Generation of hapten-specific recombinant antibodies: antibody phage display technology: a review

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ABSTRACT: Production of antibodies has been revolutionized by the development of modern molecular biology methods for the expression of recombinant DNA. Phage display technology represents one of the most powerful tools for production and selection of recombinant antibodies and has been recognized as a valuable alternative way for the preparation of antibodies of a desired specificity. In comparison to poly- and monoclonal antibodies, recombinant antibodies using the phage display technology can be prepared faster, in more automatic process and with reduced consumption of laboratory animals. This review summarizes current trends of phage display technology with focus on the generation of hapten-specific recombinant antibodies and gives the examples of successful applications of phage display in the environmental analysis of low molecular weight compound.

Keywords: antibody phage display; recombinant antibodies; hapten; antibody phage libraries; immunochemistry

List of abbreviations

2,4-D = 2,4-dichlorophenoxyacetic acid, **aa** = amino acid, **BSA** = bovine serum albumin, **CAMAL** = Combined Antibody Modelling Algorithm, **CDR** = complementarity determining region, $\mathbf{C_L}$ = constant domain of light chain, $\mathbf{C_{H1–3}}$ = constant domains 1–3 of heavy chain, **CRAbs** = chelating recombinant antibodies, **DNA** = deoxyribonucleic acid, **DTPA** = diethylenetriaminepentaacetic acid, **ELISA** = enzyme linked immunoassay, **Fab** = antibody fragment consisting of C_L, C_{H1},V_L and V_H antibody domains, **FR** = framework region, **Fv** = antigen binding fragment variable consisting of V_L and V_H antibody domains, **HPLC** = high-performance liquid chromatography, **IgG** = immunoglobuline G class, **IgM** = immunoglobuline M class, **IMAC** = immobilized metal affinity chromatography, **Ka** = association constant, **NIP** = 3-indolo-4hydroxy-5-nitrophenyl-acetate, **PCR** = polymerase chain reaction, **phOx** = 2-phenyloxazol-5-one, **QCM** = quartz crystal microbalance, **RACE** = rapid amplification of cDNA, **scFv** = single chain fragment variable, **SPR** = surface plasmon resonance, **TEL** = turkey egg-white lysozyme, **TNF** = tumor necrosis factor, **V** = genes variable antibody subgenes, $\mathbf{V_L}$ = variable domain of light chain, $\mathbf{V_H}$ = variable domain of heavy chain

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Supported by the Grant Agency of the Czech Republic (Project No. 525/03/0747) and partially supported by the Ministry of Agriculture of the Czech Republic (Project No. MZE 0002716201).

1. Introduction

Until the late 1980s the production of antibodies relied primarily on animal immunisation. The development of molecular methods for the expression of recombinant antibody fragments in bacteria and techniques for production and screening of combinatorial libraries has opened a wide range of opportunities for the selection of recombinant antibodies and their engineering (Maynard and Georgiou, 2000). The first reports of recombinant antibody fragments produced in bacteria appeared only 17 years ago (Bird et al., 1988; Huston et al., 1988).

Several *in vitro* methods have been developed for production of antibodies (Bradbury et al., 2003) in contrast to *in vivo* animal-based methods. The most commonly used technology among the various *in vitro* strategies is phage display (Hust and Dubel, 2004), recognized as a powerful tool for selecting recombinant antibody fragments with specific binding properties from a vast number of variants against a wide range of target molecules, such as proteins, glycoproteins, oligosacharides, nucleic acids, toxins or low molecular weight compounds – haptens (Willats, 2002; Yau et al., 2003). Phage display, first described by George P. Smith in 1985, refers to the display of functional foreign peptides, proteins or antibody fragments on the surface of a bacteriophage (Smith, 1985; Clackson et al., 1991). This is accomplished by fusion of the DNA coding sequences of the protein to be displayed into the phage genome to the gene encoding one of the phage surface proteins (Gao et al., 1999). It means that the gene fusion product of antibody fragments together with a phage coat protein is presented on the phage surface, while its coding genetic material resides within the phage particle. The direct link between genotype and phenotype of the antibody being displayed is the common feature among the various display technologies (Yau et al., 2003). Antibodies have been displayed as functional binding molecules in different formats of antibody fragments (McCafferty et al., 1990; Hoogenboom et al., 1991; Griffiths et al., 1993).

Surface display of the antibodies allows affinity selection by the antigen *in vitro* – an analogue of selection by antigen in natural immunity (Petrenko and Vodyanoy, 2003). Affinity selection of antibodies is accomplished by exposing the phage library to immobilized antigen molecules (binding clones are captured and non-binding clones are washed away). The captured phage particles are eluted from antigen, amplified by infecting *Escherichia coli* host cells and used in a subsequent round of affinity selection. After the final round of affinity selection phage particles are amplified in order to prepare and characterize their displayed antibodies individually (Petrenko and Vodyanoy, 2003). Finally, the monoclonal phage population with the desired binding specificities can be isolated (Willats, 2002).

With regard to antigenic specificity different types of phage libraries are currently used for selection of specific recombinant antibodies: (1) a specific library sourced from immunised animals, (2) a single-pot (general) library created with no specificity against a particular analyte. Moreover, a secondary mutant antibody phage library from randomized (mutagenized) DNA sequences of a monoclonal hybridoma line or single phage clone can be constructed and antibodies of higher affinity can be selected (Marks et al., 1991; Hawkins et al., 1992). Although high affinity recombinant antibodies can be generated from a library derived from immunised animals, selections have yielded antibody fragments with dissociation constants in the nanomolar range, this approach has several drawbacks. One of serious problems is the necessity to construct a new library for each antigen (Hust and Dubel, 2004). Also the problem associated with immunisation of animals is not avoided in these cases. In the past decade, the limitations mentioned above lead to the creation of highly diverse and universal single-pot libraries from which antibody fragments with binding affinities to a wide range of antigens can be isolated. Such single-pot libraries completely avoid the use of laboratory animals (Willats, 2002). The affinity and specificity of recombinant antibody fragments selected from single-pot libraries is directly linked

to the size of the library repertoire (Hoogenboom et al., 1998).

The main advantage of phage display technology is the large diversity of variant antibodies that can be represented and screened (for instance phage display antibody libraries with diversities 10^{10} can be constructed). Moreover, when a large library is prepared the use of the phage display technique is relatively simple, cheap, rapid, by-passing immunisation (single-pot libraries) and it is possible to significantly improve the throughput of its selection/screening process via automatized robotic workstations (Willats, 2002).

Several general reviews of phage display technology have been recently published (Gavilondo and Larrick, 2000; Maynard and Georgiou, 2000; Azzazy and Highsmith, 2002). Others deal with biotechnological applications (Benhar, 2001), stability of recombinant fragments (Worn and Pluckthun, 2001), applications in cancer therapy and diagnosis (Hudson and Souriau, 2001), immunotherapeutic applications (de Haard et al., 1998), multifunctional antibodies (Kriangkum et al., 2001), hapten-specific recombinant antibodies (Yau et al., 2003) or recombinant antibodies for environmental analysis (Kramer and Hock, 2003). This article presents a methodical overview for the generation of recombinant antibodies and engineering antibodies for improved antigen binding properties, especially against low molecular weight analytes i.e. haptens, with a general introduction to antibody phage display technology.

Recombinant antibodies generated via phage display have been mainly utilized in therapeutic and biomedical applications, but most recently recombinant technology has also expanded to other applications including immunochemistry diagnostics of low molecular weight compounds – haptens. The main application of hapten-specific recombinant antibodies may be as an immunoanalytical agent in analytical or screening assays based on various formats of ELISA and/or as an active compound of biosensor surfaces, mainly for routine monitoring of pesticides, antibiotics or other drug contamination in water, food and environment. The generation of high-affinity antibodies against hapten targets (molecular weights below 1000 Da) presents a particular problem, because haptens are not recognized by the host immune system unless presented as an epitope linked to a suitable immunogenic carrier. Conventionally, hapten-specific antibodies are produced from hyperimmunised animals or hybridoma cell lines. However, monoclonal antibody production is technically demanding and sometimes not straightforward enough (Yau et al., 2003). Therefore, recombinant DNA technology is one of the ways to complement and even partly replace hybridoma technology in production of monoclonal antibodies.

2. Recombinant antibody fragments

Recombinant antibodies include wide spectra of formats. The most popular format appears to be the single chain variable fragment (scFv) and its variants: disulfide stabilised variable fragment (dsFv) and the bare variable fragment (Fv), or fragments of antibodies described as Fab – containing variable domains plus the first constant domains or $F(ab')_2$ consisting of two disulfide linked Fab fragments, as depicted in figure Figure 1.

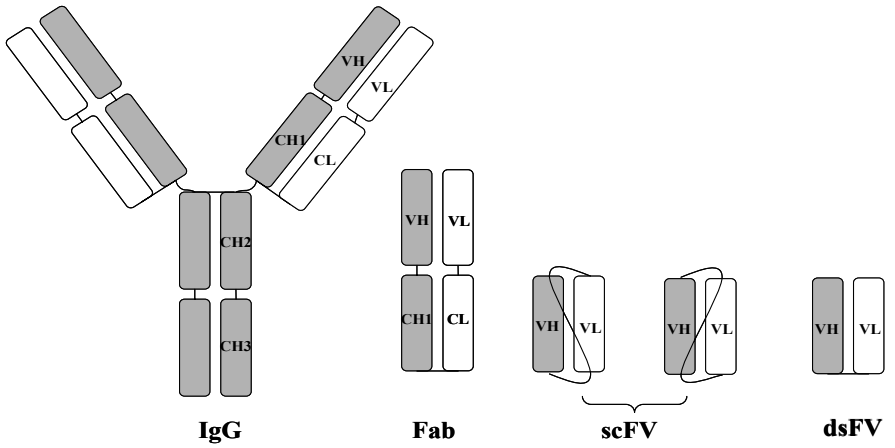

Figure 1. Schematic picture describing IgG antibody molecule and various recombinant formats

Further variants include combinations of antibody domain modular structures represented by minibodies, chelating recombinant antibodies (CRAbs), multifunctional and multispecific diabodies, triabodies, tetrabodies and fusion constructs of antibody fragments and toxins called immunotoxins or antibody fragments and other pharmaceutically active compounds called immunodrugs.

In regards to avidity, antibody fragments are typically produced with half avidity of the whole antibody molecule as a monovalent fragment but can be prepared with similar or increased avidity (multivalent fragments) when compared to the original IgG molecule. Size of the fragment plays an important role particularly in therapeutic applications. Smaller fragments have improved pharmacokinetic properties, because of their lower retention times in non target tissues, better target tissue penetration and clearance (Colcher et al., 1998). Due to their properties, antibody fragments can perform significantly better in therapeutic applications, regardless of the structure of their binding site.

In the immunochemistry field, mono- or polyvalent modification of respective antibodies has an influence on assay sensitivity; although for low molecular weight analysis the valency of antibodies does not play significant role. Therefore, the applications of single valent fragments predominate see Table 1.

2.1. Single-chain Fv fragments (scFv)

ScFv molecules are the smallest antibody fragments (26–27 kDa). They contain a complete binding site and consist of the individual heavy and light chain V domain (12–14 kDa each) and are typically linked to a single protein by a 15aa long hydrophilic and flexible polypeptide linker, which can additionally include also a His tag, an immunodetection epitope and a protease specific cleavage site. The linker of the variable region domains (carboxyl terminus of the V_L sequence to the amino terminus of the V_H sequence) has to be long enough to span the distance from the C-terminus of one domain to the N-terminus of the second domain (approx. 3.5 nm). It was reported that longer linkers can increase the antibody fragment's affinity and also decrease the formation of aggregates (Whitlow et al., 1993).

Suitable linking of domain is critical for correct conformation, expression and for proteolytic

Table l. Various antibody formats and biotechnological or therapeutic applications

Antibody format	Application	Reference
scFv	Hepatocelullar carcinoma therapy	Yeung et al., 2004
	DNA vaccination anti BCL1 lyphoma	Cesco-Gaspere et al., 2005
	Structural and functional analysis	Midelfort et al., 2004
	Self assembling monolayers- antibody arrays	Kwon et al., 2004
Multivalent scFv	Carbohydrate specific antibodies	Ravn et al., 2004
Fab	Anti platelet IIb/IIIa – inhibition of platelet aggregation	Yang et al., 2004
	Shiga Toxin 1 neutralization	Inoue et al., 2004
	Study of the retro-Diels-Alderase catalytic antibody	Zheng et al., 2004
dsFv	Cytotoxic recombinant anti-mesothelin immunotoxin	Li et al., 2004
	Anti-CD22 recombinant immunotoxin in chemotherapy of resistant hairy-cell leukemia	Kreitman et al., 2001
Minibody	Antiidiotopic antibody – cancer therapy	Chang et al., 2003
Bispecific minibody	Cure of trombocytopenia	Orita et al., 2005
	Tumor lysis	Shahied et al., 2004
IgG	Anti HbsAg neutralizing activity	Guo et al., 2004
IgG, IgE	Birch pollen allergen-specific Fab fragments isolated from combinatorial IgE and IgG libraries	Jakobsen et al., 2004

stability issues. Moreover, the linker can influence domain orientation, which consequently influences the affinity of the binding site. The extent of scFv multimerization is determined by structure and even more by length of the linker. These two parameters of the linker also influence refolding kinetics of the scFv protein (Desplancq et al., 1994; Whitlow et al., 1994). The linker $((Gly)_4Ser)_3$ was originally designed to fulfill these requirements. The glycine residues confer the flexibility of peptide bonds while the serines provide hydrophilicity of side-chains. However, the interactions among linker and antibody domains are difficult to predict, therefore other approaches of constructing and optimizing linker length and structure were used. For instance Hennecke et al. (1998) randomized the linker structure and used selectively infecting phage particles for the selection of scFv with optimal linker structure. An alternative approach used for domain linkage is to employ artificially positioned cysteines forming disulphide bond between the domains, which suppress domain dissociation (Kipriyanov et al., 1997). This dsFv (disulphide stabilised Fv) may have some advantages over the scFv in stability and aggregation of the molecule (Reiter et al., 1994). Generally scFv´s are stable at 37°C and are easier to express than recombinant Fab.

2.2. Multivalent Fv fragments

Multivalent forms of recombinant antibody molecules extend functional repertoire of single valent fragments by increasing avidity of the molecule. These molecules can be prepared by several methods. One option is to pair the domains of one scFv molecule with domains of another scFv(s) thus making bivalent or even trivalent fragments (Whitlow et al., 1993). Another option is disulfide linking of dimeric scFvs by site-specific dimerization of scFv containing unpaired C-terminal cysteines (Kipriyanov et al., 1992). Covalently linked chimeric scFvs, non-covalently linked scFv dimeric (diabodies) and trimeric molecules also have been studied. Kipriyanov et al. (1996) reported the production of artificial multivalent antibodies by fusion of scFv to streptavidin. This tetravalent fusion protein isolated from periplasmic proteins of *E. coli* was shown to retain biotin and antigen-binding activity.

2.3. Multifunctional scFv fragments

Multifunctional scFv can be constructed with various functional properties such as enzyme labels – peroxidase, alkaline phosphatase (Boulain and Ducancel, 2004; Takazawa et al., 2004), functionally labelled by streptavidin (Dubel et al., 1995), metal binding site (Malecki et al., 2002) or by leucine zippers (de Kruif and Logtenberg, 1996). Bispecific antibodies can be also prepared as single-chain constructs by linking two single-chain antibodies using a flexible linker (Schmiedl et al., 2000). The described scFv-scFv molecule might be of particular value for therapeutic *in vivo* applications because of its stability, minimal immunogenicity and more over its bispecificity enables catalysis of novel immune system reactions.

3. Recombinant antibody technology – Antibody Phage display

Incorporation of the recombinant antibody fragment and coat protein fusion product into mature phage coat exposes the antibody on the phage surface while its coding material resides within the phage particle. During the affinity selection (bio-panning) process phage populations are exposed to the tested targets in order to selectively capture binding phage. Throughout repetitive rounds of binding, washing, elution and amplification, the original diverse phage population is increasingly enriched by phages with a potential binding affinity to the tested target (Willats, 2002).

3.1. Bacteriophage M13

Different types of phage have been used as vehicles for phage display including Ff filamentous phage, Lambda and T7 (Rodi and Makowski, 1999; Danner and Belasco, 200; Willats, 2002). However, the most commonly used phage for display systems is the Ff phage family (e.g. M13, fd-phage). They are excellent cloning vehicles because the insertion of foreign DNA within their genome results in simple assembly of longer phage particles (Willats, 2002). Non-lytic M13 phage (Figure 2) is a cylinder particle (~880 nm long and 6.6 nm wide) and consists of a circular single-stranded DNA packed into a protein coat capsule. The single-stranded DNA genome of filamentous phage encodes nine different proteins, five

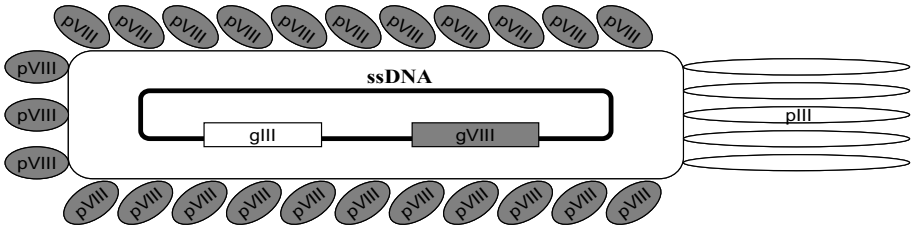

Figure 2. Schematic picture of filamentous phage M13, white areas represent gene (gIII) encoding coat protein pIII (pIII) and grey areas represent gene (gVIII) encoding coat protein pVIII (pVIII)

of them responsible for forming the phage coat capsule. Among these, pVIII is the major coat protein (with ~2700 copies per phage), while pIII, pIV, pIV, pVII and pIX are minor coat proteins (with ~5 copies per phage). All of coat proteins have been used for the display of foreign proteins (Hoogenboom et al., 1998; Sidhu, 2001; Willats, 2002; Yau et al., 2003). The different display systems were classified by (Smith and Petrenko, 1997) according to the fusion protein, type of cloning vectors, and the final fusion product used. Phage display system for fusion to pIII is illustrated on Figure 3. The simplest way of achieving the expression of foreign protein is creation of a fusion between the DNA sequence encoding protein to be displayed and chosen coat protein gene within the viral protein. This direct fusion causes all the copies of chosen coat proteins to become fusion proteins. The main drawback of this system is that fused scFvs compromise coat protein function (i.e. infection of *E. coli*) (Winter et al., 1994; Sidhu, 2001; Willats, 2002). This mentioned limitation has been resolved by development of hybrid phage display. In hybrid phage system two copies of gene coding phage coat protein exist; one with the scFv fusion and additional coding natural non-fusion protein. Thus a wild type copy of the coat protein is retained in the phage particle and phage particles display both wild type and fusion proteins (Sidhu, 2001). Phage systems displaying both wild type and fusion proteins can be also created using phagemid-based system. In this system phagemids (plasmids with a phage origin of replication) carry sequences encoding fusion proteins. Helper phage, that are co-infected together with phagemids into host bacteria, carry the majority of the genes required for the formation of phage

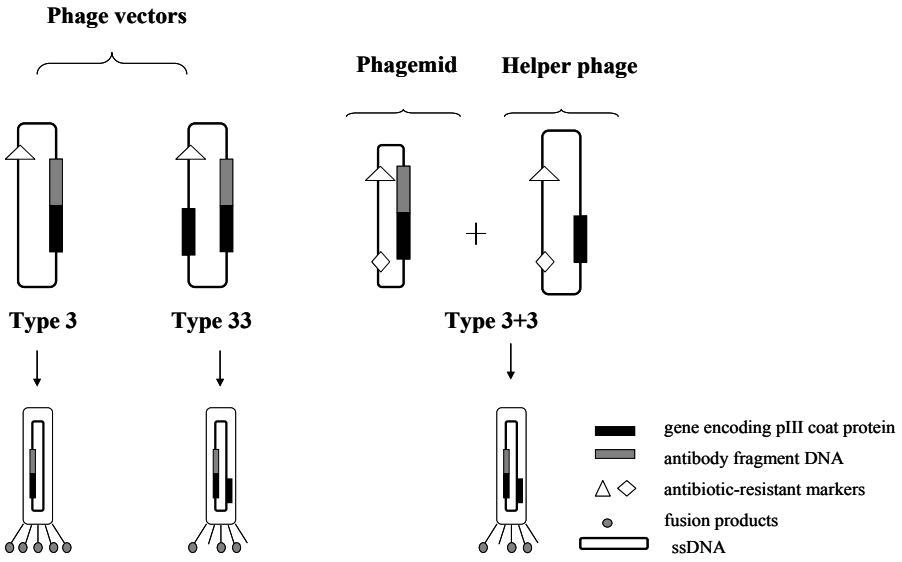

Figure 3. Various phage display systems based on pIII coat protein, gene pIII is represented as a black box, the foreign DNA insert as a grey box, and the fusion products as a grey circle (based on Yau et al., 2003)

particles (Willats, 2002). For selection of a high affinity antibody, it is reasonable to employ pIII based systems since pVIII systems produce truly multivalent phage interacting on multiple sites of active surface on the surface of selection tube, which decreases selection pressure and effectiveness of such systems (Little et al., 1994).

3.2. Phage antibody libraries

Libraries displaying antibodies can be prepared from natural, synthetic or mixed DNA sources. With regard to antigenic specificity, different types of libraries are currently in use: (1) a specific library sourced from immunised animals or a specific secondary library prepared from randomized (mutagenized) DNA sequence of a monoclonal hybridoma line or single phage clone (2) a single-pot (general) library created with no specificity against particular analyte.

3.2.1. Phage libraries derived from immunised animals

An immune antibody library has two basic characteristics: (1) its pool of antibodies is enriched with antigen-specific antibody and (2) a significant portion of these antibodies have undergone affinity maturation by the immune system (Clackson et al., 1991; Azzazzy and Highsmith, 2002).

Immunised animals are used in these libraries to isolate V-genes from the IgG mRNA of B-cells. Under some circumstances the recombinant antibody selected from the immune antibody library can be better than monoclonal antibodies obtained from hybridomas technology (Verhaar et al., 1995). Generally, these libraries do not need to be as large as the naive ones; sufficient is 10^{6-7} of different clones with unique and functional scFv´s. High-affinity antibodies against wide spectrum of antigens were derived from various immunised animals, such as mice (Tout et al., 2001), chickens (Andris-Widhopf et al., 2000), rabbits (Li et al., 2000; Kramer, 2002a,b), and also from sheep (Charlton et al., 2000, 2001) and nonhuman primates (Azzazzy and Highsmith, 2002). Advantages and disadvantages of immune libraries include: (1) the long time requirement for animal immunisation, (2) easier preparation than naive libraries (only medium sized repertoires, 10^6–10^7 of different phage particles are desired), (3) the unpredictability of the immune response of the animal to an antigen of interest, (4) the lack of immune response to some antigens, (5) the restrictions in generating human antibodies, (6) construction of a new library for each new antigen (which increases the total time of the procedure by 1–3 month). For rapid and easy construction of new phage libraries there are some commercially kits available (e.g. RPAS from Pharmacia Biotech for the creation of scFv repertoires from mouse or rat spleen cells).

3.2.2. Single-pot (general) libraries

A. Naive libraries

Naive libraries containing the V-gene repertoires can be created from collections of V-genes from the IgM/IgG mRNA of B cells of the non-immunised human donors, isolated from peripheral blood lymphocytes, tonsils, bone marrow, spleen cells or from animal and human sources, e.g. mice (Gram et al., 1992), shark (Dooley et al., 2003), and human (Clackson et al., 1991; Knappik et al., 2000). Using specific primer sets and PCR, IgM and/or IgG variable regions are amplified and cloned into the vectors designed for selection and screening. It is important for naive library to be as large as possible. The number of at least 10^8 individual clones is necessary to prepare of a representative sample of binding repertoire. Recently it has been reported the largest libraries contain about 10^{11} individual clones (Sblattero and Bradbury, 2000). An ideal naive library is expected to contain a representative sample of the primary repertoire of immune system, although it does not contain great proportion of antibodies with somatic hypermutations produced by natural immunisation.

The major advantages of using a very large naive library is: (1) it contains greater antibody sequence diversity and multiple antibodies against numerous targets (antigens) – increasing its chance to obtain a valuable antibody with a desired cross-reactivity profile to similar compounds, (2) one general library can be used for all antigens, (3) antibodies to self, non- immunogenic or toxic substances can be generated using phage libraries circumventing limitations of *in vivo* immunisation of animals, (4) antibodies reacting with human antigens can be isolated, (5) short time (down to two weeks) of antibody generations (2–4 rounds of selection)

can be automated using high throughput methods, (6) high affinity antibodies can be isolated directly from the library (Vaughan et al., 1996; de Haard et al., 1999), (7) and by-passing immunisation.

Disadvantages and hindrances in certain applications have been shown: (1) inclusion body formation can occur during production, which possibly results in poor expressivity and toxicity to host bacteria, (2) a production host can induce different glycosylation patterns of recombinant antibodies, which influence their properties in *in vivo* applications, (3) lower affinity antibodies are obtained when small sized repertoires are used, (4) there is limited information about detailed contents of naive libraries, (5) and it is technically demanding to construct these repertoires. These disadvantages may be bypassed by using synthetic antibody repertoire libraries.

B. Synthetic libraries

Synthetic repertoires are created by mutational modification of the CDR sequence of selected frameworks coding antibody variable domains. CDRs are modified by predetermined level of randomisation. These repertoires are then cloned into an antibody domain framework and subcloned to a phage display vector to generate a library of about 10^7–10^{10} clones (Griffiths et al., 1994).

C. Semi-synthetic libraries

Semi-synthetic repertoires also can be generated by selecting one or more antibody frameworks within the CDR loops. The CDR regions can be partially or completely randomised using appropriately designed randomised oligonucleotides.

Both, synthetic or semi-synthetic libraries can be constructed either with restricted V-gene usage or by all of the known V-gene segments of the species. High diversity in composition and in length is found particularly in the CDR3 H3, which significantly contributes to antigen-binding, making this particular CDR an attractive target for randomisation. Efficient cloning system (*in vivo* Cre/loxP site specific recombination) and a combination of the dual antibody cloning strategy allows construction of very large repertoires with about 10^{9-11} individual clones (Hoogenboom and Winter, 1991; Griffiths et al., 1994; Sblattero and Bradbury, 2000; Krebs et al., 2001). The main advantage of these repertoires over the naive repertoires is that the contents, local variability and overall diversity can be controlled and defined.

3.3. Construction and Production of Phage Libraries

The PCR has greatly simplified the cloning of V region genes. To amplify the V_L and V_H immunoglobulin regions, cDNA is produced by reverse transcription from B-lymphocyte mRNA, and used as a template for PCR. The genetic material can also be isolated from the hybridoma cell lines, single clones obtained from primary library or from B-lymphocytes of immunised animals.

PCR amplification requires oligonucleotide primers recognizing antibody gene sequences. Several primer sets have been published up until today with various target sequences for primer annealing. The primers on the 5´ ends, published by Benhar and Pastan (1994), were based on the N-terminal sequence of mouse monoclonal antibodies. Ruberti et al. (1994) published primers annealing to the antibody leading sequences. Sequences obtained via rapid amplification of cDNA ends (RACE) were used for primer design by Larrick et al. (1989) and primers based on the known variable region framework of amino acid sequences from V-base databases were deduced by Sblattero and Bradbury (1998).

For amplification of V regions from antibodies of different subclasses a set of downstream primers corresponding to sequences encoding part of the first constant region domain of heavy (C_H1) and light (C_L) chains are used. Together with downstream primers selected for the type of antibody chain, the degenerate upstream primers (corresponding to sequences encoding parts of the signal peptide or FR1 regions) are used. Degenerate primers often cause mutations, although not in binding site those can hamper stability or level of expression of the antibody.

The two variable domains can be assembled into a single gene using a DNA linker either in the V_H- linker- V_L or V_L- linker- V_H form (Tsumoto et al., 1994; Ayala et al., 1995; Merk et al., 1999). Merk et al., 1999 presented that the expression level of scFv in N-termini-V_L domain-linker- V_H domain-C-termini orientation was relatively high but changing the order of variable domains causing a significant decrease in yield during *in vitro* expres-

sion. Therefore testing a suitable order of antibody domains is logical when considering the product yield. Prepared DNA fragments are inserted into a suitable vector for cloning and display of antibody fragment libraries.

Phagemids, the most popular vector for display, are hybrids of phage and plasmid vectors. These vectors contain the origins of replications for both M13 phage and *E. coli*, gene coding part of pIII protein with the appropriate cloning site, and an antibiotic-resistance gene (Azzazzy and Highsmith, 2002). The biggest advantage of phagemids in comparison to a single stranded phage genom is their small size (3–5 kb) and their higher bacterial transformation efficiency. Typically, the recombinant phagemid is introduced into competent *E. coli* by $CaCl_2$ transformation or electroporation. Phagemid-containing bacterial cells are grown and then infected with a helper phage (M13VCS or K07) to yield recombinant phage that display antibody fragments as fusion to one of the phage coat proteins.

3.4. Selection of binders – "Biopanning"

Antibody variants expressed on the surface of bacteriophage are selected on basis of their affinity for a tested antigen by technique known as bio-panning (Figure 4). The selection process is achieved by repetitive rounds of phage binding to the target, washing to remove non-binding phage and elution steps to get binding phage and reamplification of the phage pool enriched for specific binding phage.

Any methods that are able to separate the specific binding clones from non-binding clones can be used as a selection method. The frequently used *in vitro* selection methods are: bio-panning on immobilized antigen coated onto plastic tubes or Petri dishes (Marks et al., 1991), columns with antigen activated matrix (McCafferty et al., 1990), or specifically coated BIAcore sensorchip (Hoogenboom et al., 1998), selection using biotinylated antigen (Hawkins et al., 1992) or direct selection on fixed prokaryotic cells respectively mammalian cells (de Kruif et al., 1995).

After washing away a large portion of non-specific phage, the phage clones bearing antibodies with specific binding properties to the matrix can be eluted in different ways. Changing binding conditions with acidic solution i.e. HCl, glycine buffers (Parmley and Smith, 1988; Kang et al., 1991; Hoogenboom et al., 1998), or with basic solution like triethylamine (Marks et al., 1991) or reducing agent for disrupting disulphide bridges between supports and targets are often used for non-specific elution. In case the binding phage are challenged by antibodies directed against an epitope on antigens (Meulemans et al., 1994), a competition elution by excess concentration of antigen (Clackson et al., 1991) or antibodies to the antigen may be used for selecting the phage. A more gentle approach can be employed by using enzymatic cleavage of a protease site engineered between the antibody and gene III, in case of the concerns about the effects of elution condition on phage integrity (Ward et al., 1996; Willats, 2002).

Ideally, only one cycle of selection would be required, however the binding of non-specific phage

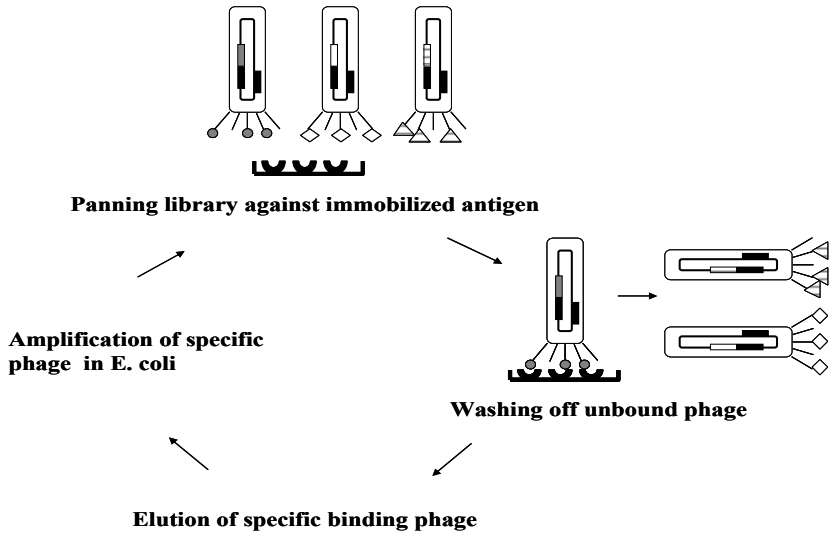

Figure 4. Schematic picture of bio-panning process – the selection of specific-binding phage from phage libraries

limits the enrichment that can be achieved by one cycle, so in practice several rounds of selection are necessary (McCafferty et al., 1990; Clackson et al., 1991; Griffiths et al., 1994; Winter et al., 1994; Hoogenboom et al., 1998).

Choice of selection conditions, the concentration of antigen and its density on the surface of solid phase influences the efficiency of selection and must be optimized during the course of selection procedure. Initial rounds of selection are typically performed under low stringency conditions (high antigen concentration, short washing times, elution) that allow keeping the rare binders in the selectable pool. Stringent conditions are applied gradually along with the growing number of selection cycle. The phage antibody with higher affinity may be enriched during rounds of selection by decreasing the concentration of antigen and by using combinations of different elution steps.

Fast and robust screening assay for binding affinity is applied after the end of selection process. A different basic methods ranging from a simple ELISA with coated antigen using phage antibodies known as "phage ELISA" or ELISA with coated antigen using the soluble antibody fragments (scFv) to various bioassays are used as screening assays for determination of binding affinity of selected recombinant antibodies to the antigen. The specificity of selected antibodies may be also tested by a fluorescent particle sorter (FAPS), or immunocyto- and histo-chemistry. For the further screening of affinity and kinetics of binding, biosensor devices are often capable of detecting mass shift upon specific binding of phage antibodies on active surface of sensor, such as surface plasmon resonance (SPR) (Emanuel et al., 2000), or quartz crystal microbalance (QCM) (Hengerer et al., 1999a,b).

3.5. Expression and purification of recombinant phage antibodies

Recombinant antibody fragments (scFv, Fab) are expressed efficiently in many microorganisms, particularly in selected strains of *E. coli*. Especially, *E. coli* host strains such as BL21 and their derivatives become especially attractive for economical production of large amounts of sole scFv fragment (An et al., 2002; Kramer et al., 2002).

Soluble antibody fragments can be produced in culture supernatant, bacterial periplasm, and/or inside the bacterial cytoplasm, depending on the particular signal sequences on N termini of recombinant antibody. While, signal sequences enable secretion to periplasm and media the overall yield is limited, but the oxidative environment outside cell cytoplasm confers to correct formation of disulfidic bonds demanded for natural folding of antibody domains. Antibodies expressed into the periplasm are recovered by osmotic shock, extruding functional molecules outside the cells. Alternatively, high-level expression of antibody fragments in the cytoplasm of *E. coli* often results in extensive aggregation and formation of inclusion bodies. These can be purified from other cellular components by centrifugation. The aggregated antibody mass can be solubilized by adding strong denaturants such as urea or guanidiun hydrochloride including some oxido-reduction system. Native antibody fragments are recovered by refolding upon removal of the denaturants. Guidelines for antibody refolding have been reviewed by Maynard and Georgiou (2000).

E. coli is considered as the basic organism for antibody production. However, it should be noted that antibody fragments also have been expressed in mammalian cells (Dorai et al., 1994; Uppala and Koivunen, 2000), insect cells (Mahiouz et al., 1998; Ailor et al., 1999), yeasts like *Saccharomyces cerevisiae* (Frenken et al., 2000), *Schizosaccharomyces pombe* (Davis et al., 1991), *Hansenula polymorpha* (Abdel-Salam et al., 2001), recently also in *Pichia pastoris* (Marty et al., 2001) and in bacterial host strains such as *Bacillus brevis* (Shiroza et al., 2003). Generally, choice of the expression hosts (bacteria, yeast, plants, insect or mammalian cells) must be consistent with final application of antibody molecule. For instance, pharmaceutically active compounds in some cases must contain completely glycosylated or phosphorylated proteins, which is possible only with eucaryotic (preferably mammalian) cell lines. However, prokaryotic organisms can be preferred with respect to possible risk of BSA transfer. Moreover therapeutic antibodies must be rigorously purified with respect to possible endotoxin contamination. High yields of antibody fragments in *E. coli* (up to 1–2 g/l) of functional antibody were reported by authors (Carter et al., 1992; Shibui et al., 1993), but in most cases production typically ranges between 20 and 200 mg of pure and active antibody fragment per litre of culture (Kipriyanov et al., 1997).

Results concerning yeast production systems confirm that the *Pichia pastoris* is a suitable host for

high-level production of scFv antibody fragments with potential *in vivo* diagnostic and therapeutic applications directly into the growth media (Freyre et al., 2000), with fast growth and low levels of contaminant proteins in the medium. An interesting point is that there is no advantage to be gained by expressing antibody fragments, which have a relatively high yield in *E. coli* and are functional, in *P. pastoris*. On the other hand, *P. pastoris* represent an attractive alternative for the antibodies, which are poorly expressed in *E. coli* (Cupit et al., 1999).

Insect cells also have shown high-level expression of scFv fragments (Kretzschmar et al., 1996) and become an efficient alternative to *E. coli* expression. However if sufficient expression characteristic is obtained by *E. coli*, there is no apparent need for using alternative hosts. Recombinant antibody fragments can be also expressed in plants. The first functional antibodies were reported in 1989 (Hiatt et al., 1989), and in 1995 high-level production of engineered antibodies was obtained in tobacco seeds (Fiedler and Conrad, 1995). Because of low cost, improvement of vectors for plantibodies, purification strategies and increase in transformable crop species, production of antibodies in plants is under intense investigation as a alternative way for production of large amounts of immunoglobulins. Genetically modified plants serving as bioreactors and providing yields over 10 kg of therapeutic antibody per acre has been developed (Gavilondo and Larrick, 2000). The differences in glycosylation of plant antibodies is significant and the difference lies mainly in plants glycosylates, which contain sugars: β (1,2) xylose and α (1,3) fructose which are not found in animal proteins (Maynard and Georgiou, 2000).

A general way to purify recombinant protein is to use one of available purification tags added either to N- or C-termini of the protein and it is necessary to use two to three purification steps to achieve desired purity. Immobilized metal ion affinity chromatography (IMAC) has proved to be the most common way for purification of bacterially produced antibodies, because of its favourable cost/efficiency ratio (Porath, 1992; Blank et al., 2002). This method is based on the binding affinity of metal ions such as Ni^{2+}, Cu^{2+} or Zn^{2+}, chelated to a chromatography matrix (typically nitrilotriacetic acid), which forms complexes with 5–6 histidine residues on the N- or C-terminus of an antibody fragment (Skerra et al., 1991).

3.6. Improving affinity of recombinant antibodies

The affinity of recombinant antibodies selected from medium sized single-pot or immune phage libraries is sufficient for use as research reagents, but often rather not optional for therapeutic applications (Hoogenboom, 1997) and the affinity maturation of the selected recombinant antibodies is required. The modification technology uses a non-localized mutagenesis strategy, which can be used in absence of a known 3-D structure of recombinant antibody binding site. For this purpose is commonly used error-prone PCR, random mutagenesis over the entire V-gene and chain-shuffing, which pairs and combines the V_L and V_H genes in number of different ways.

The advancement of protein modelling technology has made it possible to construct 3D models, which helps form hypotheses about binding site functionality. Sequencing data from an antibody gene of interest can be used to produce a computer-generated model of the binding site (Lee and Morgan, 1993). Modelling of antibody binding sites represents a combination of protein homology modelling, where a large number of antibody structure datasets can serve as a knowledge database and *ab initio* modelling used for parts of the antibody with high variability. The modelling procedure is started at the variable domain framework region (FW), which is well conserved in structure among different antibodies. The framework regions can be modelled by selection of several similar known structures in protein structure database repository (Protein Database at RCSB). A structure that is sequentially most homologous to the studied protein is used as template for FW modelling. In the case antibody sequence, structural variability is restricted to the hypervariable regions. CDRs are described as CDR-L1, L2 and L3 in the light chain and CDR-H1, H2 and H3 for the heavy chain. All CDRs except for CDR H3 are classified to the structural canonical classes, which facilitates modelling of their structures (Chothia and Lesk, 1987; Al-Lazikani et al., 1997; Decanniere et al., 2000).

Building of the canonical loops employs the known structures of highly homologous loops of the same canonical class. These are searched from database and respective amino acid changes are modelled. For non-canonical loops, the CAMAL (Combined Antibody Modelling Algorithm) by Martin et al. (1989, 1991) can be used. It consists

of a combined database/conformational search. Nowadays similar approaches are described in works of Mandal et al. (1996) and Whitelegg and Rees (2000). It should always be respected that a model represents only hypothetical structure unless it is validated by independent method. But despite the limited accuracy of antibody structure prediction, a structural model of the molecule is a useful tool for interpretation of experimental results and formulation of new hypotheses.

These hypotheses can be used in mutagenesis experiments, which are targeted to particular aminoacid residues or limited region responsible for antigen binding. These regions mostly lie within the CDR loops, although several residues flanking CDR were confirmed to also play a significant role. Mutations in binding site may affect the affinity indirectly by influencing the position of side chains contacting the antigen, by providing new contact residues or by replacing residues with low interaction energy by residues with more favourable energetics. The affinity maturation process can also involve: introduction of diversity in antibody V-genes, creation of a secondary mutant library from selected low affinity/specificity clone and selection of higher-affinity from low-affinity variants.

4. Anti-Hapten recombinant antibodies

The analytical immunochemistry dealing with low molecular weight molecules – haptens has been influenced by recombinant antibody technology, although primarily recombinant antibodies have been evaluated especially for therapeutic and biomedical application. The recent approaches of generation hapten-specific recombinant antibodies are successfully using both types (immunised/single-pot) of phage display libraries.

4.1. Immunised libraries against haptens

Antibody-producing cells, such as hybridoma cells, splenocytes or lymphocytes from the immunised donors can be used as sources of genetic material for libraries. Li et al. (2000) can serve as a successful example who used for construction of immunised phage library splenocytes of rabbit immunised simultaneously with 4 different herbicide-protein immunogens. The high affinity scFv antibodies specific for mecoprop, atrazine, simazine and isoproturon were produced within one procedure. A library for picloram-selective scFv was established by Tout et al. (2001) using the same kind of immunised source of genetic material. Kramer (2002a) developed a strategy to generate a group-selective phage library. This library was prepared from B-cells of mice immunised with different *s*-triazine immunogens secreting *s*-triazine-selective antibodies, which were then separated by immunomagnetic separation subtracting active B-cells. Specific phages enriched by three repetitive cycles of selection showed IC_{50} sensitivities in the nanomolar range.

The shift in antibody specificity was achieved by Kramer (2002b) using the sebuthylazine specific antibody, initially derived from a triazine-selective antibody library (Kramer, 2002a). DNA coding this molecule was used as a template to generate mutant antibodies that preferentially recognized atrazine. The employed method used a combination of random point mutations with V-chain shuffling of a triazine-selective immunoglobulin repertoire. The contribution of engineered antibody variants to the assay improvements was reflected by a shift of the equilibrium of dissociation constant K_D (atrazine) from 1.27×10^{-8} of the template antibody to 7.46×10^{-10} of the engineered variants.

An immunised phage library was also used for production of several other hapten-specific antibodies fragments, such as antibody fragment against ampicilin (Burmester et al., 2001) and atrazine (Charlton et al., 2000, 2001). Andris-Widhopf et al. (2000) constructed and screened combinatorial phage library against hapten fluorescein using scFv, diabody and Fab fragment library from immunised chickens.

An alternative way of preparing recombinant antibodies was used by Chiu et al. (2000) who used parental monoclonal antibody to generate PCB (polychlorinated biphenyl) specific recombinant fragment. This can serve as a general method of re-cloning and expression of the antibody genes from hybridomas into the suitable vector, which allows direct and convenient production of specific recombinant antibody fragments. Moreover, monoclonal hybridoma can be used as source for creating specific secondary library from a randomized (mutagenized) DNA sequence of parental cell line. A secondary library can be created and screened for antibodies with improved characteristics (Yau et al., 2003). Modification of specificity of generated antibodies was achieved in work by Korpimaki

et al. (2002, 2003, and 2004) who improved broad specificity recognition of monoclonal antibodies against sulphonamides antibiotics using phage display technology. A monoclonal antibody gene with high a cross-reactivity profile was cloned as a scFv fragment, and random mutant libraries were created with error-prone PCR over the whole scFv coding region. The libraries were selected using the phage display. Several of obtained mutants had significantly altered binding properties and improved broad specificity. In the following study these authors generated new antibody libraries by recombining the previously enriched libraries with DNA-shuffling and introducing new mutations with error-prone PCR and oligonucleotide-directed mutagenesis. The selected antibodies provided broad specificity to all 13 tested sulphonamides with relative high affinity and sensitivity. The different selected examples of recombinant antibodies for haptens selected from phage libraries derived from immunised animals or hybridoma cell lines are listed in Table 2.

4.2. Single-pot libraries

The robust potential in production of recombinant antibodies against wide spectra of antigens was demonstrated using large single-pot (non-immunised) phage libraries (McCafferty et al., 1990; Clackson et al., 1991; Marks et al., 1991; Griffits et al., 1994). Although single-pot libraries have been particularly used for isolation protein-selective phage clones, there are published some successful works in the selection of anti-hapten phage clones from these types' of libraries. One of the first attempts in the production of recombinant antibodies using phage libraries from non-immunised donors was made by Marks et al. (1991). The authors prepared a combinatorial phage library (with over 10^7 members) from peripheral blood lymphocytes of non-immunised donors using polymerase chain reaction (PCR). Phage with antigen-binding activities were selected by four rounds of growth and panning with either protein antigen (turkey egg-white lysozyme (TEL) or bovine serum albumin (BSA)) or with hapten (2-phenyloxazol-5-one (phOx)), and the encoding heavy and light chain genes were sequenced. Prepared soluble antibody fragment were shown to bind specifically to protein antigen or haptens with affinities, K_a (TEL) = 10^7 M^{-1}, K_a (phOx) = 2×10^6 M^{-1}, respectively, marking thus the first successful preparation of an anti-hapten recombinant antibody from a phage library. Griffiths et al. (1994) created a large synthetic repertoire (close

Table 2. Selected examples of recombinant antibodies for haptens selected from hybridoma cell lines or from phage libraries derived from immunised animals

Haptens	Antibody fragments/Source	References
Paraquat	scFv/hybridoma	Graham et al., 1995
2,4-D	Fab/hybridoma	Gerdes et al., 1997, 1999
Cyclohexanendione	scFv/hybridoma	Webb et al., 1997
Atrazine	scFv/hybridoma	Strachan et al., 1998
Atrazine, paraquat	scFv/hybridoma	Longstaff et al., 1998
Mercoprop	scFv/hybridoma	Strachan et al., 1998
Picloram	Fab/hybridoma	Yau et al., 1998
Atrazine	scFv/rabbit	Li et al., 2000
Fluorescein	scFv, Fab, diabody/chicken	Andris-Widhopf et al., 2000
Mecoprop	scFv/rabbit	Li et al., 2000
Atrazine	scFv/sheep	Charlton et al., 2000
PCBs	Fab/hybridoma	Chiu et al., 2000
Picloram	scFv/mouse	Tout et al., 2001
s-Atrazine derivates	scFv/rabbits	Kramer, 2002a,b
Sulfonamides	scFv/hybridoma	Korpimaki et al., 2002, 2003, 2004

to 6.5×10^{10}) of Fab fragments phage library. The Fab fragments with antigen-binding activity to a range of different protein antigens and haptens (e.g. fluorescein, 2-phenyloxazol-5-one (phOx), 3-indolo-4hydroxy-5-nitrophenyl-acetate (NIP)) were isolated with affinities comparable with those of antibodies from a secondary immune response in mice (up to 4 nM). de Haard et al. (1999) reported design and construction of large phage antibody library in Fab format from non-immunised donors (total clones 3.7×10^{10}). The suitability of using this phage library was determined by the successful selection of antigen-specific antibody fragments against six antigens including hapten, 2-phenyloxazol-5-one (phOx). The different large phage display libraries from forty-three non-immunised donors constructed by Vaughan et al. (1996) was used to isolate scFv fragments with antigen-binding activity against different protein antigens or haptens. The best of isolated scFv fragments, an anti-fluorescein antibody (0.3 nM) and an antibody directed against hapten diethylenetriaminepentaacetic acid (DTPA, 0.8 nM), were the first antibodies with subnanomolar binding affinities isolated from a naive library at that time. The different naive libraries were successfully used for the isolation of phage clones producing hapten-specific fragments against various steroid hormones. Gram et al. (1992) constructed a combinatorial Fab library expressing immunoglobulin μ and κ light-chain fragments from bone marrow of non-immunised BALB/c mice on the surface of filamentous phage. Phage displaying low affinity Fabs (binding constant, 10^4–10^5 M^{-1}) binding to progesterone-bovine serum albumin conjugate were isolated from the constructed library. Then random mutagenesis of the heavy- and light-chain variable regions of selected clones was performed by error-prone PCR, and subsequent clones with improved hapten-binding activity were selected. Dorsam et al. (1997) isolated human antibodies specific for digoxigenin, estradiol, testosterone and progesterone from a small combinatorial IgM repertoire (4×10^7) of single-chain antibodies. The determined affinities (K_a) of both anti-estradiol and anti-progesterone scFv were approximately 10^8 M^{-1}. Little et al. (1999) generated a large complex library of single-chain antibodies based on four individual libraries from each of 50 non-immunised donors, the final combined library comprised 4×10^9 of members. The DNA encoding the heavy and light-chain variable domains of the IgM and IgG repertoires was amplified by PCR using two different sets of primers for each individual library. Specific antibodies to a wide spectrum of antigens including small haptens (e.g. progesterone, testosterone), peptides, proteins and viruses were obtained by screening the final combined library. Moghaddam et al. (2001) screened and panned two phage antibody libraries, the human lymphocyte antibody library and the semi-synthetic antibody library previously described (Braunagel and Little, 1997; Little et al., 1999), against AflatoxinB1-BSA and screened single-chain antibody fragments for binding to AflatoxinB1-BSA and soluble AflatoxinB1. It was found that many of the isolated antibodies specifically bound to AflatoxinB1-BSA, but not soluble AflatoxinB1 or BSA. Modification of the protocol by using different elution strategies led to the isolation of single-chain fragment (scFv) antibodies with specific binding to soluble AflatoxinB1 with affinity of 6×10^{-9} M (K_D). The isolation of single-chain fragment antibodies with binding activity to 6-monoacetylmorphine, the major metabolite of heroine, with affinities (K_D) of $1–3 \times 10^{-7}$ M and with no cross-reactivity to morphine was reported by Moghaddam et al. (2003). The chain shuffling of scFv antibodies, isolated from single-pot antibody library previously described (Little et al., 1999; Moghaddam et al., 2001) was used for isolation of specific antibodies to 6-monoacetylmorphine but not to morphine. Isolation of single-chain antibody fragments against the cyanobacterial hepatotoxin microcystin-LR from a synthetic human phage library (Tomlinson library) was reported in paper by McElhiney et al. (2000). The most sensitive antibody clone selected from the library detected free microcystin-LR with an IC_{50} of 4 μM. The suitability of different synthetic and semi-synthetic antibody phage libraries (Griffin and Tomlinson) for generation specific antibodies to the hapten target microcystin-LR was tested by Strachan et al. (2002). In a competition enzyme-linked immunosorbent assay, bacterially expressed antibodies selected via the Griffin library were found to show at least 300 times greater sensitivity than those isolated from the Tomlinson library for free microcystin. In the most recent published paper by McElhiney et al. (2002) the human semi-synthetic library (Griffin library) was employed to isolate recombinant antibody fragments against the same cyanobacterial hepatotoxin microcystin-LR. It was reported that the most sensitive single-chain antibody (scAb) isolated was capable of detecting

microcystin-LR at levels below the World Health Organisation limit in drinking water (1 μg/l) and cross-reacted with three other purified microcystin variants (microcystin-RR, -LW, and -LF) and the related cyanotoxin nodularin. The quantification of microcystins in toxic samples assayed by ELISA showed good correlation with analysis by high-performance liquid chromatography. Bruggeman et al. (1996, 1997) used a single-pot human synthetic phage antibody library for isolation antibody fragments against oxygen sensitive reduced flavin by phage selection under anaerobic and reducing conditions at pH 5 and pre-elution step with the oxidized flavin. The binding of selected antibody fragment to the hapten, reduced flavin, was characterized by time-resolved polarized fluorescence, and was reported to be highly specific for the reduced flavin. The Griffin1 library was also used for preparation of phage antibodies against herbicide 2,4-dichlorophenoxyacetic acid. Brichta et al. (2003) panned the Griffin library against 2,4-D herbicide. Four cycles of panning were used in the selection process and free 2,4-D hapten molecules was used during the elution step. Specificity testing of scFv revealed that antibody binding moiety of 2,4-D molecule consists of a 2,4-dichlorophenyl fragment of the molecule. However, the scFv ELISA assay sensitivity was one order of magnitude lower in comparison to monoclonal ELISA for 2,4-D (Franek et al., 1994). This results indicate contemporary naive phage libraries are capable of producing hapten-specific scFv but in some cases with limited sensitivity.

5. CONCLUSION

Several recombinant strategies for the generation of anti-hapten antibodies have been tested in contrast to over-dominating mono and polyclonal methods. However, among the various *in vitro* methods such as ribosome, phage and bacterial display systems being developed the preparation of novel recombinant antibodies is mainly based on phage display systems. One of the main advantages of phage display technology is its flexibility, enabling to clone optimized repertoire according to the latest developments, and that its selection and screening procedures can be easily automated and thus can reach high throughput status.

The phage display technology has been developed from original simple libraries to more sophisticated production system, where antibody frameworks displayed in the library have been optimized with respect to their expression levels. Mutations hampering production and/or leading to aggregation and inclusion body formation have been eliminated. Optimal numbers of selection cycles and use of free haptens as elution agents have been reported to improve the library selection output (McElhiney et al., 2002; Strachan et al., 2002; Brichta et al., 2003). Moreover the efficiency of selection process can be largely increased by using selectively infecting phage particles. In regards to the production of recombinant fragments, recloning systems have been developed for simplification of recombinant fragment format shift from scFv to Fab or to the whole IgG format, thus restoring the original effector functions of the complete antibody molecule.

The generation of high-affinity recombinant antibodies against hapten targets represents a set of problems particularly because of hapten molecular weight. Hapten-specific recombinant antibodies generated from the immune libraries play a dominant role over the single-pot (general) libraries, whereas the generation of highly specific recombinant antibodies against high molecular weight compounds successfully using both types of phage libraries. The results in published papers indicate that the use of immune phage libraries leads to generation of anti-hapten recombinant antibodies with higher specificity and affinity in comparison to those selected using single-pot (general) libraries. Examples of published ELISA assays based on hapten-specific recombinant antibodies derived from immunised donors and/or hybridoma are listed in Table 2. Both types of recombinant antibody production methods are opened to modification/mutation and secondary library creation from primary clones enabling further improvement of antibody.

Never the less, the recombinant approach represents attractive methodological alternative to polyclonal and monoclonal antibodies and can complement these technologies especially in the therapeutic applications where it tends to predominate. Concerning low molecular weight compound analysis, significant technological and knowledge progress must be done before high quality anti-hapten libraries can be constructed.

The creation of high quality anti-hapten libraries can be conditioned by decryption of structural rules forming antibody sites prone to bind low molecular weight compound, and by preserv-

ing the wide structural variability, that enable the binding of different types of haptenic molecules. These two crucial requirements might be in some cases unsupportive; therefore it would be essential to decrypt aminoacid residues responsible for forming cavity for hapten in the centre of antigen binding site. Although numerous binding sites for particular hapten molecules were described a more detailed concept of forming hapten binding sites is still required.

Acknowledgement

The authors wish to thank Maria Vass for critical English revision of the manuscript.

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Received: 04–12–20
Accepted after corrections: 05–05–13

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