected from the primary library. The designed diversity of this library was 1.8×10^{9} and the size of the transformed library was 6.6×10^{8}. Both of the mbLib and amLib libraries were displayed on phage as a fusion to phage coat protein p9.

From the primary binding library new specific antibodies were successfully isolated for several protein antigens. For each antigen, several unique binders were isolated after only 2 – 3 phage display selection cycles (Table 18).

5.4.2 Selection for improved affinity

The pool of binders enriched for lysozyme was affinity matured by light chain shuffling with the amLib library. Solid phase panning with and without off-rate selection was compared with the selection in solution. For off-rate selection excess of unconjugated lysozyme was added after the phage had been bound to the beads. In solution panning, where the phage were first incubated with the antigen in solution, a marked amount (20 – 100 %) of streptavidin binding antibodies was enriched especially in the off-rate selection. Subtractive selection with streptavidin was not sufficient to prevent enrichment of the streptavidin binders. However, enrichment of streptavidin binders was largely prevented when selection was carried out on solid phase, using streptavidin beads saturated with the biotinylated antigen.

Antibodies with improved affinities were isolated by all the methods, but the highest affinity binders were isolated using solid phase panning together with two rounds of off-rate selection (Fig.11). The lysozyme-binding antibodies enriched from the light chain shuffled library had affinities of Kd = 0.8 – 218 nM (n = 19), whereas affinities before affinity maturation were Kd = 14 – 176 nM (n=4). Thus, affinity maturation resulted in all in 17.5-fold improvement in affinity. However, affinity of a single clone was improved 70-fold, from 280 nM to 4 nM. The V_H found in the 4 nM binder was found after affinity maturation in combination with altogether eight different V_L sequences. The same V_H was selected both in solution panning (4 / 8 clones, Kd = 8 – 122 nM) and by solid phase capture (12/20 clones, Kd = 4 – 14 nM).

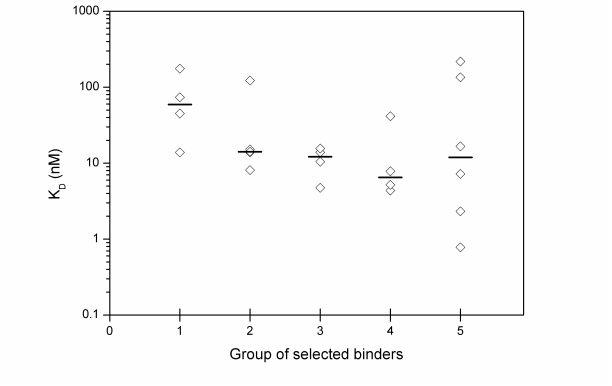

Figure 11. Affinity of lysozyme-binding clones (**V**/Fig.3). (**1**) Binders selected from the primary library. (**2-5**) Binders selected from the light chain shuffled library for improved affinity. Selections by solution panning (**2**), solid phase panning (**3**), solid phase panning with off-rate selection either on the 3rd or 4th round (**4**) and solid phase panning with off-rate selection on both the 3rd and 4th round (**5**).

6 DISCUSSION

6.1 Binding scaffolds

6.1.1 Recombinant antibodies (II, III, V)

ScFv fragments are often preferred over Fab fragments as frameworks in antibody libraries because they are simpler to display on phage (Hust & Dubel, 2004). However, Fab fragments are favoured in several applications. ScFv fragments are often less stable than Fab fragments (Quintero-Hernandez *et al.*, 2007) and several scFv antibody fragments have needed engineering to improve stability (Worn & Pluckthun, 2001; Honegger, 2008). Lower stability of scFvs compared to Fab is caused by the lack the stabilising interactions from the constant domains (Rothlisberger *et al.*, 2005). Moreover, in scFv fragments the two variable domains are linked through a flexible polypeptide linker, which may allow temporal opening of the domain interface (Arndt *et al.*, 1998; Jager & Pluckthun, 1999) and predispose to a formation of multimers (Desplancq *et al.*, 1994). In Fab fragments, the heavy and light chains are covalently associated through a disulfide bond, which prevents dissociation of the two chains.

The anti-TSH scFv fragment 5404, cloned from a mouse monoclonal antibody (**III**), was very unstable and aggregated in concentrated solutions. The tendency to aggregate is not necessarily associated with stability, but high stability could protect against unfolding, because it makes aggregation less likely (Jespers *et al.*, 2004). The stabilised anti-TSH scFv variants were still prone to aggregation, but the level of aggregation is difficult to measure quantitatively. However, higher expression level and improved stability eased production and purification of the engineered anti-TSH scFv fragments.

Aggregation is not common for all scFv fragments and the anti-sulfonamide antibody (**II**) did not have any problems related to expression, aggregation or stability. Aggregation may depend on the variable domains. Some antibody domains have large hydrophobic interface surface and are more prone to aggregation than others (Ewert *et al.*, 2003). Because the Fab fragment of 5404 did not aggregate, aggregation tendency of the 5404 scFv fragment might be associated with opening of a (relatively weak) V_L/V_H interface surface or exposed constant/variable domain interface.

For synthetic antibody libraries, the framework can be selected to be optimal. In the synthetic mbLib human antibody library (**V**), V_H and V_L were used that are common in nature and known to be stable and well expressing in *E. coli* (Knappik *et al.*, 2000); (Ewert *et al.*, 2003). The same variable domains that were exploited in the mbLib library have also been used previously in other antibody libraries (Pini *et al.*, 1998; Azriel-Rosenfeld *et al.*, 2004; Silacci *et al.*, 2005). Because natural antibodies exploit several frameworks, using only a single framework in a universal antibody library can limit some of the conformational diversity. However, it has been shown that it is possible to generate high-affinity antibodies for various targets also from libraries that exploit only a single framework (Fellouse *et al.*, 2007). In addition, in natural antibodies certain V_L and V_H genes are found more frequently than others (Griffiths *et al.*, 1994; Schofield *et al.*, 2007), and limiting the number of frameworks in a library allows presentation of higher diversity at the binding site.

6.1.2 Alternative binding scaffold (I)

Antibodies are not optimal for binding small molecules or haptens which themselves are not immunogenic. Instead, several enzymes catalyse reactions of small molecules and thus could be suitable frameworks for engineered hapten binders. TIM ($(\beta\alpha)_8$) barrel (Braenden, 1991; Reardon & Farber, 1995; Wierenga, 2001; Nagano *et al.*, 2002) is the most frequently occurring folding motif in proteins. It is a stable fold and active centre is often located at the loop regions that should be rather freely mutable without affecting the framework structure. DHPS, which is a TIM barrel enzyme taking part in the biosynthesis of folic acid in bacteria (Talarico *et al.*, 1991; Achari *et al.*, 1997; Vinnicombe & Derrick, 1999; Baca *et al.*, 2000), was intended to be used as an alternative binding scaffold.

An optimal binding scaffold should be small, stable, monomeric, devoid of cysteines, well expressed in bacteria and compatible with some display technique. DHPS does not fulfill all these criteria, because it is homodimeric, both of the subunits contain three unpaired cysteine residues and it could not be displayed on phage. Display techniques are required for efficient selection of binders from complex libraries. Filamentous phage display (Carmen & Jermutus, 2002; Bradbury & Marks, 2004) is a robust and the most commonly used display technique in antibody engineering. Periplasmic expression is a prerequisite for filamentous phage display. Even though DHPS is naturally expressed in cytoplasm, it could also be expressed in periplam. Compared to antibody fragments, the periplasmic expression level of DHPS should have been sufficient for a phage display. However, practically no DHPS was observed on the surface of filamentous phage when it was expressed as a fusion to phage coat protein p3.

The main cause for the poor phage display of DHPS was assumed to be in the cysteine residues. Unpaired cysteine residues not only reduce periplasmic expression (Schmiedl *et al.*, 2000) but also interfere with the phage display if the wrong disulfide bonds are formed (Wind *et al.*, 1999). The Phage display of another cytoplasmic protein, Ras, has been established by cysteine replacement (Wind *et al.*, 1999). Cysteine replacement was also required with DHPS for an efficient phage display. However, it was not sufficient alone and an additional D96N mutation was needed to establish a functional phage display of DHPS. As with the wild type Ras, the cysteine containing DHPS was also found to be in a corrupt conformation on the phage (Wind *et al.*, 1999). Thus, replacement of unpaired cysteine residues might be more widely useful, if there are problems in the phage display of cytoplasmic protein.

Difficulties to display DHPS on a phage could also have been associated with the dimeric nature of DHPS, because a minimum of two subunits should be displayed on each phage particle in order to enable formation of the homodimer. However, the dimeric form appeared not to be responsible for the low phage display efficiency of DHPS, because the engineered DHPS, which still formed a dimer, was efficiently displayed on the surface of the phage.

6.2 Optimising framework by phage display

6.2.1 Selection for improved phage display (I)

Random mutagenesis followed by a phage display selection can be used to improve phage display efficiency and periplasmic expression in *E.coli* (Coia *et al.*, 1997; Tuckey & Noren, 2002). Because of the growth advantage, well expressing clones tend to be overpopulated and

enrich in phage display even when not specifically selected (Jung & Pluckthun, 1997). As a result, in affinity selection the highest affinity binders can even be overcome by some well-expressing but lower affinity clones. When well-expressing clones are intentionally selected from randomly mutated antibody libraries, there is a risk that also the antigen binding site is mutated. However, selection with the specific antigen also guarantees that antigen binding is maintained (Coia *et al.*, 1997; Tuckey & Noren, 2002).

Because DHPS is an enzyme, there was no existing antigen or high-affinity ligand that could have been used to select the well displayed mutants. Instead, selection was carried out using an anti-DHPS antibody. Because DHPS was intended for use as an alternative binding scaffold, maintaining the existing enzymatic activity and specificity was not relevant, and the antibody selected the mutants that, on the whole, were expressed on phage. When enrichment was observed, the amount of the selecting antibody was reduced to favour the most prominent DHPS variants. Some clones might have been selected because of increased affinity to the collecting antibody and not solely by the display efficiency. However, mutating the epitope in DHPS was not a concern in selection, because any clone that is displayed on phage would be practical. To find the best expressing clones, individual clones were analysed using antibodies directed against different epitopes. The D96N mutation that was found to be responsible for the improved phage display, located inside the barrel, and did not change the epitope.

6.2.2 Selection for stability of scFv fragment (III)

Stability selection by phage display is based on inactivation of unstable variants by heat, chaotope (e.g. GdmCl) or protease (Sieber *et al.*, 1998; Jung *et al.*, 1999; Martin *et al.*, 2001; Martin *et al.*, 2001). Stable variants are then collected based on antigen binding activity (Jung *et al.*, 1999) or phage infectivity, if p3 is lost as a result of protease digestion of the displayed protein (Sieber *et al.*, 1998; Martin *et al.*, 2001). To capture the still active binders, denaturation pressure needs to be released by lowering the temperature or diluting the chaotrope concentration. If denaturation is reversible, the antibodies may refold during the capture step, which complicates the selection. Thermal denaturation is usually considered to be irreversible, and therefore selects more efficiently for stability than reversible chemical denaturation (Jung *et al.*, 1999). However, it was found that GdmCl denaturation could be made practically irreversible, if denaturation was carried out under reducing conditions. Refolding was also observed after thermal denaturation, which could as well be made irreversible in a reducing environment.

In scFv fragments, there is a structural disulfide bond inside both variable domains. These disulfide bonds are important for the integrity and stability of the domains and usually only extremely stable scFv fragments can fold correctly if the disulfide bridges are removed (Frisch *et al.*, 1996; Worn & Pluckthun, 1998a; Worn & Pluckthun, 1998b; Proba *et al.*, 1998; Ohage & Steipe, 1999). Accordingly, folding under reducing conditions has been used as a basis for stability selection by ribosome display (Jermutus *et al.*, 2001). Therefore, if any refolding occurs after full denaturation in the reducing environment, the clones are supposed to be stable.

Improved refolding from a partially denatured state could help to avoid aggregation, because rapid refolding might help to regain the correct structure. Indeed, antibody domains that resist aggregation have been selected based on the ability to refold after heat denaturation (Jespers *et al.*, 2004). The 5404 scFv refolded on phage after denaturation even though the antibody was prone to aggregation in solution. Monovalent phage display probably promoted refolding, because a single unfolded antibody on the phage capsid is less likely to aggregate than free

antibodies in concentrated solutions. Accordingly, multivalent phage display and high phage concentration has been required to select against aggregation by phage display (Jespers *et al.*, 2004).

6.3 Selection for affinity and specificity by phage display

6.3.1 Engineering broad-specificity (II)

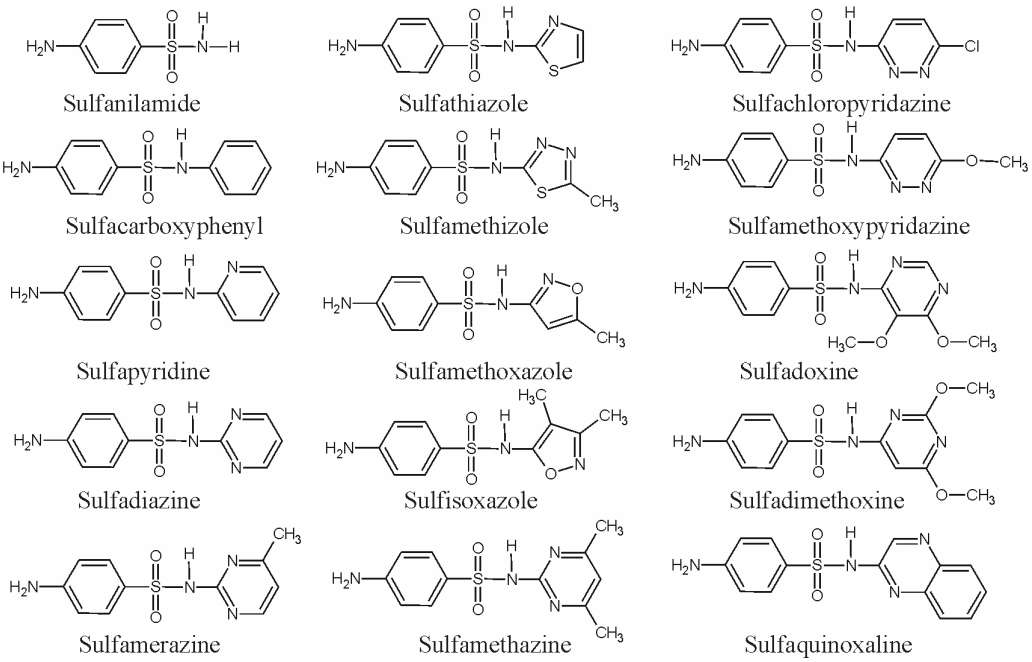

Figure 12. Sulfonamide structures (**II**/Fig.1).

Group-specific antibodies bind antigens that share a common chemical structure. Sulfonamides are antibiotics that have a common p-aminobenzene sulfonic acid (sulfanilamide) moiety and differ with the substituent in the N1 position (Fig.12). To create antibodies against sulfonamides by immunisation, they need to be conjugated to a carrier protein. Conjugation through the generic moiety generates focused antibodies specific to a single sulfonamide. Instead, conjugating at the heterogeneous group or the N1 position gives rise to antibodies that recognise the generic portion and thus multiple sulfonamides (Haasnoot *et al.*, 2000a). However, creating a truly generic antibody that would bind all sulfonamides with similar affinity has been difficult (Nesterenko *et al.*, 2009; Zhang & Wang, 2009).

Antibody engineering has been used to improve the broad-specificity and affinity of a monoclonal antibody 27G3. The antibody was originally raised by immunising mice with a protein-conjugated sulfathiazole (Haasnoot *et al.*, 2000a). The antibody was cloned into scFv

format and had been improved previously by two rounds of *in vitro* evolution, which increased the affinity and altered the recognition to be more broad-specific. The 1st generation library was created by random mutagenesis with error-prone PCR (Korpimaki *et al.*, 2002). In the 2nd round of affinity maturation, mutations in the improved clones found in the 1st round were recombined by DNA shuffling (Korpimaki *et al.*, 2003). Higher affinity mutants with more generic cross-reaction profile had been selected from the libraries by phage display using the following selection strategies. 1) Reducing the amount of selection antigen to favour the best-affinity binders. 2) Alternating the sulfonamides used in selection and 3) selection with competing antigen to favour broad-specific binders.

In the third round of engineering of the anti-sulfonamide antibody, described here, targeted random mutations were introduced to the previously engineered scFv A.3.5. Randomised positions were selected based on molecular model and experience from previous rounds of mutagenesis. The three sulfonamides (sulfaquinoxaline, sulfadimethoxine and sulfamethazine) that were detected most poorly by the scFv A.3.5 have larger heteronegnous groups than sulfathiazole, which was used to raise the parental monoclonal antibody, or sulfamerazine and sulfacarboxyphenyl, which had been used in evolution of the A3.5 scFv. To improve binding especially to these bulky sulfonamides, sulfamethazine with the weakest affinity was used as a selection antigen.

When affinity is improved, the antibody usually becomes structurally more rigid (Manivel *et al.*, 2002). Because specificity and affinity are somewhat opposing properties, affinity of generic antibodies is often compromised. Multivalent phage display (Rondot *et al.*, 2001) was therefore exploited in the selection to efficiently capture even the low and medium-affinity binders. Multivalent display guaranteed a similar display level between clones and thus differences in avidity were avoided. Selections on microtiter wells densely coated with the antigen supported multivalent binding. The bound phage could be eluted by protease digestion independently of the antibody affinity. For the third and fourth round of selection, the antigen concentration was reduced to better discriminate between the highest affinity binders. In the fourth round the low antigen density abolished multivalent binding (Fig.6).

6.3.2 Affinity improvement of synthetic binders by light chain shuffling (V)

In nature, the antibodies selected by primary response have low affinity and are then matured through somatic hypermutagenesis to higher affinity. Similarly, the affinities of antibodies isolated from synthetic antibody libraries may be unoptimal and require affinity maturation. The synthetic antibody library described here was designed so that the isolated binders could be affinity-matured rapidly through light chain shuffling with a separate light chain library. Affinity maturation for whole enriched pool eliminates the need to screen and isolate individual clones from the primary library. Moreover, it allows affinity maturation of even lower-affinity binders, which might have higher potential to develop into high-affinity binders than the most enriched clones.

Affinities of several antibodies have been improved by light chain shuffling. Because the heavy chain CDRs, especially the CDR-H3, usually make most contacts with the antigen, optimising the light chain CDRs can result in improved affinity. When antibodies have been affinity-matured *in vitro* through recombination with a naïve V_L library, the selected light chains have often been homologous to the parental antibody, belonged to the same germline family or had identical canonical loop structures (Marks *et al.*, 1992; Ohlin *et al.*, 1996; Osbourn *et al.*, 1996; Schier *et al.*, 1996a; Lu *et al.*, 2003). Therefore, light chain shuffling is extremely compatible

with the single-framework strategy. The single-framework approach also established development of a rapid and simple method for chain shuffling. The method also allows parallel switching of the antibody format from scFv to Fab, depending on the format of the premade light chain library used as a template for chain shuffling.

6.3.3 Selection for affinity by p9 display (V)

Filamentous phage display using the phage coat protein p9 has not been exploited to any great extent, even though it has shown great promise in selections (Gao *et al.*, 2002). In p9 display, phage can be efficiently rescued by an infection process, because p9 is located at the opposite end of the threadlike M13 phage as p3, which is responsible for phage infection. In p9 display it is not even necessary to elute the bound antibody phage, because p3 is freely available and indicates the solution. Therefore, phage can be rescued by direct infection simply by adding the bacterial cells (Gao *et al.*, 2002). This is practical especially when optimising affinity, because the highest affinity antibodies are probably most difficult to be eluted by acid or base. Moreover, the cells expressing p9 protein from a phagemid vector can be efficiently infected with helper phage as the presence of p9 does not interfere with the superinfection as does the p3 (Scott & Barbas, 2001).

Isolating the highest-affinity binders among thousands of moderate-affinity binders is a challenge because in addition to affinity, expression, toxicity and avidity also contribute to enrichment. Binding in solution usually selects more efficiently for affinity than binding to immobilised antigen (Schier *et al.*, 1996a) because on solid phase capture variations in phage display and antibody valency increase avidity and ruin the selection for affinity. p9 display is potentially useful for affinity maturation because it produces monovalent phage display and therefore allows selection for improved affinity, also on solid phase.

Selection on solid phase was advantageous compared to the binding in solution, because the immobilised antigen blocked streptavidin sites and thereby largely prevented enrichment of unspecific clones. The highest affinity binders could then be selected by off-rate incubation with antigen. Off-rate selection has been shown to select efficiently for high affinity (Hawkins *et al.*, 1992; Yang *et al.*, 1995), and accordingly, the highest affinity lysozyme-binders were enriched by two rounds of off-rate selection. Blocking the streptavidin sites was especially important in off-rate selection, because the lowest affinity binders were competed out from the surface by addition of excess of free antigen, while the unspecific streptavidin binders remained bound. Alternative options to limit enrichment of streptavidin binders when exploiting biotin-streptavidin interaction for antigen capture include antigen-specific elution for example through cleavable biotin linkers (Fellouse *et al.*, 2007; Kobayashi *et al.*, 2009).

6.4 Immunoassays exploting engineered and recombinant antibodies

6.4.1 Recombinant antibody fragments as capture reagents in immunoassays (IV)

An analyte-binding surface is an essential determinant of immunoassay performance in heterogeneous immunoassays. Recombinant antibodies can provide higher analyte-binding capacity than monoclonal antibodies because of their smaller size (scFv 29 kDa, Fab 49 kDa vs Mab 155 kDa) and possibility for site-specific immobilisation. The small size allows dense coating and site-specific immobilisation guarantees correct orientation where the antigen-binding site indicates the solution (Peluso *et al.*, 2003).

In this study, site-specific immobilisation of the recombinant antibodies was established through a genetically introduced single cysteine residue at the C-terminus. It provides homogeneous conjugated product with a single biotin in a defined position of antibody. Similar site-specific biotinylation is not available for monoclonal antibodies, and therefore Mabs are usually biotinylated by random chemical biotinylation at amino groups of lysine residues. Random biotinylation at multiple sites may reduce the affinity to antigen or result in misorientation of some of the Mabs, which reduces the antigen binding capacity by blocking some the binding sites. Unfavourable orientation and inactivation of the antibody may be more prominent with heavily biotinylated Mabs. Blocking some of the binding sites of Mab is suggested, because in the wells coated with recombinant antibody fragments, the number of available binding sites was increased more compared to Mab than suggested by the number of immobilised binding sites. Moreover, in the two-step TSH assay the signal measured from the Mab wells was lower than in Fab and scFv wells, possibly because the second antibody could not detect all TSH bound to unfavourably orientated Mab. In the one-step assay, the maximum signal was similar in all wells, because TSH first mainly binds the labelled antibody in the solution. With recombinant antibody fragments random biotinylation would not be practical, because, due to their small size, a significant amount of the antibodies would be affected (Saviranta *et al*., 1998).

6.4.2 Use of recombinant antibodies in a rapid TSH immunoassay (IV)

TSH is a glycoprotein hormone secreted by the pituitary (Szkudlinski *et al.*, 2002). It stimulates the synthesis of thyroid hormones from thyroid, which in turn gives negative feed-back when regulating the synthesis of TSH. Normal serum TSH concentrations range from 0.3 – 0.5 to 3 – 5 mIU/L (Stockigt, 2002), but the level is changed in hypo- and hyperthyroidism, which are common thyroid disorders (Tunbridge *et al.*, 1977; Sawin *et al.*, 1985; Bjoro *et al.*, 2000; Vanderpump & Tunbridge, 2002). Measurement of serum TSH has therefore been recommended as a first-line test in screening for these disorders (Ladenson *et al.*, 2000; Helfand & Redfern, 1998; Stockigt, 2002) and a rapid assay would allow accurate diagnosis during a patient visit.

In rapid immunoassays requiring short turnaround time, binding equilibrium is frequently not achieved. In such assays fast kinetics results in more analyte bound during the short incubation time, which in principle should establish detection of fewer antigens in the sample and, thus, improve assay sensitivity. It is possible to improve the association rate constant (k_{on}) of recombinant antibodies by antibody engineering (Katakura *et al.*, 2004; Duenas *et al*., 1996). However, the rate of binding antigen from solution to solid surface is also limited by diffusion (Nygren & Stenberg, 1989). Therefore, improvement in k_{on} would propably not significantly improve the immunoassay kinetics. Instead, the rate of antigen binding and thus the reaction kinetics of a two-site immunoassay should be improved if the concentration of the capturing antibody is increased. This principle was exploited in a rapid immunoassay for TSH. In the assay, analyte-binding capacity and thus also kinetics was maximised using small-sized correctly oriented recombinant antibody fragments. However, despite similar kinetics in the scFv and Fab based assay, the scFv-based assay was less sensitive because of higher background and variability. The performance of the Mab based assay was limited mostly by slow kinetics. The scFv fragment used in the assay was a stabilised variant, because the original scFv cloned from monoclonal antibody partially denatured at the temperature used in the immunoanalyser.

With analytical sensitivity of 0.09 mIU/L and functional sensitivity of 0.12 mIU/L TSH, the Fab based assay is sufficiently sensitive to diagnose hypothyroidism. The assays were not finally optimised for maximal sensitivity, but rather provide information on the performance of recombinant antibodies in a simple and rapid assay setup. To also allow accurate diagnosis of hyperthyroidism, a condition in which TSH concentrations are decreased, the assay should me more sensitive. Sensitivity can be improved for example by using nanoparticle labels (Pelkkikangas *et al.*, 2004) or limiting coating only to the site at the bottom of the assay well where the signal is measured (Ylikotila *et al.*, 2005). However, both of these approaches also slow down the kinetics.

The Fab based assay performed well in all aspects and the results indicate that a Fab fragment derived from even a very unstable scFv can be a good alternative to Mab. As a homogeneous, stable and small-sized binding molecule with optimal surface coating properties and a reduced risk of interference by heterophilic antibodies, Fab fragment is a promising immunoreagent for the future.

6.4.3 Generic antibody for sulfonamide drug residue screening (II)

Sulfonamides are antibiotics that are used in veterinary and also in human medicine as therapeutic and prophylactic agents. It has been estimated that > 5 % of the human patients experience unwanted side effects from sulfonamides (Dibbern & Montanaro, 2008). If proper withdrawal periods are not followed in animals, drug residues can be found in meat and milk products, causing symptoms in sulfonamide sensitive individuals. Legislation has therefore set the allowed maximum residue limit for sulfonamides, which is 100 µg/kg in EU and USA (Food and Drug Regulation, 1991; EU regulation, 1999).

Several immuoassays have been developed for the detection of sulfonamides. Some assays that use polyclonal antibodies are capable of recognising several sulfonamides. However, the supply of polyclonal antisera is limited. Creating broad-specific monoclonal antibodies has been more difficult than polyclonal antibodies (Cliquet *et al.*, 2003). Typically monoclonal anti-sulfonamide antibodies are capable of binding the target sulfonamide with good affinity and also cross-react with some other sulfonamides (Sheth & Sporns, 1991; Haasnoot *et al*., 2000b; Muldoon *et al*., 2000; Spinks *et al*., 1999; Spinks *et al*., 2002; Cliquet *et al*., 2003; Wang *et al*., 2007). Truly generic monoclonal anti-sulfonamide antibodies that would recognise all sulfonamides with equal sensitivity are lacking.

As far as we know, the M.3.4 scFv antibody described here (**II**) is the most generic monoclonal anti-sulfonamide antibody in the sense that it has recognised all the tested sulfonamides below the concentration of MRL (Korpimaki *et al.*, 2004a; Korpimaki *et al.*, 2004b). Moreover, the monoclonal antibody 27G3 that was the parental monoclonal antibody to the scFv M.3.4, is thus far the only anti-sulfonamide antibody that has been improved by genetic engineering for more sensitive detection and broader binding specificity for sulfonamides. The generic M.3.4 scFv was shown to be able to detect eighteen different sulfonamides below the MRL in a time-resolved fluorescent immunoassay which uses only a single 15 min incubation (Korpimaki *et al*., 2004b).The antibody, therefore, provides a valuable tool for screening sulfonamide drug residues. The screening assay would be more qualitative than quantitative, because of the variable sensitivity for different sulfonamides. Additionally, the M.3.4 antibody has been exploited in a multi-sulfonamide biosensor immunoassay (Bienenmann-Ploum *et al*., 2005) and flow cytometric immunoassay (de Keizer *et al.*, 2008). In a study, where biosensor sulfonamide assays were compared, the assay using the scFv M.3.4 was the most sensitive towards most of

the sulfonamides, but another assay (Qflex Kit) detected the five sulfonamides registered for application in poultry in the Netherlands within the narrowest measurement range (Bienenmann-Ploum *et al*., 2005).

7 CONCLUSIONS

Antibody fragments are interesting alternatives to intact mononclonal antibodies in immunoassays and have the option to be improved further by antibody engineering. Evolutionary methods, which combine random or targeted mutagenesis with guided selection, are powerful tools in antibody and protein engineering, overcoming the need for rational design. Screening was exploited in the selection of cysteine-free DHPS variants from a small library (**I**), but for large libraries efficient selection by display techniques, such as phage display, is needed.

A prerequisite for efficient selection is a functional phage display of the protein to be engineered. We managed to establish functional phage display for DHPS by combining replacement of the cysteine residues with additional random mutagenesis and phage display selection. The obtained DHPS variant should allow further engineering of DHPS, for example, to an alternative binding scaffold (**I**). Phage display was also used to select for improved stability (**III**) and antigen binding (**II**, **V**). For stability selection, an optimised selection technique based on denaturation under reducing conditions was established. The method allows more efficient selection of stabilised scFv fragments by phage display than a previously used method (**III**). For affinity selection, monovalent p9 based phage display was studied. The p9 display, which has not been exploited very widely earlier, showed great promise in affinity selection. High affinity clones were obtained even when the p9 display was combined with solid phase capture, which is usually not recommended for affinity selection because of the risk of avidity when the conventional monovalent display techniques are used (**V**).

The binding properties of an anti-sulfonamide antibody recognising the p-aminobenzene sulfonamide moiety, general to all sulfonamides, were fine-tuned. Improved detection of the three sulfonamides that were previously not detected very well was achieved by mutations at the binding site and phage display selection by one of the weakly binding sulfonamides. Altogether, the anti-sulfonamide antibody has been optimised at three subsequent steps towards more broad-specific and sensitive binding in a manner that would be difficult to realise *in vivo*. With the capability to recognise different sulfonamides, the resulting antibody is one of the most generic group-specific antibodies in the world. The antibody establishes rapid screening for different sulfonamide drug residues in a single test and is a valuable tool for future assays (**II**).

Recombinant antibody fragments have not been exploited widely in immunoassays and the performance of recombinant immunoassays have not been thoroughly characterised. We exploited site-specifically biotinylated recombinant scFv and Fab antibody fragments as capture reagents in a rapid immunoassay for TSH and compared the performance of the assay to the parental monoclonal antibody. Fast kinetics was achieved by maximising analyte-binding capacity with the small-sized, correctly oriented recombinant scFv and Fab antibody fragments (**IV**). Despite the benefits in capacity and kinetics, the anti-TSH scFv was found to be rather unstable and it had a tendency to aggregate in solution, which limis its usability. Engineering significantly improved the stability of the scFv (**III**), but despite the apparently sufficient stability, the stabilized anti-TSH scFv did not perform in the assay as well as the unmodified, inherently stable Fab fragment. Indeed, the anti-TSH Fab fragment appeared to be a very promising immunoreagent providing an assay with fast kinetics, sensitive detection and reliable performance (**IV)**.

Finally, we constructed a synthetic antibody library. The library uses a scFv framework, which consists of a stable and well expressing V_L and V_H domain, which are often also enriched from naive libraries. Together with a successful diversification strategy, the framework should provide good performance for the library. The library allows isolation of new, specific high-affinity antibodies within a relatively short time scale. From the library, binders can also be potentially obtained for difficult antigens such as toxic and self-antigens. The library design establishes affinity maturation by light chain shuffling. The affinities of the isolated antibodies were similar to the antibodies from the secondary immune response in nature or other synthetic libraries of similar size (**V**).

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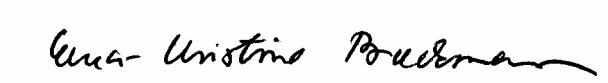

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REFERENCES

Achari A., Somers D.O., Champness J.N., Bryant P.K., Rosemond J. and Stammers D.K. (1997) Crystal structure of the anti-bacterial sulfonamide drug target dihydropteroate synthase. *Nat.Struct.Biol.* **4:** 490-497.

Ali S.A., Joao H.C., Hammerschmid F., Eder J. and Steinkasserer A. (1999) Transferrin trojan horses as a rational approach for the biological delivery of therapeutic peptide domains. *J.Biol.Chem.* **274:** 24066-24073.

Almagro J.C. (2004) Identification of differences in the specificity-determining residues of antibodies that recognize antigens of different size: implications for the rational design of antibody repertoires. *J.Mol.Recognit.* **17:** 132-143.

Ames R.S., Tornetta M.A., Jones C.S. and Tsui P. (1994) Isolation of neutralizing anti-C5a monoclonal antibodies from a filamentous phage monovalent Fab display library. *J.Immunol.* **152:** 4572-4581.

Arai R., Ueda H., Tsumoto K., Mahoney W.C., Kumagai I. and Nagamune T. (2000) Fluorolabeling of antibody variable domains with green fluorescent protein variants: application to an energy transfer-based homogeneous immunoassay. *Protein Eng.* **13:** 369-376.

Arai R., Nakagawa H., Tsumoto K., Mahoney W., Kumagai I., Ueda H. and Nagamune T. (2001) Demonstration of a homogeneous noncompetitive immunoassay based on bioluminescence resonance energy transfer. *Anal.Biochem.* **289:** 77-81.

Arbabi Ghahroudi M., Desmyter A., Wyns L., Hamers R. and Muyldermans S. (1997) Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett.* **414:** 521-526.

Arndt K.M., Muller K.M. and Pluckthun A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry* **37:** 12918-12926.

Azriel-Rosenfeld R., Valensi M. and Benhar I. (2004) A human synthetic combinatorial library of arrayable single-chain antibodies based on shuffling in vivo formed CDRs into general framework regions. *J.Mol.Biol.* **335:** 177-192.

Baca A.M., Sirawaraporn R., Turley S., Sirawaraporn W. and Hol W.G. (2000) Crystal structure of Mycobacterium tuberculosis 7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. *J.Mol.Biol.* **302:** 1193-1212.

Baggio R., Burgstaller P., Hale S.P., Putney A.R., Lane M., Lipovsek D., Wright M.C., Roberts R.W., Liu R., Szostak J.W., *et al.* (2002) Identification of epitope-like consensus motifs using mRNA display. *J.Mol.Recognit.* **15:** 126-134.

Balass M., Morag E., Bayer E.A., Fuchs S., Wilchek M. and Katchalski-Katzir E. (1996) Recovery of high-affinity phage from a nitrostreptavidin matrix in phage-display technology. *Anal.Biochem.* **243:** 264-269.

Balint R.F. and Larrick J.W. (1993) Antibody engineering by parsimonious mutagenesis. *Gene* **137:** 109-118.

Barbas C.F.,3rd, Kang A.S., Lerner R.A. and Benkovic S.J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc.Natl.Acad.Sci.U.S.A.* **88:** 7978-7982.

Barbas C.F.,3rd, Bain J.D., Hoekstra D.M. and Lerner R.A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc.Natl.Acad.Sci.U.S.A.* **89:** 4457-4461.

Barbas C.F.,3rd, Amberg W., Simoncsits A., Jones T.M. and Lerner R.A. (1993a) Selection of human anti-hapten antibodies from semisynthetic libraries. *Gene* **137:** 57-62.

Barbas C.F.,3rd, Collet T.A., Amberg W., Roben P., Binley J.M., Hoekstra D., Cababa D., Jones T.M., Williamson R.A. and Pilkington G.R. (1993b) Molecular profile of an antibody response to HIV-1 as probed by combinatorial libraries. *J.Mol.Biol.* **230:** 812-823.

Barbas C.F.,3rd, Hu D., Dunlop N., Sawyer L., Cababa D., Hendry R.M., Nara P.L. and Burton D.R. (1994) In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc.Natl.Acad.Sci.U.S.A.* **91:** 3809-3813.

Barderas R., Desmet J., Timmerman P., Meloen R. and Casal J.I. (2008) Affinity maturation of antibodies assisted by in silico modeling. *Proc.Natl.Acad.Sci.U.S.A.* **105:** 9029-9034.

Batista F.D. and Neuberger M.S. (1998) Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. *Immunity* **8:** 751-759.

Benhar I. (2007) Design of synthetic antibody libraries. *Expert Opin.Biol.Ther.* **7:** 763-779.

Beste G., Schmidt F.S., Stibora T. and Skerra A. (1999) Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc.Natl.Acad.Sci.U.S.A.* **96:** 1898-1903.

Better M., Chang C.P., Robinson R.R. and Horwitz A.H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. *Science* **240:** 1041-1043.

Bienenmann-Ploum M., Korpimäki T., Haasnoot W. and Kohen F. (2005) Comparison of multi-sulfonamide biosensor immunoassays. *Anal.Chim.Acta* **529:** 115-122.

Biles B.D. and Connolly B.A. (2004) Low-fidelity Pyrococcus furiosus DNA polymerase mutants useful in error-prone PCR. *Nucleic Acids Res.* **32:** e176.

Binz H.K., Stumpp M.T., Forrer P., Amstutz P. and Pluckthun A. (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J.Mol.Biol.* **332:** 489-503.

Binz H.K., Amstutz P., Kohl A., Stumpp M.T., Briand C., Forrer P., Grutter M.G. and Pluckthun A. (2004) High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat.Biotechnol.* **22:** 575-582.

Binz H.K., Amstutz P. and Pluckthun A. (2005) Engineering novel binding proteins from nonimmunoglobulin domains. *Nat.Biotechnol.* **23:** 1257-1268.

Birtalan S., Zhang Y., Fellouse F.A., Shao L., Schaefer G. and Sidhu S.S. (2008) The intrinsic contributions of tyrosine, serine, glycine and arginine to the affinity and specificity of antibodies. *J.Mol.Biol.* **377:** 1518-1528.

Bjerner J., Nustad K., Norum L.F., Olsen K.H. and Bormer O.P. (2002) Immunometric assay interference: incidence and prevention. *Clin.Chem.* **48:** 613-621.

Bjoro T., Holmen J., Kruger O., Midthjell K., Hunstad K., Schreiner T., Sandnes L. and Brochmann H. (2000) Prevalence of thyroid disease, thyroid dysfunction and thyroid peroxidase antibodies in a large, unselected population. The Health Study of Nord-Trondelag (HUNT). *Eur.J.Endocrinol.* **143:** 639-647.

Bloom L. and Calabro V. (2009) FN3: a new protein scaffold reaches the clinic. *Drug Discov.Today* **14:** 949-955.

Boder E.T. and Wittrup K.D. (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat.Biotechnol.* **15:** 553-557.

Boder E.T., Midelfort K.S. and Wittrup K.D. (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc.Natl.Acad.Sci.U.S.A.* **97:** 10701-10705.

Boder E.T. and Wittrup K.D. (1998) Optimal Screening of Surface-Displayed Polypeptide Libraries. *Biotechnol. Prog.* **14:** 55-62.

Borghouts C., Kunz C. and Groner B. (2005) Peptide aptamers: recent developments for cancer therapy. *Expert Opin.Biol.Ther.* **5:** 783-797.

Bork P. (1993) Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally? *Proteins* **17:** 363-374.

Borrebaeck C.A. and Ohlin M. (2002) Antibody evolution beyond Nature. *Nat.Biotechnol.* **20:** 1189-1190.

Bothmann H. and Pluckthun A. (2000) The periplasmic Escherichia coli peptidylprolyl cis,trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. *J.Biol.Chem.* **275:** 17100-17105.

Bowley D.R., Labrijn A.F., Zwick M.B. and Burton D.R. (2007) Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. *Protein Eng.Des.Sel.* **20:** 81-90.

Bradbury A.R. and Marks J.D. (2004) Antibodies from phage antibody libraries. *J.Immunol.Methods* **290:** 29-49.

Braenden C.I. (1991) The TIM barrel. The most frequently occurring folding motif in proteins. *Curr. Opin. Struct. Biol.* **1:** 978-983.

Braunagel M. and Little M. (1997) Construction of a semisynthetic antibody library using trinucleotide oligos. *Nucleic Acids Res.* **25:** 4690-4691.

Breitling F., Dubel S., Seehaus T., Klewinghaus I. and Little M. (1991) A surface expression vector for antibody screening. *Gene* **104:** 147-153.

Brockmann E.C., Cooper M., Stromsten N., Vehniainen M. and Saviranta P. (2005) Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions. *J.Immunol.Methods* **296:** 159-170.

Brocks B., Kraft S., Zahn S., Noll S., Pan C., Schauer M. and Krebs B. (2006) Generation and optimization of human antagonistic antibodies against TIMP-1 as potential therapeutic agents in fibrotic diseases. *Hum.Antibodies* **15:** 115-124.

Burks E.A., Chen G., Georgiou G. and Iverson B.L. (1997) In vitro scanning saturation mutagenesis of an antibody binding pocket. *Proc.Natl.Acad.Sci.U.S.A.* **94:** 412-417.

Burton D.R., Barbas C.F.,3rd, Persson M.A., Koenig S., Chanock R.M. and Lerner R.A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc.Natl.Acad.Sci.U.S.A.* **88:** 10134-10137.

Cabilly S., Riggs A.D., Pande H., Shively J.E., Holmes W.E., Rey M., Perry L.J., Wetzel R. and Heyneker H.L. (1984) Generation of antibody activity from immunoglobulin polypeptide chains produced in Escherichia coli. *Proc.Natl.Acad.Sci.U.S.A.* **81:** 3273-3277.

Cadwell R.C. and Joyce G.F. (1992) Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* **2:** 28-33.

Carlsson A., Wingren C., Ingvarsson J., Ellmark P., Baldertorp B., Ferno M., Olsson H. and Borrebaeck C.A. (2008) Serum proteome profiling of metastatic breast cancer using recombinant antibody microarrays. *Eur.J.Cancer* **44:** 472-480.

Carmen S. and Jermutus L. (2002) Concepts in antibody phage display. *Brief Funct.Genomic Proteomic* **1:** 189-203.

Chames P., Coulon S. and Baty D. (1998) Improving the affinity and the fine specificity of an anti-cortisol antibody by parsimonious mutagenesis and phage display. *J.Immunol.* **161:** 5421-5429.

Charlton K., Harris W.J. and Porter A.J. (2001) The isolation of super-sensitive anti-hapten antibodies from combinatorial antibody libraries derived from sheep. *Biosens.Bioelectron.* **16:** 639-646.

Chen C., Roberts V.A., Stevens S., Brown M., Stenzel-Poore M.P. and Rittenberg M.B. (1995) Enhancement and destruction of antibody function by somatic mutation: unequal occurrence is controlled by V gene combinatorial associations. *EMBO J.* **14:** 2784-2794.

Chen G., Dubrawsky I., Mendez P., Georgiou G. and Iverson B.L. (1999) In vitro scanning saturation mutagenesis of all the specificity determining residues in an antibody binding site. *Protein Eng.* **12:** 349-356.

Chen W., Zhu Z., Feng Y., Xiao X. and Dimitrov D.S. (2008) Construction of a large phage-displayed human antibody domain library with a scaffold based on a newly identified highly soluble, stable heavy chain variable domain. *J.Mol.Biol.* **382:** 779-789.

Chen W., Zhu Z., Feng Y. and Dimitrov D.S. (2009) A large human domain antibody library combining heavy and light chain CDR3 diversity. *Mol.Immunol.* **:** .

Chen Y., Wiesmann C., Fuh G., Li B., Christinger H.W., McKay P., de Vos A.M. and Lowman H.B. (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J.Mol.Biol.* **293:** 865-881.

Cheng X.J., Hayasaka H., Watanabe K., Tao Y.L., Liu J.Y., Tsukamoto H., Horii T., Tanabe K. and Tachibana H. (2007) Production of high-affinity human monoclonal antibody fab fragments to the 19-kilodalton C-terminal merozoite surface protein 1 of Plasmodium falciparum. *Infect.Immun.* **75:** 3614-3620.

Chlewicki L.K., Holler P.D., Monti B.C., Clutter M.R. and Kranz D.M. (2005) High-affinity, peptide-specific T cell receptors can be generated by mutations in CDR1, CDR2 or CDR3. *J.Mol.Biol.* **346:** 223-239.

Chmura A.J., Orton M.S. and Meares C.F. (2001) Antibodies with infinite affinity. *Proc.Natl.Acad.Sci.U.S.A.* **98:** 8480-8484.

Chodorge M., Fourage L., Ravot G., Jermutus L. and Minter R. (2008) In vitro DNA recombination by L-Shuffling during ribosome display affinity maturation of an anti-Fas antibody increases the population of improved variants. *Protein Eng.Des.Sel.* **21:** 343-351.

Chothia C. and Lesk A.M. (1987) Canonical structures for the hypervariable regions of immunoglobulins. *J.Mol.Biol.* **196:** 901-917.

Chothia C., Lesk A.M., Tramontano A., Levitt M., Smith-Gill S.J., Air G., Sheriff S., Padlan E.A., Davies D. and Tulip W.R. (1989) Conformations of immunoglobulin hypervariable regions. *Nature* **342:** 877-883.

Chothia C., Lesk A.M., Gherardi E., Tomlinson I.M., Walter G., Marks J.D., Llewelyn M.B. and Winter G. (1992) Structural repertoire of the human VH segments. *J.Mol.Biol.* **227:** 799-817.

Chowdhury P.S. and Pastan I. (1999) Improving antibody affinity by mimicking somatic hypermutation in vitro. *Nat.Biotechnol.* **17:** 568-572.

Christensen P.A., Danielczyk A., Ravn P., Larsen M., Stahn R., Karsten U. and Goletz S. (2009) Modifying antibody specificity by chain shuffling of V / V between antibodies with related specificities. *Scand.J.Immunol.* **69:** 1-10.

Christmann A., Walter K., Wentzel A., Kratzner R. and Kolmar H. (1999) The cystine knot of a squash-type protease inhibitor as a structural scaffold for Escherichia coli cell surface display of conformationally constrained peptides. *Protein Eng.* **12:** 797-806.

Clackson T., Hoogenboom H.R., Griffiths A.D. and Winter G. (1991) Making antibody fragments using phage display libraries. *Nature* **352:** 624-628.

Clark M.A., Hawkins N.J., Papaioannou A., Fiddes R.J. and Ward R.L. (1997) Isolation of human anti-c-erbB-2 Fabs from a lymph node-derived phage display library. *Clin.Exp.Immunol.* **109:** 166-174.

Cliquet P., Cox E., Haasnoot W., Schacht E. and Goddeeris B.M. (2003) Generation of group-specific antibodies against sulfonamides. *J.Agric.Food Chem.* **51:** 5835-5842.

Cobaugh C.W., Almagro J.C., Pogson M., Iverson B. and Georgiou G. (2008) Synthetic antibody libraries focused towards peptide ligands. *J.Mol.Biol.* **378:** 622-633.

Coco W.M., Encell L.P., Levinson W.E., Crist M.J., Loomis A.K., Licato L.L., Arensdorf J.J., Sica N., Pienkos P.T. and Monticello D.J. (2002) Growth factor engineering by degenerate homoduplex gene family recombination. *Nat.Biotechnol.* **20:** 1246-1250.

Coia G., Ayres A., Lilley G.G., Hudson P.J. and Irving R.A. (1997) Use of mutator cells as a means for increasing production levels of a recombinant antibody directed against Hepatitis B. *Gene* **201:** 203-209.

Collis A.V., Brouwer A.P. and Martin A.C. (2003) Analysis of the antigen combining site: correlations between length and sequence composition of the hypervariable loops and the nature of the antigen. *J.Mol.Biol.* **325:** 337-354.

Cornish-Bowden A. (1985) Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. *Nucleic Acids Res.* **13:** 3021-3030.

Dallas W.S., Gowen J.E., Ray P.H., Cox M.J. and Dev I.K. (1992) Cloning, sequencing, and enhanced expression of the dihydropteroate synthase gene of Escherichia coli MC4100. *J.Bacteriol.* **174:** 5961-5970.

Daugherty P.S., Chen G., Iverson B.L. and Georgiou G. (2000) Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc.Natl.Acad.Sci.U.S.A.* **97:** 2029-2034.

Davies J. and Riechmann L. (1996) Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability. *Protein Eng.* **9:** 531-537.

de Bruin R., Spelt K., Mol J., Koes R. and Quattrocchio F. (1999) Selection of high-affinity phage antibodies from phage display libraries. *Nat.Biotechnol.* **17:** 397-399.

de Haard H.J., van Neer N., Reurs A., Hufton S.E., Roovers R.C., Henderikx P., de Bruine A.P., Arends J.W. and Hoogenboom H.R. (1999) A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J.Biol.Chem.* **274:** 18218-18230.

de Keizer W., Bienenmann-Ploum M.E., Bergwerff A.A. and Haasnoot W. (2008) Flow cytometric immunoassay for sulfonamides in raw milk. *Anal.Chim.Acta* **620:** 142-149.

de Kruif J., Boel E. and Logtenberg T. (1995) Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J.Mol.Biol.* **248:** 97-105.

de Wildt R.M., Tomlinson I.M., van Venrooij W.J., Winter G. and Hoet R.M. (2000) Comparable heavy and light chain pairings in normal and systemic lupus erythematosus IgG(+) B cells. *Eur.J.Immunol.* **30:** 254-261.

Dennis M.S. and Lazarus R.A. (1994) Kunitz domain inhibitors of tissue factor-factor VIIa. I. Potent inhibitors selected from libraries by phage display. *J.Biol.Chem.* **269:** 22129-22136.

Desiderio A., Franconi R., Lopez M., Villani M.E., Viti F., Chiaraluce R., Consalvi V., Neri D. and Benvenuto E. (2001) A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J.Mol.Biol.* **310:** 603-615.

Desplancq D., King D.J., Lawson A.D. and Mountain A. (1994) Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Eng.* **7:** 1027-1033.

Dexlin L., Ingvarsson J., Frendeus B., Borrebaeck C.A. and Wingren C. (2008) Design of recombinant antibody microarrays for cell surface membrane proteomics. *J.Proteome Res.* **7:** 319-327.

Dibbern D.A.,Jr and Montanaro A. (2008) Allergies to sulfonamide antibiotics and sulfur-containing drugs. *Ann.Allergy Asthma Immunol.* **100:** 91-100; quiz 100-3, 111.

Dinh Q., Weng N.P., Kiso M., Ishida H., Hasegawa A. and Marcus D.M. (1996) High affinity antibodies against Lex and sialyl Lex from a phage display library. *J.Immunol.* **157:** 732-738.

Ditzel H.J., Binley J.M., Moore J.P., Sodroski J., Sullivan N., Sawyer L.S., Hendry R.M., Yang W.P., Barbas C.F.,3rd and Burton D.R. (1995) Neutralizing recombinant human antibodies to a conformational V2- and CD4-binding site-sensitive epitope of HIV-1 gp120 isolated by using an epitope-masking procedure. *J.Immunol.* **154:** 893-906.

Dolezal O., Pearce L.A., Lawrence L.J., McCoy A.J., Hudson P.J. and Kortt A.A. (2000) ScFv multimers of the anti-neuraminidase antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V(L) to V(H) orientation drives the formation of dimers, trimers, tetramers and higher molecular mass multimers. *Protein Eng.* **13:** 565-574.

Dooley H., Flajnik M.F. and Porter A.J. (2003) Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. *Mol.Immunol.* **40:** 25-33.

Drummond D.A., Iverson B.L., Georgiou G. and Arnold F.H. (2005) Why high-error-rate random mutagenesis libraries are enriched in functional and improved proteins. *J.Mol.Biol.* **350:** 806-816.

Duenas M. and Borrebaeck C.A. (1994) Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. *Biotechnology (N.Y)* **12:** 999-1002.

Duenas M., Malmborg A.C., Casalvilla R., Ohlin M. and Borrebaeck C.A. (1996) Selection of phage displayed antibodies based on kinetic constants. *Mol.Immunol.* **33:** 279-285.

Dufner P., Jermutus L. and Minter R.R. (2006) Harnessing phage and ribosome display for antibody optimisation. *Trends Biotechnol.* **24:** 523-529.

Eggel A., Baumann M.J., Amstutz P., Stadler B.M. and Vogel M. (2009) DARPins as bispecific receptor antagonists analyzed for immunoglobulin E receptor blockage. *J.Mol.Biol.* **393:** 598-607.

Eriksson S., Vehniainen M., Jansen T., Meretoja V., Saviranta P., Pettersson K. and Lovgren T. (2000) Dual-label time-resolved immunofluorometric assay of free and total prostate-specific antigen based on recombinant Fab fragments. *Clin.Chem.* **46:** 658-666.

Essen L.O. and Skerra A. (1993) Single-step purification of a bacterially expressed antibody Fv fragment by immobilized metal affinity chromatography in the presence of betaine. *J.Chromatogr.A* **657:** 55-61.

Eu regulation no. 508/1999, L60(9-3-1999), 1999; pp 16-52.

Ewert S., Huber T., Honegger A. and Pluckthun A. (2003) Biophysical properties of human antibody variable domains. *J.Mol.Biol.* **325:** 531-553.

Famm K. and Winter G. (2006) Engineering aggregation-resistant proteins by directed evolution. *Protein Eng.Des.Sel.* **19:** 479-481.

Famm K., Hansen L., Christ D. and Winter G. (2008) Thermodynamically stable aggregation-resistant antibody domains through directed evolution. *J.Mol.Biol.* **376:** 926-931.

Fellouse F.A., Wiesmann C. and Sidhu S.S. (2004) Synthetic antibodies from a four-amino-acid code: a dominant role for tyrosine in antigen recognition. *Proc.Natl.Acad.Sci.U.S.A.* **101:** 12467-12472.

Fellouse F.A., Li B., Compaan D.M., Peden A.A., Hymowitz S.G. and Sidhu S.S. (2005) Molecular recognition by a binary code. *J.Mol.Biol.* **348:** 1153-1162.

Fellouse F.A., Esaki K., Birtalan S., Raptis D., Cancasci V.J., Koide A., Jhurani P., Vasser M., Wiesmann C., Kossiakoff A.A., *et al.* (2007) High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries. *J.Mol.Biol.* **373:** 924-940.

Figini M., Obici L., Mezzanzanica D., Griffiths A., Colnaghi M.I., Winter G. and Canevari S. (1998) Panning phage antibody libraries on cells: isolation of human Fab fragments against ovarian carcinoma using guided selection. *Cancer Res.* **58:** 991-996.

Finlay W.J., Cunningham O., Lambert M.A., Darmanin-Sheehan A., Liu X., Fennell B.J., Mahon C.M., Cummins E., Wade J.M., O'Sullivan C.M., *et al.* (2009) Affinity maturation of a humanized rat antibody for anti-RAGE therapy: comprehensive mutagenesis reveals a high level of mutational plasticity both inside and outside the complementarity-determining regions. *J.Mol.Biol.* **388:** 541-558.

Food and Drug Regulation, Canad Gazette Part II, Table 3, Division 15, Part B., 1991; 125, pp. 1478-1480.

Foote J. and Eisen H.N. (1995) Kinetic and affinity limits on antibodies produced during immune responses. *Proc.Natl.Acad.Sci.U.S.A.* **92:** 1254-1256.

Foote J. and Eisen H.N. (2000) Breaking the affinity ceiling for antibodies and T cell receptors. *Proc.Natl.Acad.Sci.U.S.A.* **97:** 10679-10681.

Forrer P., Stumpp M.T., Binz H.K. and Pluckthun A. (2003) A novel strategy to design binding molecules harnessing the modular nature of repeat proteins. *FEBS Lett.* **539:** 2-6.

Fowler R.G., Schaaper R.M. and Glickman B.W. (1986) Characterization of mutational specificity within the lacI gene for a mutD5 mutator strain of Escherichia coli defective in 3'----5' exonuclease (proofreading) activity. *J.Bacteriol.* **167:** 130-137.

Francisco J.A., Campbell R., Iverson B.L. and Georgiou G. (1993) Production and fluorescence-activated cell sorting of Escherichia coli expressing a functional antibody fragment on the external surface. *Proc.Natl.Acad.Sci.U.S.A.* **90:** 10444-10448.

Frippiat J.P., Williams S.C., Tomlinson I.M., Cook G.P., Cherif D., Le Paslier D., Collins J.E., Dunham I., Winter G. and Lefranc M.P. (1995) Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum.Mol.Genet.* **4:** 983-991.

Frisch C., Kolmar H., Schmidt A., Kleemann G., Reinhardt A., Pohl E., Uson I., Schneider T.R. and Fritz H.J. (1996) Contribution of the intramolecular disulfide bridge to the folding stability of REIv, the variable domain of a human immunoglobulin kappa light chain. *Fold.Des.* **1:** 431-440.

Fromant M., Blanquet S. and Plateau P. (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal.Biochem.* **224:** 347-353.

Fuchs P., Dubel S., Breitling F., Braunagel M., Klewinghaus I. and Little M. (1992) Recombinant human monoclonal antibodies. Basic principles of the immune system transferred to E. coli. *Cell Biophys.* **21:** 81-91.

Fukuda I., Kojoh K., Tabata N., Doi N., Takashima H., Miyamoto-Sato E. and Yanagawa H. (2006) In vitro evolution of single-chain antibodies using mRNA display. *Nucleic Acids Res.* **34:** e127.

Furuta M., Ito T., Eguchi C., Tanaka T., Wakabayashi-Takai E. and Kaneko K. (2002) Two-dimensional electrophoresis/phage panning (2D-PP): a novel technology for direct antibody selection on 2-D blots. *J.Biochem.* **132:** 245-251.

Gao C., Mao S., Kaufmann G., Wirsching P., Lerner R.A. and Janda K.D. (2002) A method for the generation of combinatorial antibody libraries using pIX phage display. *Proc.Natl.Acad.Sci.U.S.A.* **99:** 12612-12616.

Garrard L.J., Yang M., O'Connell M.P., Kelley R.F. and Henner D.J. (1991) Fab assembly and enrichment in a monovalent phage display system. *Biotechnology (N.Y)* **9:** 1373-1377.

Gilbreth R.N., Esaki K., Koide A., Sidhu S.S. and Koide S. (2008) A dominant conformational role for amino acid diversity in minimalist protein-protein interfaces. *J.Mol.Biol.* **381:** 407-418.

Goletz S., Christensen P.A., Kristensen P., Blohm D., Tomlinson I., Winter G. and Karsten U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J.Mol.Biol.* **315:** 1087-1097.

Gonzalez-Techera A., Umpierrez-Failache M., Cardozo S., Obal G., Pritsch O., Last J.A., Gee S.J., Hammock B.D. and Gonzalez-Sapienza G. (2008) High-throughput method for ranking the affinity of peptide ligands selected from phage display libraries. *Bioconjug.Chem.* **19:** 993-1000.

Graff C.P., Chester K., Begent R. and Wittrup K.D. (2004) Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. *Protein Eng.Des.Sel.* **17:** 293-304.

Gram H., Marconi L.A., Barbas C.F.,3rd, Collet T.A., Lerner R.A. and Kang A.S. (1992) In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc.Natl.Acad.Sci.U.S.A.* **89:** 3576-3580.

Green N.M. (1990) Avidin and streptavidin. *Methods Enzymol.* **184:** 51-67.

Griffiths A.D., Malmqvist M., Marks J.D., Bye J.M., Embleton M.J., McCafferty J., Baier M., Holliger K.P., Gorick B.D. and Hughes-Jones N.C. (1993) Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* **12:** 725-734.

Griffiths A.D., Williams S.C., Hartley O., Tomlinson I.M., Waterhouse P., Crosby W.L., Kontermann R.E., Jones P.T., Low N.M. and Allison T.J. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13:** 3245-3260.

Griffiths A.D. and Duncan A.R. (1998) Strategies for selection of antibodies by phage display. *Curr.Opin.Biotechnol.* **9:** 102-108.

Gronwall C. and Stahl S. (2009) Engineered affinity proteins--generation and applications. *J.Biotechnol.* **140:** 254-269.

Guchhait P., Lopez J.A. and Thiagarajan P. (2004) Characterization of autoantibodies against sulfatide from a V-gene phage-display library derived from patients with systemic lupus erythematosus. *J.Immunol.Methods* **295:** 129-137.

Haasnoot W., Du Pre J., Cazemier G., Kemmers-Voncken A., Verheijen R. and Jansen B.J.M. (2000a) Monoclonal Antibodies Against a Sulfathiazole Derivative for the Immunochemical Detection of Sulfonamides. *Food Agric.Immunol.* **12:** 127-138.

Haasnoot W., Cazemier G., Du Pre J., Kemmers-Voncken A., Bienenmann-Ploum M. and Verheijen R. (2000b) Sulphonamide antibodies: from specific polyclonals to generic monoclonals. *Food Agric. Immunol.* **12:** 15-30.

Hackel B.J., Kapila A. and Wittrup K.D. (2008) Picomolar affinity fibronectin domains engineered utilizing loop length diversity, recursive mutagenesis, and loop shuffling. *J.Mol.Biol.* **381:** 1238-1252.

Hamers-Casterman C., Atarhouch T., Muyldermans S., Robinson G., Hamers C., Songa E.B., Bendahman N. and Hamers R. (1993) Naturally occurring antibodies devoid of light chains. *Nature* **363:** 446-448.

Hanes J. and Pluckthun A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc.Natl.Acad.Sci.U.S.A.* **94:** 4937-4942.

Hanes J., Jermutus L., Weber-Bornhauser S., Bosshard H.R. and Pluckthun A. (1998) Ribosome display efficiently selects and evolves high-affinity antibodies in vitro from immune libraries. *Proc.Natl.Acad.Sci.U.S.A.* **95:** 14130-14135.

Hanes J., Schaffitzel C., Knappik A. and Pluckthun A. (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat.Biotechnol.* **18:** 1287-1292.

Harma H., Tarkkinen P., Soukka T. and Lovgren T. (2000) Miniature single-particle immunoassay for prostate-specific antigen in serum using recombinant Fab fragments. *Clin.Chem.* **46:** 1755-1761.

Hawkins R.E., Russell S.J. and Winter G. (1992) Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J.Mol.Biol.* **226:** 889-896.

Hawkins R.E., Russell S.J., Baier M. and Winter G. (1993) The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen. The interaction of mutant D1.3 antibodies with lysozyme. *J.Mol.Biol.* **234:** 958-964.

Hawlisch H., Meyer zu Vilsendorf A., Bautsch W., Klos A. and Kohl J. (2000) Guinea pig C3 specific rabbit single chain Fv antibodies from bone marrow, spleen and blood derived phage libraries. *J.Immunol.Methods* **236:** 117-131.

He M. and Taussig M.J. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. *Nucleic Acids Res.* **25:** 5132-5134.

Helfand M. and Redfern C.C. (1998) Clinical guideline, part 2. Screening for thyroid disease: an update. American College of Physicians. *Ann.Intern.Med.* **129:** 144-158.

Hellman J., Wahlberg M., Karp M., Korpela T., Mäntsälä P. (1990) Effects of modifications at the C-terminus of cyclomaltodextrin glucanotransferase from Bacillus circulans var alkalophilus on catalytic activity. *Biotechnol. Appl. Biochem.* **12:** 387-396.

Hemminki A., Niemi S., Hautoniemi L., Soderlund H. and Takkinen K. (1998) Fine tuning of an anti-testosterone antibody binding site by stepwise optimisation of the CDRs. *Immunotechnology* **4:** 59-69.

Hey T., Fiedler E., Rudolph R. and Fiedler M. (2005) Artificial, non-antibody binding proteins for pharmaceutical and industrial applications. *Trends Biotechnol.* **23:** 514-522.

Ho M., Kreitman R.J., Onda M. and Pastan I. (2005) In vitro antibody evolution targeting germline hot spots to increase activity of an anti-CD22 immunotoxin. *J.Biol.Chem.* **280:** 607-617.

Ho M. and Pastan I. (2009) In vitro antibody affinity maturation targeting germline hotspots. *Methods Mol.Biol.* **525:** 293-308, xiv.

Hoet R.M., Cohen E.H., Kent R.B., Rookey K., Schoonbroodt S., Hogan S., Rem L., Frans N., Daukandt M., Pieters H., *et al.* (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat.Biotechnol.* **23:** 344-348.

Holt L.J., Herring C., Jespers L.S., Woolven B.P. and Tomlinson I.M. (2003) Domain antibodies: proteins for therapy. *Trends Biotechnol.* **21:** 484-490.

Honegger A. (2008) Engineering antibodies for stability and efficient folding. *Handb.Exp.Pharmacol.* **(181):** 47-68.

Hoogenboom H.R., Griffiths A.D., Johnson K.S., Chiswell D.J., Hudson P. and Winter G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res.* **19:** 4133-4137.

Hoogenboom H.R. and Winter G. (1992) By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J.Mol.Biol.* **227:** 381-388.

Hoogenboom H.R. (2005) Selecting and screening recombinant antibody libraries. *Nat.Biotechnol.* **23:** 1105-1116.

Horton R.M., Hunt H.D., Ho S.N., Pullen J.K. and Pease L.R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77:** 61-68.

Hosse R.J., Rothe A. and Power B.E. (2006) A new generation of protein display scaffolds for molecular recognition. *Protein Sci.* **15:** 14-27.

Hufton S.E., van Neer N., van den Beuken T., Desmet J., Sablon E. and Hoogenboom H.R. (2000) Development and application of cytotoxic T lymphocyte-associated antigen 4 as a protein scaffold for the generation of novel binding ligands. *FEBS Lett.* **475:** 225-231.

Huovinen T., Sanmark H., Yla-Pelto J., Vehniainen M. and Lamminmaki U. (2010) Oligovalent Fab display on M13 phage improved by directed evolution. *Mol.Biotechnol.* **44:** 221-231.

Huse W.D., Sastry L., Iverson S.A., Kang A.S., Alting-Mees M., Burton D.R., Benkovic S.J. and Lerner R.A. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* **246:** 1275-1281.

Hust M. and Dubel S. (2004) Mating antibody phage display with proteomics. *Trends Biotechnol.* **22:** 8-14.

Hust M., Meysing M., Schirrmann T., Selke M., Meens J., Gerlach G.F. and Dubel S. (2006) Enrichment of open reading frames presented on bacteriophage M13 using hyperphage. *BioTechniques* **41:** 335-342.

Huston J.S., Levinson D., Mudgett-Hunter M., Tai M.S., Novotny J., Margolies M.N., Ridge R.J., Bruccoleri R.E., Haber E. and Crea R. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc.Natl.Acad.Sci.U.S.A.* **85:** 5879-5883.

Ignatovich O., Tomlinson I.M., Jones P.T., Winter G. (1997) The creation of diversity in the human immunoglobulin V(lambda) repertoire. *J.Mol.Biol.* **268:** 69-77.

Ihara M., Suzuki T., Kobayashi N., Goto J. and Ueda H. (2009) Open-sandwich enzyme immunoassay for one-step noncompetitive detection of corticosteroid 11-deoxycortisol. *Anal.Chem.* **81:** 8298-8304.

Ingvarsson J., Larsson A., Sjoholm A.G., Truedsson L., Jansson B., Borrebaeck C.A. and Wingren C. (2007) Design of recombinant antibody microarrays for serum protein profiling: targeting of complement proteins. *J.Proteome Res.* **6:** 3527-3536.

Irving R.A., Kortt A.A. and Hudson P.J. (1996) Affinity maturation of recombinant antibodies using E. coli mutator cells. *Immunotechnology* **2:** 127-143.

Jager M. and Pluckthun A. (1999) Domain interactions in antibody Fv and scFv fragments: effects on unfolding kinetics and equilibria. *FEBS Lett.* **462:** 307-312.

Janeway C.A., Travers P., Walport M., Shlomchik M. (2001) *Immunobiology. The immune system in health and disease.* 5th ed., Current Biology Publications, London

Jarutat T., Nickels C., Frisch C., Stellmacher F., Hofig K.P., Knappik A. and Merz H. (2007) Selection of vimentin-specific antibodies from the HuCAL phage display library by subtractive panning on formalin-fixed, paraffin-embedded tissue. *Biol.Chem.* **388:** 651-658.

Jermutus L., Honegger A., Schwesinger F., Hanes J. and Pluckthun A. (2001) Tailoring in vitro evolution for protein affinity or stability. *Proc.Natl.Acad.Sci.U.S.A.* **98:** 75-80.

Jespers L., Schon O., Famm K. and Winter G. (2004) Aggregation-resistant domain antibodies selected on phage by heat denaturation. *Nat.Biotechnol.* **22:** 1161-1165.

Jung S. and Pluckthun A. (1997) Improving in vivo folding and stability of a single-chain Fv antibody fragment by loop grafting. *Protein Eng.* **10:** 959-966.

Jung S., Honegger A. and Pluckthun A. (1999) Selection for improved protein stability by phage display. *J.Mol.Biol.* **294:** 163-180.

Kang A.S., Barbas C.F., Janda K.D., Benkovic S.J. and Lerner R.A. (1991a) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc.Natl.Acad.Sci.U.S.A.* **88:** 4363-4366.

Kang A.S., Jones T.M. and Burton D.R. (1991b) Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc.Natl.Acad.Sci.U.S.A.* **88:** 11120-11123.

Katakura Y., Zhuang G., Nakatani T., Furuta T., Omasa T., Kishimoto M., Suga K. and Shioya S. (2004) A practical kinetic model for efficient isolation of useful antibodies from phage display libraries. *J. Mol. Catal. B: Enzym.* **28:** 191-200.

Kim S.J. and Hong H.J. (2007) Guided selection of human antibody light chains against TAG-72 using a phage display chain shuffling approach. *J.Microbiol.* **45:** 572-577.

Kimura R.H., Levin A.M., Cochran F.V. and Cochran J.R. (2009) Engineered cystine knot peptides that bind alphavbeta3, alphavbeta5, and alpha5beta1 integrins with low-nanomolar affinity. *Proteins* **77:** 359-369.

Kjaer S., Stausbol-Gron B., Wind T., Ravn P., Hobolt Jensen K., Kahns L. and Clark B.F. (1998) Glycerol diversifies phage repertoire selections and lowers non-specific phage absorption. *FEBS Lett.* **431:** 448-452.

Knappik A., Ge L., Honegger A., Pack P., Fischer M., Wellnhofer G., Hoess A., Wolle J., Pluckthun A. and Virnekas B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J.Mol.Biol.* **296:** 57-86.

Kobayashi N., Oyama H., Nakano M., Kanda T., Banzono E., Kato Y., Karibe T., Nishio T. and Goto J. (2009) "Cleavable" hapten-biotin conjugates: preparation and use for the generation of anti-steroid single-domain antibody fragments. *Anal.Biochem.* **387:** 257-266.

Kohler G. and Milstein C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495-497.

Koide A., Bailey C.W., Huang X. and Koide S. (1998) The fibronectin type III domain as a scaffold for novel binding proteins. *J.Mol.Biol.* **284:** 1141-1151.

Koide A., Abbatiello S., Rothgery L. and Koide S. (2002) Probing protein conformational changes in living cells by using designer binding proteins: application to the estrogen receptor. *Proc.Natl.Acad.Sci.U.S.A.* **99:** 1253-1258.

Koide A., Tereshko V., Uysal S., Margalef K., Kossiakoff A.A. and Koide S. (2007a) Exploring the capacity of minimalist protein interfaces: interface energetics and affinity maturation to picomolar KD of a single-domain antibody with a flat paratope. *J.Mol.Biol.* **373:** 941-953.

Koide A., Gilbreth R.N., Esaki K., Tereshko V., Koide S. (2007b) High-affinity single-domain binding proteins with a binary-code interface. *Proc Natl Acad Sci U S A* **104:** 6632-6637.

Koide A., Wojcik J., Gilbreth R.N., Reichel A., Piehler J. and Koide S. (2009) Accelerating phage-display library selection by reversible and site-specific biotinylation. *Protein Eng.Des.Sel.* **22:** 685-690.

Kokko L., Sandberg K., Lovgren T. and Soukka T. (2004) Europium(III) chelate-dyed nanoparticles as donors in a homogeneous proximity-based immunoassay for estradiol. *Anal. Chim. Acta* **503:** 155-162.

Korpimaki T., Rosenberg J., Virtanen P., Karskela T., Lamminmaki U., Tuomola M., Vehniainen M. and Saviranta P. (2002) Improving broad specificity hapten recognition with protein engineering. *J.Agric.Food Chem.* **50:** 4194-4201.

Korpimaki T., Rosenberg J., Virtanen P., Lamminmaki U., Tuomola M. and Saviranta P. (2003) Further improvement of broad specificity hapten recognition with protein engineering. *Protein Eng.* **16:** 37-46.

Korpimaki T., Brockmann E.C., Kuronen O., Saraste M., Lamminmaki U. and Tuomola M. (2004a) Engineering of a broad specificity antibody for simultaneous detection of 13 sulfonamides at the maximum residue level. *J.Agric.Food Chem.* **52:** 40-47.

Korpimaki T., Hagren V., Brockmann E.C. and Tuomola M. (2004b) Generic lanthanide fluoroimmunoassay for the simultaneous screening of 18 sulfonamides using an engineered antibody. *Anal.Chem.* **76:** 3091-3098.

Krag D.N., Shukla G.S., Shen G.P., Pero S., Ashikaga T., Fuller S., Weaver D.L., Burdette-Radoux S. and Thomas C. (2006) Selection of tumor-binding ligands in cancer patients with phage display libraries. *Cancer Res.* **66:** 7724-7733.

Kramer K. (2002a) Synthesis of a group-selective antibody library against haptens. *J.Immunol.Methods* **266:** 209-220.

Kramer K. (2002b) Evolutionary affinity and selectivity optimization of a pesticide-selective antibody utlizing a hapten-selective immunoglobulin repertoire. *Environ.Sci.Technol.* **36:** 4892-4898.

Krebber A., Bornhauser S., Burmester J., Honegger A., Willuda J., Bosshard H.R. and Pluckthun A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J.Immunol.Methods* **201:** 35-55.

Krebs B., Rauchenberger R., Reiffert S., Rothe C., Tesar M., Thomassen E., Cao M., Dreier T., Fischer D., Hoss A., *et al.* (2001) High-throughput generation and engineering of recombinant human antibodies. *J.Immunol.Methods* **254:** 67-84.

Kretzschmar T., Zimmermann C. and Geiser M. (1995) Selection procedures for nonmatured phage antibodies: a quantitative comparison and optimization strategies. *Anal.Biochem.* **224:** 413-419.

Kunkel T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc.Natl.Acad.Sci.U.S.A.* **82:** 488-492.

Ladenson P.W., Singer P.A., Ain K.B., Bagchi N., Bigos S.T., Levy E.G., Smith S.A., Daniels G.H. and Cohen H.D. (2000) American Thyroid Association guidelines for detection of thyroid dysfunction. *Arch.Intern.Med.* **160:** 1573-1575.

Laffly E., Pelat T., Cedrone F., Blesa S., Bedouelle H. and Thullier P. (2008) Improvement of an antibody neutralizing the anthrax toxin by simultaneous mutagenesis of its six hypervariable loops. *J.Mol.Biol.* **378:** 1094-1103.

Lamminmaki U., Pauperio S., Westerlund-Karlsson A., Karvinen J., Virtanen P.L., Lovgren T. and Saviranta P. (1999) Expanding the conformational diversity by random insertions to CDRH2 results in improved anti-estradiol antibodies. *J.Mol.Biol.* **291:** 589-602.

Lamminmaki U., Westerlund-Karlsson A., Toivola M. and Saviranta P. (2003) Modulating the binding properties of an anti-17beta-estradiol antibody by systematic mutation combinations. *Protein Sci.* **12:** 2549-2558.

Laukkanen M.L., Makinen-Kiljunen S., Isoherranen K., Haahtela T., Soderlund H. and Takkinen K. (2003) Hevein-specific recombinant IgE antibodies from human single-chain antibody phage display libraries. *J.Immunol.Methods* **278:** 271-281.

Lee C.V., Liang W.C., Dennis M.S., Eigenbrot C., Sidhu S.S. and Fuh G. (2004a) High-affinity human antibodies from phage-displayed synthetic Fab libraries with a single framework scaffold. *J.Mol.Biol.* **340:** 1073-1093.

Lee C.V., Sidhu S.S. and Fuh G. (2004b) Bivalent antibody phage display mimics natural immunoglobulin. *J.Immunol.Methods* **284:** 119-132.

Lee K.J., Mao S., Sun C., Gao C., Blixt O., Arrues S., Hom L.G., Kaufmann G.F., Hoffman T.Z., Coyle A.R., *et al.* (2002) Phage-display selection of a human single-chain fv antibody highly specific for melanoma and breast cancer cells using a chemoenzymatically synthesized G(M3)-carbohydrate antigen. *J.Am.Chem.Soc.* **124:** 12439-12446.

Lee W., Oh B.K., Lee W.H. and Choi J.W. (2005) Immobilization of antibody fragment for immunosensor application based on surface plasmon resonance. *Colloids Surf.B Biointerfaces* **40:** 143-148

Leung D.W., Chen E., Goeddel D.V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1:** 11-15.

Li Y., Cockburn W., Kilpatrick J.B. and Whitelam G.C. (2000a) High affinity ScFvs from a single rabbit immunized with multiple haptens. *Biochem.Biophys.Res.Commun.* **268:** 398-404.

Li Y., Kilpatrick J. and Whitelam G.C. (2000b) Sheep monoclonal antibody fragments generated using a phage display system. *J.Immunol.Methods* **236:** 133-146.

Lin P.K. and Brown D.M. (1989) Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues. *Nucleic Acids Res.* **17:** 10373-10383.

Ling M.M. (2003) Large antibody display libraries for isolation of high-affinity antibodies. *Comb.Chem.High Throughput Screen.* **6:** 421-432.

Lipovsek D., Lippow S.M., Hackel B.J., Gregson M.W., Cheng P., Kapila A. and Wittrup K.D. (2007) Evolution of an interloop disulfide bond in high-affinity antibody mimics based on fibronectin type III domain and selected by yeast surface display: molecular convergence with single-domain camelid and shark antibodies. *J.Mol.Biol.* **368:** 1024-1041.

Liu B. and Marks J.D. (2000) Applying phage antibodies to proteomics: selecting single chain Fv antibodies to antigens blotted on nitrocellulose. *Anal.Biochem.* **286:** 119-128.

Liu B., Huang L., Sihlbom C., Burlingame A. and Marks J.D. (2002) Towards proteome-wide production of monoclonal antibody by phage display. *J.Mol.Biol.* **315:** 1063-1073.

Liu J.L., Anderson G.P., Delehanty J.B., Baumann R., Hayhurst A. and Goldman E.R. (2007) Selection of cholera toxin specific IgNAR single-domain antibodies from a naive shark library. *Mol.Immunol.* **44:** 1775-1783.

Liu Z., Song D., Kramer A., Martin A.C., Dandekar T., Schneider-Mergener J., Bautz E.K. and Dubel S. (1999) Fine mapping of the antigen-antibody interaction of scFv215, a recombinant antibody inhibiting RNA polymerase II from Drosophila melanogaster. *J.Mol.Recognit.* **12:** 103-111.

Lorimer I.A. and Pastan I. (1995) Random recombination of antibody single chain Fv sequences after fragmentation with DNaseI in the presence of Mn2+. *Nucleic Acids Res.* **23:** 3067-3068.

Loset G.A., Lobersli I., Kavlie A., Stacy J.E., Borgen T., Kausmally L., Hvattum E., Simonsen B., Hovda M.B. and Brekke O.H. (2005) Construction, evaluation and refinement of a large human antibody phage library based on the IgD and IgM variable gene repertoire. *J.Immunol.Methods* **299:** 47-62.

Lou J., Marzari R., Verzillo V., Ferrero F., Pak D., Sheng M., Yang C., Sblattero D. and Bradbury A. (2001) Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. *J.Immunol.Methods* **253:** 233-242.

Low N.M., Holliger P.H. and Winter G. (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J.Mol.Biol.* **260:** 359-368.

Lu D., Shen J., Vil M.D., Zhang H., Jimenez X., Bohlen P., Witte L. and Zhu Z. (2003) Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity. *J.Biol.Chem.* **278:** 43496-43507.

Lu J. and Sloan S.R. (1999) An alternating selection strategy for cloning phage display antibodies. *J.Immunol.Methods* **228:** 109-119.

Luginbuhl B., Kanyo Z., Jones R.M., Fletterick R.J., Prusiner S.B., Cohen F.E., Williamson R.A., Burton D.R. and Pluckthun A. (2006) Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation. *J.Mol.Biol.* **363:** 75-97.

MacCallum R.M., Martin A.C. and Thornton J.M. (1996) Antibody-antigen interactions: contact analysis and binding site topography. *J.Mol.Biol.* **262:** 732-745.

Malmborg A.C., Duenas M., Ohlin M., Soderlind E. and Borrebaeck C.A. (1996) Selection of binders from phage displayed antibody libraries using the BIAcore biosensor. *J.Immunol.Methods* **198:** 51-57.

Malone J. and Sullivan M.A. (1996) Analysis of antibody selection by phage display utilizing anti-phenobarbital antibodies. *J.Mol.Recognit.* **9:** 738-745.

Manivel V., Bayiroglu F., Siddiqui Z., Salunke D.M. and Rao K.V. (2002) The primary antibody repertoire represents a linked network of degenerate antigen specificities. *J.Immunol.* **169:** 888-897.

Mao S., Gao C., Lo C.H., Wirsching P., Wong C.H. and Janda K.D. (1999) Phage-display library selection of high-affinity human single-chain antibodies to tumor-associated carbohydrate antigens sialyl Lewisx and Lewisx. *Proc.Natl.Acad.Sci.U.S.A.* **96:** 6953-6958.

Marks J.D., Hoogenboom H.R., Bonnert T.P., McCafferty J., Griffiths A.D. and Winter G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J.Mol.Biol.* **222:** 581-597.

Marks J.D., Griffiths A.D., Malmqvist M., Clackson T.P., Bye J.M. and Winter G. (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N.Y)* **10:** 779-783.

Martin A., Sieber V. and Schmid F.X. (2001) In-vitro selection of highly stabilized protein variants with optimized surface. *J.Mol.Biol.* **309:** 717-726.

Martineau P. and Betton J.M. (1999) In vitro folding and thermodynamic stability of an antibody fragment selected in vivo for high expression levels in Escherichia coli cytoplasm. *J.Mol.Biol.* **292:** 921-929.

McCafferty J., Griffiths A.D., Winter G. and Chiswell D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348:** 552-554.

McConnell S.J., Dinh T., Le M.H. and Spinella D.G. (1999) Biopanning phage display libraries using magnetic beads vs. polystyrene plates. *BioTechniques* **26:** 208-10, 214.

Men R., Yamashiro T., Goncalvez A.P., Wernly C., Schofield D.J., Emerson S.U., Purcell R.H. and Lai C.J. (2004) Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized immunoglobulin G1 antibody that is highly efficient for neutralization of dengue type 4 virus. *J.Virol.* **78:** 4665-4674.

Merz T., Wetzel S.K., Firbank S., Pluckthun A., Grutter M.G. and Mittl P.R. (2008) Stabilizing ionic interactions in a full-consensus ankyrin repeat protein. *J.Mol.Biol.* **376:** 232-240.

Meulemans E.V., Slobbe R., Wasterval P., Ramaekers F.C. and van Eys G.J. (1994) Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. *J.Mol.Biol.* **244:** 353-360.

Midelfort K.S. and Wittrup K.D. (2006) Context-dependent mutations predominate in an engineered high-affinity single chain antibody fragment. *Protein Sci.* **15:** 324-334.

Moghaddam A., Lobersli I., Gebhardt K., Braunagel M. and Marvik O.J. (2001) Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naive recombinant antibody libraries. *J.Immunol.Methods* **254:** 169-181.

Mondon P., Souyris N., Douchy L., Crozet F., Bouayadi K. and Kharrat H. (2007) Method for generation of human hyperdiversified antibody fragment library. *Biotechnol.J.* **2:** 76-82.

Monroe J.G. and Dorshkind K. (2007) Fate decisions regulating bone marrow and peripheral B lymphocyte development. *Adv.Immunol.* **95:** 1-50.

Mukkala V.M., Mikola H. and Hemmila I. (1989) The synthesis and use of activated N-benzyl derivatives of diethylenetriaminetetraacetic acids: alternative reagents for labeling of antibodies with metal ions. *Anal.Biochem.* **176:** 319-325.

Mukkala V.-M., Hänninen E., Hemmilä I. (1993) Synthesis of two biotin isotiosyanate derivatives. *Proceedings of the 8yh European Symposium on Organic Chemistry*. P422, Sitges, Spain.

Muldoon M.T., Holtzapple C.K., Deshpande S.S., Beier R.C. and Stanker L.H. (2000) Development of a monoclonal antibody-based cELISA for the analysis of sulfadimethoxine. 1. Development and characterization of monoclonal antibodies and molecular modeling studies of antibody recognition. *J.Agric.Food Chem.* **48:** 537-544.

Muyldermans S., Atarhouch T., Saldanha J., Barbosa J.A. and Hamers R. (1994) Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng.* **7:** 1129-1135.

Nagano N., Orengo C.A. and Thornton J.M. (2002) One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. *J.Mol.Biol.* **321:** 741-765.

Nagy Z.A., Hubner B., Lohning C., Rauchenberger R., Reiffert S., Thomassen-Wolf E., Zahn S., Leyer S., Schier E.M., Zahradnik A., *et al.* (2002) Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells. *Nat.Med.* **8:** 801-807.

Nakamura M., Watanabe H., Nishimiya Y., Tsumoto K., Ishimura K. and Kumagai I. (2001) Panning of a phage VH library using nitrocellulose membranes: application to selection of a human VH library. *J.Biochem.* **129:** 209-212.

Nesterenko I.S., Nokel M.A. and Eremin S.A. (2009) Immunochemical methods for the detection of sulfanylamide drugs. *J. Anal. Chem.* **64:** 435-444.

Neuberger M.S., Ehrenstein M.R., Klix N., Jolly C.J., Yelamos J., Rada C. and Milstein C. (1998) Monitoring and interpreting the intrinsic features of somatic hypermutation. *Immunol.Rev.* **162:** 107-116.

Nguyen V.K., Hamers R., Wyns L. and Muyldermans S. (2000) Camel heavy-chain antibodies: diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. *EMBO J.* **19:** 921-930.

Nissim A., Hoogenboom H.R., Tomlinson I.M., Flynn G., Midgley C., Lane D. and Winter G. (1994) Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J.* **13:** 692-698.

Nord K., Nilsson J., Nilsson B., Uhlen M. and Nygren P.A. (1995) A combinatorial library of an alpha-helical bacterial receptor domain. *Protein Eng.* **8:** 601-608.

Nord K., Gunneriusson E., Ringdahl J., Stahl S., Uhlen M. and Nygren P.A. (1997) Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. *Nat.Biotechnol.* **15:** 772-777.

Nuttall S.D. and Walsh R.B. (2008) Display scaffolds: protein engineering for novel therapeutics. *Curr.Opin.Pharmacol.* **8:** 609-615.

Nygren H. and Stenberg M. (1989) Immunochemistry at interfaces. *Immunology* **66:** 321-327.

Nygren P.A. and Skerra A. (2004) Binding proteins from alternative scaffolds. *J.Immunol.Methods* **290:** 3-28.

Nygren P.A. (2008) Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold. *FEBS J.* **275:** 2668-2676.

O'Brien P.M., Aitken R., O'Neil B.W. and Campo M.S. (1999) Generation of native bovine mAbs by phage display. *Proc.Natl.Acad.Sci.U.S.A.* **96:** 640-645.

O'Connell D., Becerril B., Roy-Burman A., Daws M. and Marks J.D. (2002) Phage versus phagemid libraries for generation of human monoclonal antibodies. *J.Mol.Biol.* **321:** 49-56.

Ohage E. and Steipe B. (1999) Intrabody construction and expression. I. The critical role of VL domain stability. *J.Mol.Biol.* **291:** 1119-1128.

Ohiro Y., Arai R., Ueda H. and Nagamune T. (2002) A homogeneous and noncompetitive immunoassay based on the enhanced fluorescence resonance energy transfer by leucine zipper interaction. *Anal.Chem.* **74:** 5786-5792.

Ohiro Y., Ueda H., Shibata N. and Nagamune T. (2007) Enhanced fluorescence resonance energy transfer immunoassay with improved sensitivity based on the Fab'-based immunoconjugates. *Anal.Biochem.* **360:** 266-272.

Ohlin M., Owman H., Mach M. and Borrebaeck C.A. (1996) Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. *Mol.Immunol.* **33:** 47-56.

Okamoto T., Mukai Y., Yoshioka Y., Shibata H., Kawamura M., Yamamoto Y., Nakagawa S., Kamada H., Hayakawa T., Mayumi T., *et al.* (2004) Optimal construction of non-immune scFv phage display libraries from mouse bone marrow and spleen established to select specific scFvs efficiently binding to antigen. *Biochem.Biophys.Res.Commun.* **323:** 583-591.

Orlandi R., Gussow D.H., Jones P.T. and Winter G. (1989) Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc.Natl.Acad.Sci.U.S.A.* **86:** 3833-3837.

Orlova A., Magnusson M., Eriksson T.L., Nilsson M., Larsson B., Hoiden-Guthenberg I., Widstrom C., Carlsson J., Tolmachev V., Stahl S., *et al.* (2006) Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Res.* **66:** 4339-4348.

Orum H., Andersen P.S., Oster A., Johansen L.K., Riise E., Bjornvad M., Svendsen I. and Engberg J. (1993) Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucleic Acids Res.* **21:** 4491-4498.

Osbourn J.K., Field A., Wilton J., Derbyshire E., Earnshaw J.C., Jones P.T., Allen D. and McCafferty J. (1996) Generation of a panel of related human scFv antibodies with high affinities for human CEA. *Immunotechnology* **2:** 181-196.

Padlan E.A., Abergel C. and Tipper J.P. (1995) Identification of specificity-determining residues in antibodies. *FASEB J.* **9:** 133-139.

Pansri P., Jaruseranee N., Rangnoi K., Kristensen P. and Yamabhai M. (2009) A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol.* **9:** 6.

Parhami-Seren B., Viswanathan M. and Margolies M.N. (2002) Selection of high affinity p-azophenyarsonate Fabs from heavy-chain CDR2 insertion libraries. *J.Immunol.Methods* **259:** 43-53.

Park S.G., Lee J.S., Je E.Y., Kim I.J., Chung J.H. and Choi I.H. (2000) Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in Escherichia coli. *Biochem.Biophys.Res.Commun.* **275:** 553-557.

Parsons H.L., Earnshaw J.C., Wilton J., Johnson K.S., Schueler P.A., Mahoney W. and McCafferty J. (1996) Directing phage selections towards specific epitopes. *Protein Eng.* **9:** 1043-1049.

Pedrazzi G., Schwesinger F., Honegger A., Krebber C. and Pluckthun A. (1997) Affinity and folding properties both influence the selection of antibodies with the selectively infective phage (SIP) methodology. *FEBS Lett.* **415:** 289-293.

Pelkkikangas A.M., Jaakohuhta S., Lovgren T. and Harma H. (2004) Simple, rapid, and sensitive thyroid-stimulating hormone immunoassay using europium(III) nanoparticle label. *Anal.Chim.Acta* **517:** 169-176.

Peluso P., Wilson D.S., Do D., Tran H., Venkatasubbaiah M., Quincy D., Heidecker B., Poindexter K., Tolani N., Phelan M., *et al.* (2003) Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal.Biochem.* **312:** 113-124.

Persson H., Lantto J. and Ohlin M. (2006) A focused antibody library for improved hapten recognition. *J.Mol.Biol.* **357:** 607-620.

Persson H., Wallmark H., Ljungars A., Hallborn J. and Ohlin M. (2008) In vitro evolution of an antibody fragment population to find high-affinity hapten binders. *Protein Eng.Des.Sel.* **21:** 485-493.

Pini A., Viti F., Santucci A., Carnemolla B., Zardi L., Neri P. and Neri D. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J.Biol.Chem.* **273:** 21769-21776.

Proba K., Worn A., Honegger A. and Pluckthun A. (1998) Antibody scFv fragments without disulfide bonds made by molecular evolution. *J.Mol.Biol.* **275:** 245-253.

Proulx C., Boyer L., St-Amour I., Bazin R. and Lemieux R. (2002) Higher affinity human D MoAb prepared by light-chain shuffling and selected by phage display. *Transfusion* **42:** 59-65.

Pulli T., Hoyhtya M., Soderlund H. and Takkinen K. (2005) One-step homogeneous immunoassay for small analytes. *Anal.Chem.* **77:** 2637-2642.

Quintero-Hernandez V., Juarez-Gonzalez V.R., Ortiz-Leon M., Sanchez R., Possani L.D. and Becerril B. (2007) The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization capacity of recombinant antibodies. *Mol.Immunol.* **44:** 1307-1315.

Raats J., van Bree N., van Woezik J. and Pruijn G. (2003a) Generating recombinant anti-idiotypic antibodies for the detection of haptens in solution. *J.Immunoassay Immunochem* **24:** 115-146.

Raats J.M., Wijnen E.M., Pruijn G.J., van den Hoogen F.H. and van Venrooij W.J. (2003b) Recombinant human monoclonal autoantibodies specific for citrulline-containing peptides from phage display libraries derived from patients with rheumatoid arthritis. *J.Rheumatol.* **30:** 1696-1711.

Rajpal A., Beyaz N., Haber L., Cappuccilli G., Yee H., Bhatt R.R., Takeuchi T., Lerner R.A. and Crea R. (2005) A general method for greatly improving the affinity of antibodies by using combinatorial libraries. *Proc.Natl.Acad.Sci.U.S.A.* **102:** 8466-8471.

Rasila T.S., Pajunen M.I. and Savilahti H. (2009) Critical evaluation of random mutagenesis by error-prone PCR protocols, Escherichia coli mutator strain and hydroxylamine treatment. *Anal.Biochem.* **:** .

Rauchenberger R., Borges E., Thomassen-Wolf E., Rom E., Adar R., Yaniv Y., Malka M., Chumakov I., Kotzer S., Resnitzky D., *et al.* (2003) Human combinatorial Fab library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3. *J.Biol.Chem.* **278:** 38194-38205.

Ravn P., Danielczyk A., Jensen K.B., Kristensen P., Christensen P.A., Larsen M., Karsten U. and Goletz S. (2004) Multivalent scFv display of phagemid repertoires for the selection of carbohydrate-specific antibodies and its application to the Thomsen-Friedenreich antigen. *J.Mol.Biol.* **343:** 985-996.

Reardon D. and Farber G.K. (1995) The structure and evolution of alpha/beta barrel proteins. *FASEB J.* **9:** 497-503.

Rhyner C., Weichel M., Hubner P., Achatz G., Blaser K. and Crameri R. (2003) Phage display of human antibodies from a patient suffering from coeliac disease and selection of isotype-specific scFv against gliadin. *Immunology* **110:** 269-274.

Ridgway J.B., Ng E., Kern J.A., Lee J., Brush J., Goddard A. and Carter P. (1999) Identification of a human anti-CD55 single-chain Fv by subtractive panning of a phage library using tumor and nontumor cell lines. *Cancer Res.* **59:** 2718-2723.

Rojas G., Talavera A., Munoz Y., Rengifo E., Krengel U., Angstrom J., Gavilondo J. and Moreno E. (2004) Light-chain shuffling results in successful phage display selection of functional prokaryotic-expressed antibody fragments to N-glycolyl GM3 ganglioside. *J.Immunol.Methods* **293:** 71-83.

Rojas G., Lamdan H., Padron S., Munoz Y., Ayala M. and Gavilondo J.V. (2005) Efficient construction of a highly useful phage-displayed human antibody repertoire. *Biochem.Biophys.Res.Commun.* **336:** 1207-1213.

Rondot S., Koch J., Breitling F. and Dubel S. (2001) A helper phage to improve single-chain antibody presentation in phage display. *Nat.Biotechnol.* **19:** 75-78.

Rothe A., Klimka A., Tur M.K., Pfitzner T., Huhn M., Sasse S., Mallmann P., Engert A. and Barth S. (2004) Construction of phage display libraries from reactive lymph nodes of breast carcinoma patients and selection for specifically binding human single chain Fv on cell lines. *Int.J.Mol.Med.* **14:** 729-735.

Rothe C., Urlinger S., Lohning C., Prassler J., Stark Y., Jager U., Hubner B., Bardroff M., Pradel I., Boss M., *et al.* (2008) The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J.Mol.Biol.* **376:** 1182-1200.

Rothlisberger D., Honegger A. and Pluckthun A. (2005) Domain interactions in the Fab fragment: a comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. *J.Mol.Biol.* **347:** 773-789.

Roux K.H., Greenberg A.S., Greene L., Strelets L., Avila D., McKinney E.C. and Flajnik M.F. (1998) Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. *Proc.Natl.Acad.Sci.U.S.A.* **95:** 11804-11809.

Saboulard D., Dugas V., Jaber M., Broutin J., Souteyrand E., Sylvestre J. and Delcourt M. (2005) High-throughput site-directed mutagenesis using oligonucleotides synthesized on DNA chips. *BioTechniques* **39:** 363-368.

Sakamoto S., Taura F., Pongkitwitoon B., Putalun W., Tsuchihashi R., Kinjo J., Tanaka H. and Morimoto S. (2010) Development of sensitivity-improved fluorescence-linked immunosorbent assay using a fluorescent single-domain antibody against the bioactive naphthoquinone, plumbagin. *Anal.Bioanal Chem.* **396:** 2955-2963.

Sakai K., Shimizu Y., Chiba T., Matsumoto-Takasaki A., Kusada Y., Zhang W., Nakata M., Kojima N., Toma K., Takayanagi A., *et al.* (2007) Isolation and characterization of phage-displayed single chain antibodies recognizing nonreducing terminal mannose residues. 1. A new strategy for generation of anti-carbohydrate antibodies. *Biochemistry* **46:** 253-262.

Sanna P.P., Williamson R.A., De Logu A., Bloom F.E. and Burton D.R. (1995) Directed selection of recombinant human monoclonal antibodies to herpes simplex virus glycoproteins from phage display libraries. *Proc.Natl.Acad.Sci.U.S.A.* **92:** 6439-6443.

Santala V. and Saviranta P. (2004) Affinity-independent elution of antibody-displaying phages using cleavable DNA linker containing streptavidin beads. *J.Immunol.Methods* **284:** 159-163.

Saviranta P., Haavisto T., Rappu P., Karp M. and Lovgren T. (1998) In vitro enzymatic biotinylation of recombinant fab fragments through a peptide acceptor tail. *Bioconjug.Chem.* **9:** 725-735.

Sawin C.T., Castelli W.P., Hershman J.M., McNamara P. and Bacharach P. (1985) The aging thyroid. Thyroid deficiency in the Framingham Study. *Arch.Intern.Med.* **145:** 1386-1388.

Sblattero D. and Bradbury A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat.Biotechnol.* **18:** 75-80.

Schaaper R.M. (1988) Mechanisms of mutagenesis in the Escherichia coli mutator mutD5: role of DNA mismatch repair. *Proc.Natl.Acad.Sci.U.S.A.* **85:** 8126-8130.

Schaffitzel C., Hanes J., Jermutus L. and Pluckthun A. (1999) Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. *J.Immunol.Methods* **231:** 119-135.

Schier R., Bye J., Apell G., McCall A., Adams G.P., Malmqvist M., Weiner L.M. and Marks J.D. (1996a) Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J.Mol.Biol.* **255:** 28-43.

Schier R., McCall A., Adams G.P., Marshall K.W., Merritt H., Yim M., Crawford R.S., Weiner L.M., Marks C. and Marks J.D. (1996b) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J.Mol.Biol.* **263:** 551-567.

Schlehuber S., Beste G. and Skerra A. (2000) A novel type of receptor protein, based on the lipocalin scaffold, with specificity for digoxigenin. *J.Mol.Biol.* **297:** 1105-1120.

Schlehuber S. and Skerra A. (2002) Tuning ligand affinity, specificity, and folding stability of an engineered lipocalin variant -- a so-called 'anticalin' -- using a molecular random approach. *Biophys.Chem.* **96:** 213-228.

Schlehuber S. and Skerra A. (2005) Lipocalins in drug discovery: from natural ligand-binding proteins to "anticalins". *Drug Discov.Today* **10:** 23-33.

Schmiedl A., Breitling F., Winter C.H., Queitsch I. and Dubel S. (2000) Effects of unpaired cysteines on yield, solubility and activity of different recombinant antibody constructs expressed in E. coli. *J.Immunol.Methods* **242:** 101-114.

Schofield D.J., Pope A.R., Clementel V., Buckell J., Chapple S.D., Clarke K.F., Conquer J.S., Crofts A.M., Crowther S.R., Dyson M.R., *et al.* (2007) Application of phage display to high throughput antibody generation and characterization. *Genome Biol.* **8:** R254.

Schonfeld D., Matschiner G., Chatwell L., Trentmann S., Gille H., Hulsmeyer M., Brown N., Kaye P.M., Schlehuber S., Hohlbaum A.M., *et al.* (2009) An engineered lipocalin specific for CTLA-4 reveals a combining site with structural and conformational features similar to antibodies. *Proc.Natl.Acad.Sci.U.S.A.* **106:** 8198-8203.

Schoonbroodt S., Steukers M., Viswanathan M., Frans N., Timmermans M., Wehnert A., Nguyen M., Ladner R.C. and Hoet R.M. (2008) Engineering antibody heavy chain CDR3 to create a phage display Fab library rich in antibodies that bind charged carbohydrates. *J.Immunol.* **181:** 6213-6221.

Schweizer A., Rusert P., Berlinger L., Ruprecht C.R., Mann A., Corthesy S., Turville S.G., Aravantinou M., Fischer M., Robbiani M., *et al.* (2008) CD4-specific designed ankyrin repeat proteins are novel potent HIV entry inhibitors with unique characteristics. *PLoS Pathog.* **4:** e1000109.

Scott J.K., Barbas C.F., Barbas C.F., Burton D.R., Scott J.K., Silverman G.J. (2001) in *Phage Display: A Laboratory Manual*, eds Barbas C F, Burton D R, Scott J K, Silverman G J (Cold Spring Harbor Lab. Press, Plainview, NY), pp 2.1–2.19.

Seehaus T., Breitling F., Dubel S., Klewinghaus I. and Little M. (1992) A vector for the removal of deletion mutants from antibody libraries. *Gene* **114:** 235-237.

Sennhauser G. and Grutter M.G. (2008) Chaperone-assisted crystallography with DARPins. *Structure* **16:** 1443-1453.

Sergeeva A., Kolonin M.G., Molldrem J.J., Pasqualini R. and Arap W. (2006) Display technologies: application for the discovery of drug and gene delivery agents. *Adv.Drug Deliv.Rev.* **58:** 1622-1654.

Shao C.Y., Secombes C.J. and Porter A.J. (2007) Rapid isolation of IgNAR variable single-domain antibody fragments from a shark synthetic library. *Mol.Immunol.* **44:** 656-665.

Shao Z., Zhao H., Giver L. and Arnold F.H. (1998) Random-priming in vitro recombination: an effective tool for directed evolution. *Nucleic Acids Res.* **26:** 681-683.

Sheedy C., MacKenzie C.R. and Hall J.C. (2007) Isolation and affinity maturation of hapten-specific antibodies. *Biotechnol.Adv.* **25:** 333-352.

Sheets M.D., Amersdorfer P., Finnern R., Sargent P., Lindquist E., Schier R., Hemingsen G., Wong C., Gerhart J.C. and Marks J.D. (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc.Natl.Acad.Sci.U.S.A.* **95:** 6157-6162.

Shen Y., Yang X., Dong N., Xie X., Bai X. and Shi Y. (2007) Generation and selection of immunized Fab phage display library against human B cell lymphoma. *Cell Res.* **17:** 650-660.

Sheth H.B. and Sporns P. (1991) Development of a single ELISA for detection of sulfonamides. *J. Agric. Food Chem.* **39:** 1696-1700.

Shi L., Wheeler J.C., Sweet R.W., Lu J., Luo J., Tornetta M., Whitaker B., Reddy R., Brittingham R., Borozdina L., *et al.* (2010) De Novo Selection of High-Affinity Antibodies from Synthetic Fab Libraries Displayed on Phage as pIX Fusion Proteins. *J.Mol.Biol.* **:** .

Sidhu S.S., Lowman H.B., Cunningham B.C. and Wells J.A. (2000) Phage display for selection of novel binding peptides. *Methods Enzymol.* **328:** 333-363.

Sidhu S.S., Li B., Chen Y., Fellouse F.A., Eigenbrot C. and Fuh G. (2004) Phage-displayed antibody libraries of synthetic heavy chain complementarity determining regions. *J.Mol.Biol.* **338:** 299-310.

Sieber V., Pluckthun A. and Schmid F.X. (1998) Selecting proteins with improved stability by a phage-based method. *Nat.Biotechnol.* **16:** 955-960.

Siegel R.W., Baugher W., Rahn T., Drengler S. and Tyner J. (2008) Affinity maturation of tacrolimus antibody for improved immunoassay performance. *Clin.Chem.* **54:** 1008-1017.

Silacci M., Brack S., Schirru G., Marlind J., Ettorre A., Merlo A., Viti F. and Neri D. (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. *Proteomics* **5:** 2340-2350.

Sivanandam A.S., Mohan S., Kapur S., Kita H., Lau K.H., Bagi G., Baylink D.J. and Qin X. (2004) Covalent interaction between proform of eosinophil major basic protein (proMBP) and pregnancy-associated plasma protein-A (PAPP-A) is a cell-mediated event and required for proMBP inhibition of the catalytic activity of PAPP-A. *Arch.Biochem.Biophys.* **423:** 343-350.

Skerra A. and Pluckthun A. (1988) Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science* **240:** 1038-1041.

Skerra A., Pfitzinger I. and Pluckthun A. (1991) The functional expression of antibody Fv fragments in Escherichia coli: improved vectors and a generally applicable purification technique. *Biotechnology (N.Y)* **9:** 273-278.

Skerra A. (2007) Alternative non-antibody scaffolds for molecular recognition. *Curr.Opin.Biotechnol.* **18:** 295-304.

Skerra A. (2008) Alternative binding proteins: anticalins - harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *FEBS J.* **275:** 2677-2683.

Smith G.P., Patel S.U., Windass J.D., Thornton J.M., Winter G. and Griffiths A.D. (1998) Small binding proteins selected from a combinatorial repertoire of knottins displayed on phage. *J.Mol.Biol.* **277:** 317-332.

Soderlind E., Vergeles M. and Borrebaeck C.A. (1995) Domain libraries: synthetic diversity for de novo design of antibody V-regions. *Gene* **160:** 269-272.

Soderlind E., Strandberg L., Jirholt P., Kobayashi N., Alexeiva V., Aberg A.M., Nilsson A., Jansson B., Ohlin M., Wingren C., *et al.* (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat.Biotechnol.* **18:** 852-856.

Spinks C.A., Wyatt G.M., Lee H.A. and Morgan M.R.A. (1999) Molecular Modeling of Hapten Structure and Relevance to Broad Specificity Immunoassay of Sulfonamide Antibiotics. *Bioconjugate Chem.* **10:** 583-588.

Spinks C.A., Wyatt G.M., Everest S., Jackman R. and Morgan M.R.A. (2002) Atypical antibody specificity: advancing the development of a generic assay for sulphonamides using heterologous ELISA. *J. Sci. Food Agric.* **82:** 428-434.

Steidl S., Ratsch O., Brocks B., Durr M. and Thomassen-Wolf E. (2008) In vitro affinity maturation of human GM-CSF antibodies by targeted CDR-diversification. *Mol.Immunol.* **46:** 135-144.

Steiner D., Forrer P. and Pluckthun A. (2008) Efficient selection of DARPins with sub-nanomolar affinities using SRP phage display. *J.Mol.Biol.* **382:** 1211-1227.

Steinhauer C., Wingren C., Khan F., He M., Taussig M.J. and Borrebaeck C.A. (2006) Improved affinity coupling for antibody microarrays: engineering of double-(His)6-tagged single framework recombinant antibody fragments. *Proteomics* **6:** 4227-4234.

Stemmer W.P., Morris S.K. and Wilson B.S. (1993) Selection of an active single chain Fv antibody from a protein linker library prepared by enzymatic inverse PCR. *BioTechniques* **14:** 256-265.

Stemmer W.P. (1994a) DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc.Natl.Acad.Sci.U.S.A.* **91:** 10747-10751.

Stemmer W.P. (1994b) Rapid evolution of a protein in vitro by DNA shuffling. *Nature* **370:** 389-391.

Stockigt J.R. (2002) Case finding and screening strategies for thyroid dysfunction. *Clin.Chim.Acta* **315:** 111-124.

Stumpp M.T., Binz H.K. and Amstutz P. (2008) DARPins: a new generation of protein therapeutics. *Drug Discov.Today* **13:** 695-701.

Sun Y., Shukla G.S., Weaver D., Pero S.C. and Krag D.N. (2009) Phage-display selection on tumor histological specimens with laser capture microdissection. *J.Immunol.Methods* **347:** 46-53.

Suzuki C., Ueda H., Mahoney W. and Nagamune T. (2000) Open sandwich enzyme-linked immunosorbent assay for the quantitation of small haptens. *Anal.Biochem.* **286:** 238-246.

Swers J.S., Kellogg B.A. and Wittrup K.D. (2004) Shuffled antibody libraries created by in vivo homologous recombination and yeast surface display. *Nucleic Acids Res.* **32:** e36.

Szkudlinski M.W., Fremont V., Ronin C. and Weintraub B.D. (2002) Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiol.Rev.* **82:** 473-502.

Talarico T.L., Dev I.K., Dallas W.S., Ferone R. and Ray P.H. (1991) Purification and partial characterization of 7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase and 7,8-dihydropteroate synthase from Escherichia coli MC4100. *J.Bacteriol.* **173:** 7029-7032.

Tanaka T., Lobato M.N. and Rabbitts T.H. (2003) Single domain intracellular antibodies: a minimal fragment for direct in vivo selection of antigen-specific intrabodies. *J.Mol.Biol.* **331:** 1109-1120.

Tarkkinen P., Palenius T. and Lovgren T. (2002) Ultrarapid, ultrasensitive one-step kinetic immunoassay for C-reactive protein (CRP) in whole blood samples: measurement of the entire CRP concentration range with a single sample dilution. *Clin.Chem.* **48:** 269-277.

Taussig M.J., Stoevesandt O., Borrebaeck C.A., Bradbury A.R., Cahill D., Cambillau C., de Daruvar A., Dubel S., Eichler J., Frank R., *et al.* (2007) ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. *Nat.Methods* **4:** 13-17.

Thompson J., Pope T., Tung J.S., Chan C., Hollis G., Mark G. and Johnson K.S. (1996) Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. *J.Mol.Biol.* **256:** 77-88.

To R., Hirama T., Arbabi-Ghahroudi M., MacKenzie R., Wang P., Xu P., Ni F. and Tanha J. (2005) Isolation of monomeric human V(H)s by a phage selection. *J.Biol.Chem.* **280:** 41395-41403.

Tolmachev V., Orlova A., Nilsson F.Y., Feldwisch J., Wennborg A. and Abrahmsen L. (2007) Affibody molecules: potential for in vivo imaging of molecular targets for cancer therapy. *Expert Opin.Biol.Ther.* **7:** 555-568.

Tomlinson I.M., Cook G.P., Carter N.P., Elaswarapu R., Smith S., Walter G., Buluwela L., Rabbitts T.H. and Winter G. (1994) Human immunoglobulin VH and D segments on chromosomes 15q11.2 and 16p11.2. *Hum.Mol.Genet.* **3:** 853-860.

Tuckey C.D. and Noren C.J. (2002) Selection for mutants improving expression of an anti-MAP kinase monoclonal antibody by filamentous phage display. *J.Immunol.Methods* **270:** 247-257.

Tunbridge W.M., Evered D.C., Hall R., Appleton D., Brewis M., Clark F., Evans J.G., Young E., Bird T. and Smith P.A. (1977) The spectrum of thyroid disease in a community: the Whickham survey. *Clin.Endocrinol.(Oxf)* **7:** 481-493.

Ueda H., Tsumoto K., Kubota K., Suzuki E., Nagamune T., Nishimura H., Schueler P.A., Winter G., Kumagai I. and Mohoney W.C. (1996) Open sandwich ELISA: a novel immunoassay based on the interchain interaction of antibody variable region. *Nat.Biotechnol.* **14:** 1714-1718.

Uhlen M. and Hober S. (2009) Generation and validation of affinity reagents on a proteome-wide level. *J.Mol.Recognit.* **22:** 57-64.

van den Beucken T., van Neer N., Sablon E., Desmet J., Celis L., Hoogenboom H.R. and Hufton S.E. (2001) Building novel binding ligands to B7.1 and B7.2 based on human antibody single variable light chain domains. *J.Mol.Biol.* **310:** 591-601.

Vaisanen V., Peltola M.T., Lilja H., Nurmi M. and Pettersson K. (2006) Intact free prostate-specific antigen and free and total human glandular kallikrein 2. Elimination of assay interference by enzymatic digestion of antibodies to F(ab')2 fragments. *Anal.Chem.* **78:** 7809-7815.

Vallina-Garcia R., del Mar Garcia-Suarez M., Fernandez-Abedul M.T., Mendez F.J. and Costa-Garcia A. (2007) Oriented immobilisation of anti-pneumolysin Fab through a histidine tag for electrochemical immunosensors. *Biosens.Bioelectron.* **23:** 210-217.

Vanderpump M.P. and Tunbridge W.M. (2002) Epidemiology and prevention of clinical and subclinical hypothyroidism. *Thyroid* **12:** 839-847.

Vargas-Madrazo E., Lara-Ochoa F. and Almagro J.C. (1995) Canonical structure repertoire of the antigen-binding site of immunoglobulins suggests strong geometrical restrictions associated to the mechanism of immune recognition. *J.Mol.Biol.* **254:** 497-504.

Vaughan T.J., Williams A.J., Pritchard K., Osbourn J.K., Pope A.R., Earnshaw J.C., McCafferty J., Hodits R.A., Wilton J. and Johnson K.S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat.Biotechnol.* **14:** 309-314.

Vikholm-Lundin I. (2005) Immunosensing based on site-directed immobilization of antibody fragments and polymers that reduce nonspecific binding. *Langmuir* **21:** 6473-6477.

Vinnicombe H.G. and Derrick J.P. (1999) Dihydropteroate synthase: an old drug target revisited. *Biochem.Soc.Trans.* **27:** 53-58.

Vogt M. and Skerra A. (2004) Construction of an artificial receptor protein ("anticalin") based on the human apolipoprotein D. *Chembiochem* **5:** 191-199.

Volkel T., Muller R. and Kontermann R.E. (2004) Isolation of endothelial cell-specific human antibodies from a novel fully synthetic scFv library. *Biochem.Biophys.Res.Commun.* **317:** 515-521.

von Lode P., Hagren V., Palenius T. and Lovgren T. (2003a) One-step quantitative thyrotropin assay for the detection of hypothyroidism in point-of-care conditions. *Clin.Biochem.* **36:** 121-128.

von Lode P., Rosenberg J., Pettersson K. and Takalo H. (2003b) A europium chelate for quantitative point-of-care immunoassays using direct surface measurement. *Anal.Chem.* **75:** 3193-3201.

Vopel S., Muhlbach H. and Skerra A. (2005) Rational engineering of a fluorescein-binding anticalin for improved ligand affinity. *Biol.Chem.* **386:** 1097-1104.

Wang Z., Zhang S., Nesterenko I.S., Eremin S.A. and Shen J. (2007) Monoclonal antibody-based fluorescence polarization immunoassay for sulfamethoxypyridazine and sulfachloropyridazine. *J.Agric.Food Chem.* **55:** 6871-6878.

Ward E.S., Gussow D., Griffiths A.D., Jones P.T. and Winter G. (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. *Nature* **341:** 544-546.

Ward R.L., Clark M.A., Lees J. and Hawkins N.J. (1996) Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. *J.Immunol.Methods* **189:** 73-82.

Wark K.L. and Hudson P.J. (2006) Latest technologies for the enhancement of antibody affinity. *Adv.Drug Deliv.Rev.* **58:** 657-670.

Warren D.J., Bjerner J., Paus E., Bormer O.P. and Nustad K. (2005) Use of an in vivo biotinylated single-chain antibody as capture reagent in an immunometric assay to decrease the incidence of interference from heterophilic antibodies. *Clin.Chem.* **51:** 830-838.

Weaver M. and Laske D.W. (2003) Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. *J.Neurooncol.* **65:** 3-13.

White G.P., Meeusen E.N. and Newton S.E. (2001) A single-chain variable region immunoglobulin library from the abomasal lymph node of sheep infected with the gastrointestinal nematode parasite Haemonchus contortus. *Vet.Immunol.Immunopathol.* **78:** 117-129.

Wierenga R.K. (2001) The TIM-barrel fold: a versatile framework for efficient enzymes. *FEBS Lett.* **492:** 193-198.

Willats W.G. (2002) Phage display: practicalities and prospects. *Plant Mol.Biol.* **50:** 837-854.

Wind T., Stausbol-Gron B., Kjaer S., Kahns L., Jensen K.H. and Clark B.F. (1997) Retrieval of phage displayed scFv fragments using direct bacterial elution. *J.Immunol.Methods* **209:** 75-83.

Wind T., Kjaer S. and Clark B.F. (1999) Display of Ras on filamentous phage through cysteine replacement. *Biochimie* **81:** 1079-1087.

Winkler J., Martin-Killias P., Pluckthun A. and Zangemeister-Wittke U. (2009) EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins. *Mol.Cancer.Ther.* **8:** 2674-2683.

Worn A. and Pluckthun A. (1998a) An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. *FEBS Lett.* **427:** 357-361.

Worn A. and Pluckthun A. (1998b) Mutual stabilization of VL and VH in single-chain antibody fragments, investigated with mutants engineered for stability. *Biochemistry* **37:** 13120-13127.

Worn A. and Pluckthun A. (2001) Stability engineering of antibody single-chain Fv fragments. *J.Mol.Biol.* **305:** 989-1010.

Xu J.L. and Davis M.M. (2000) Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* **13:** 37-45.

Xu L., Aha P., Gu K., Kuimelis R.G., Kurz M., Lam T., Lim A.C., Liu H., Lohse P.A., Sun L., *et al.* (2002) Directed evolution of high-affinity antibody mimics using mRNA display. *Chem.Biol.* **9:** 933-942.

Yamanaka H.I., Inoue T. and Ikeda-Tanaka O. (1996) Chicken monoclonal antibody isolated by a phage display system. *J.Immunol.* **157:** 1156-1162.

Yang H.Y., Kang K.J., Chung J.E. and Shim H. (2009) Construction of a large synthetic human scFv library with six diversified CDRs and high functional diversity. *Mol.Cells* **27:** 225-235.

Yang W.P., Green K., Pinz-Sweeney S., Briones A.T., Burton D.R. and Barbas C.F.,3rd (1995) CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J.Mol.Biol.* **254:** 392-403.

Ylikotila J., Valimaa L., Vehniainen M., Takalo H., Lovgren T. and Pettersson K. (2005) A sensitive TSH assay in spot-coated microwells utilizing recombinant antibody fragments. *J.Immunol.Methods* **306:** 104-114.

Ylikotila J., Hellstrom J.L., Eriksson S., Vehniainen M., Valimaa L., Takalo H., Bereznikova A. and Pettersson K. (2006) Utilization of recombinant Fab fragments in a cTnI immunoassay conducted in spot wells. *Clin.Biochem.* **39:** 843-850.

Yoshinaga K., Matsumoto M., Torikai M., Sugyo K., Kuroki S., Nogami K., Matsumoto R., Hashiguchi S., Ito Y., Nakashima T., *et al.* (2008) Ig L-chain shuffling for affinity maturation of phage library-derived human anti-human MCP-1 antibody blocking its chemotactic activity. *J.Biochem.* **143:** 593-601.

Zaccolo M., Williams D.M., Brown D.M. and Gherardi E. (1996) An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. *J.Mol.Biol.* **255:** 589-603.

Zaccolo M. and Gherardi E. (1999) The effect of high-frequency random mutagenesis on in vitro protein evolution: a study on TEM-1 beta-lactamase. *J.Mol.Biol.* **285:** 775-783.

Zahnd C., Spinelli S., Luginbuhl B., Amstutz P., Cambillau C. and Pluckthun A. (2004) Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J.Biol.Chem.* **279:** 18870-18877.

Zahnd C., Wyler E., Schwenk J.M., Steiner D., Lawrence M.C., McKern N.M., Pecorari F., Ward C.W., Joos T.O. and Pluckthun A. (2007) A designed ankyrin repeat protein evolved to picomolar affinity to Her2. *J.Mol.Biol.* **369:** 1015-1028.

Zemlin M., Klinger M., Link J., Zemlin C., Bauer K., Engler J.A., Schroeder H.W.,Jr and Kirkham P.M. (2003) Expressed murine and human CDR-H3 intervals of equal length exhibit distinct repertoires that differ in their amino acid composition and predicted range of structures. *J.Mol.Biol.* **334:** 733-749.

Zhang H. and Wang S. (2009) Review on enzyme-linked immunosorbent assays for sulfonamide residues in edible animal products. *J. Immunol. Methods* **350:** 1-13.

Zhao H. and Arnold F.H. (1997) Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res.* **25:** 1307-1308.

Zhao H., Giver L., Shao Z., Affholter J.A. and Arnold F.H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat.Biotechnol.* **16:** 258-261.

Zhuang G., Katakura Y., Furuta T., Omasa T., Kishimoto M. and Suga K. (2001) A kinetic model for a biopanning process considering antigen desorption and effective antigen concentration on a solid phase. *J.Biosci.Bioeng.* **91:** 474-481.