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Recombinant Antibodies and Non-Antibody Scaffolds for Immunoassays

Bhupal Ban and Diane A. Blake
Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, Louisiana, USA

1. Introduction

The measurement of trace amounts of physiologically active small molecules (for example, lipids, drugs, other synthetic chemicals and metals) is critical for both clinical and environmental analyses. Most small molecules can be analyzed using highly sophisticated analytical techniques, including high pressure liquid chromatography (HPLC), gas chromatography (GC), and inductively coupled plasma atomic emission spectroscopy (ICPAES). However, these methods require extensive purification, experienced technicians, and expensive instruments and reagents. Immunoassays offer an alternative to these instrument-intensive methods. Immunoassays rely on an antibody (Ab), or mixture of antibodies, for recognition of the molecule being analyzed (the analyte). Immunoassays are frequently applied to the analysis of both low molecular ligands and macromolecular drugs, and are also applied in such important areas as the quantitation of biomarkers that indicate disease progression and immunogenicity of therapeutic drug candidates. The performance of immunoassays is critically dependent on the binding properties of the antibody used in the analysis, and identification of suitable antibodies is often a major hurdle in assay development. Recombinant antibodies will play a major role in future immunoassay development.

2. Natural and recombinant antibody fragments

The antibody is the key reagent of an immunoassay and it can be produced by animal immunization, hybridoma technology, and/or recombinant techniques. Most, but not all, production methods require immunization of an animal with an antigen. An antigen is a molecule that can be recognized by the immune system (immunogenicity) and that can be bound specifically to an antibody (reactogenicity). Molecules with both immunogenicity and reactogenicity are called “complete antigens” and molecules that possess only reactogenicity are called “incomplete antigens”. Incomplete antigens, also called haptens, encompass a wide variety of molecules, including drugs, explosives, pesticides, herbicides, polycyclic aromatic hydrocarbons, and metal ions. These haptens can induce the immune system to produce antibodies only when they are covalently conjugated to a larger carrier molecule such as a protein.

Although polyclonal antibodies hold their place as the reagents of choice for general-purpose applications in the biological sciences, the volume of serum that can be obtained

from immunized animals and batch-to-batch differences in affinity and cross-reactivity make them less attractive for quantitative immunoassays. The first milestone for the generalized the use of immunoassays was the development of hybridoma technology, which overcame problems of heterogeneity and supply (Kohler & Milstein, 1975). While traditional monoclonal antibodies are used throughout biological research, many potential applications remain unfulfilled. The production of monoclonal antibodies requires considerable time, expense and expertise, as well as specialized cell culture facilities. The use of animal immunization means that the selection for relevant binding specificities occurs in the uncontrolled serum environment. This technology is adequate for stable antigens but not for molecules that are highly toxic, not immunogenic in mammals or not stable enough to withstand the immune processing steps required for the *in vivo* immune response. Most importantly, when working with monoclonal antibodies, it is not possible to alter or improve an antibody's binding properties without cumbersome procedures that convert the molecules to recombinant forms that can be engineered. All these reasons urged the development of strategies aimed at the production of recombinant antibodies (rAbs) and alternative scaffolds (Gebauer & Skerra, 2009) of smaller dimensions that can be easily selected, manipulated and produced using standard molecular biology techniques.

There are several distinct classes of natural antibodies (IgG, IgM, IgA, and IgE) that provide animals with key defenses against pathogenic organisms and toxins. Most immunoassay systems rely upon IgG as the immunoglobulin of choice. IgG is bivalent, and its ability to bind to two antigenic sites greatly increases its functional affinity and confers high retention time on cell surface receptors. The basic structure of an IgG molecule is shown in figure 1. Most IgG molecules are composed of two heavy chains (HC) and two light chains (LC), which are stabilized and linked by inter- and intra-chain disulfide bonds. The HC and LC can be further subdivided into variable regions and constant regions. The antigen binding site is formed by the combination of the variable region of the HC and LC. Most IgG molecules have two identical antigen binding sites, which are usually flat and concave for protein antigens, but which may form a pocket when the antibody has been selected against a hapten. Within the HC and LC variable regions are 3 hypervariable regions, also called complementary determining regions (CDRs), and 4 frameworks regions (FRs). The greatest sequence variation among individual antibodies occurs within the CDRs, while the FRs are more conserved. In general, it is assumed that the CDR regions from the LC and HC associate to form the antigen binding site. The lower part of the IgG molecule contains the heavy chain domains (crystallizable fragment, Fc) that are responsible for important biological effector functions. In additional to these conventional antibodies, camelids and sharks produce unusual antibodies composed only of heavy chains, also shown in figure 1. These peculiar heavy chain antibodies lack light chains (and, in the case of camelid antibodies also CH1 domain). Therefore, the antigen binding site of heavy chain antibodies is formed only by a single domain that is linked directly via a hinge region to the Fc domain. Intact IgG molecules, the bivalent $(Fab')_2$, or the monovalent (Fab), all of which contain the antigen binding site(s), can be used in immunoassays.

Recombinant antibody forms have also been developed to facilitate antibody engineering. The single chain fragment variable (scFv) molecule is a small antibody fragment of 26-27 kDa. It contains the complete variable domain of the HC and LC, typically linked by a 15 aa long hydrophilic and flexible polypeptide linker. The scFv fragments can also include a His tag for purification, an immunodetection epitope and a protease-specific cleavage site. The

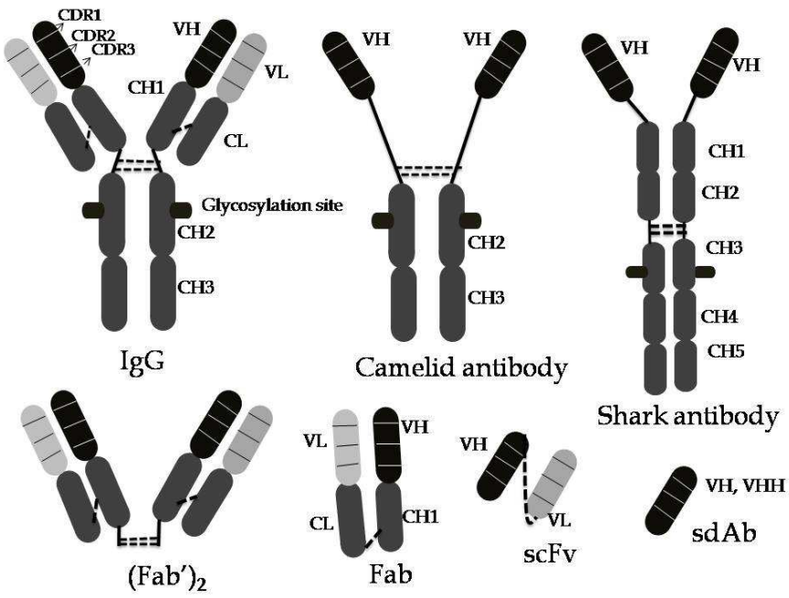

Fig. 1. Structure of conventional, camelid and shark antibodies and of antibody fragments.

orientation of the HC and LC domains is critical for binding activity, expression and proteolytic stability. Although a vast number of recombinant antibody (rAb) structures have been proposed (Holliger & Hudson, 2005), scFv fragments derived from mammalian IgGs and the single domain antibodies (sdAbs), which include the VHH from camelid and llama and the VH from shark, are the antibody fragments most widely used for both research and industrial applications (Kontermann, 2010; Wesolowski et al., 2009).

3. Principles and selection platforms of rAbs

Powerful combinatorial technologies have enabled the development of *in vitro* immune repertoires and selection methodologies that can be used to derive antibodies with or without the direct immunization of a living host (Hoogenboom, 2005; Marks & Bradbury, 2004). Recombinant antibody technology has provided an alternative method to engineer antibody fragments with the desired specificity and affinity within inexpensive and relatively simple host systems. Effective *in vitro* libraries have been constructed using either the entire antigen-binding fragment (Fab) or the single chain variable fragment (scFv), which represents the smallest domain capable of mediating antigen recognition. The simplest and most widely used antibody libraries utilize the scFv format, although single domain heavy chain libraries (VH and VHH) have also been constructed. The construction of *in vitro* libraries using different sources will be reviewed herein.

3.1 Antibodies from immune antibody libraries

The first rAbs were derived from pre-existing hybridomas; now, however, rAbs are mostly isolated from immune antibody libraries, i.e., antibody libraries generated from genetic material derived from immunized animals or naturally infected animals or humans. These libraries are biased for binding to the antigen. Thus, affinity maturation takes place *in vivo* and the chances of isolating the high–affinity antibodies are increased. Immune libraries are

constructed using HC and LC variable domain gene pools amplified directly from immune sources; lymphoid sources include peripheral blood, bone marrow, spleen and tonsil (Huse et al., 1989; Schoonbroodt et al., 2005). In contrast to hybridoma technology, which can sample no more ~10% of the immune repertoire of an animal, a recombinant immune library, when prepared with the appropriate primers, can sample >80% of the immune repertoire and the diversity of antibodies that can be derived from a single immunized donor is much higher than what is possible using hybridomas. Selection is performed *in vitro,* which enhances the ability to select for rare antibody specificities. In addition, the immune repertoires of almost any species can be trapped, even those where hybridoma technology has not been described (chicken and llama), is not freely available (rabbit), or is not very robust (sheep). Immune libraries can provide higher-affinity binders than non-immune libraries. Immunizations are generally required for each targeted antigen, although multi-antigen immunizations have been performed successfully (Li et al., 2000). Advantages and disadvantages of immune libraries include: (1) the ease of preparation compared to naïve libraries; (2) the time requirement for animal immunization; (3) the unpredictability of the immune response of the animal to an antigen of interest; (4) lack of immune response to some antigens; and (5) the necessity of construction of new libraries for each new antigen.

3.2 Antibodies from nonimmune, synthetic, and semi- synthetic libraries

Non-immune (naïve) libraries are derived from normal, unimmunized, rearranged V gene from the IgM/IgG mRNA of B cells, peripheral blood lymphocytes, bone marrow, spleen or tonsil. These libraries are not explicitly biased to contain clones binding to antigens; as such they are useful for selecting antibodies against a wide variety of antigens. Using specific sets of primers and PCR, IgM and IgG variable regions are amplified and cloned into specific vectors designed for selection and screening (Bradbury & Marks, 2004; Marks et al., 1991, 2004). An ideal naïve library is expected to contain a representative sample of the primary repertoires of the immune system, although it will not contain a large proportion of antibodies with somatic hypermutations produced by natural immunization. The major advantages and disadvantages of using very large naïve libraries are: (1) the large antibody repertoire, which can be selected for binders for all antigens including non-immunogenic and toxic agents; (2) a shorter time period to binding proteins, because selection is performed on an already existing library; (3) low affinity antibodies are obtained from these libraries; and (4) it is technically demanding to construct these large non-immune repertoires. Many of these disadvantages may be bypassed by using synthetic antibody libraries.

Synthetic antibody libraries are created by introducing degenerate, synthetic DNA into the regions encoding CDRs of the defined variable-domain frameworks. Synthetic diversity bypasses the natural biases and redundancies of antibody repertoires created *in vivo* and allows control over the genetic makeup of V genes and the introduction of diversity (Hoogenboom & Winter, 1992). A synthetic library has been described that was constructed on the basis of existing information on the structure of the antigenic site of proteins and small molecules (Persson et al., 2006; Sidhu & Fellouse 2006).

Semi-synthetic libraries have been constructed by incorporating CDR loops with both natural and synthetic diversity into one or more of the antibody framework regions. High diversity semi-synthetic repertoires have been generated by introducing partially or completely randomized sequences mainly into the CDR3 region of the heavy chain. This

process generates highly complex libraries and facilitates the selection of antibodies against self-antigens, which are normally removed by the negative selection of the immune system (Barbas et al.,1992). An efficient cloning system (*in vivo* Cre/loxP site specific recombination) combined with dual antibody cloning strategies allows construction of very large repertoires with about $10^{9\text{-}11}$ individual clones (Sblattero & Bradbury, 2000). Semi-synthetic libraries, however, have the disadvantage of always containing a certain number of non-functional clones, stemming from PCR errors, stop codons in the random sequence, or improperly folded protein products.

4. *In vitro* selection procedures for rAbs from combinatorial libraries

Recombinant antibody technologies provide the investigator with a great deal of control over selection and screening conditions and thus permit the generation of antibodies against highly specialized antigen conformations or epitopes. The most powerful methods, phage, yeast, and ribosomal display technologies, are complementary in their properties and can be used with naïve, immunized or synthetic antibody repertoires.

4.1 Phage display libraries for the isolation of antibodies

Phage display-based selections are now a relatively standard procedure in many molecular biology laboratories. The generation of antibody fragments with high specificity and affinity for virtually any antigen has been made possible using phage display. Phage display libraries are produced by cloning the pool of genes coding for antibody fragments into vectors that can be packed into the viral genome. The rAb is then expressed as an antibody fragment on the surface of mature phage particles. Selection of specific antibody fragments involves exposure to antigen, which allows the antigen-specific phage antibodies to bind their target during the bio-panning. The binding is followed by extensive wash steps and subsequent recovery of antigen-specific phage. The phage particles can then be used to infect *E. coli* bacteria. Different display systems can lead to monovalent (single copy) or to multivalent (multiple copy) display of the antibody fragment, depending on the type of anchor protein and display vector used (Sidhu et al., 2000). The most popular system uses a monovalent display vector system, which is convenient for selecting antibodies with higher affinity. Monovalent display is achieved by using a direct fusion to a minor viral coat protein (pIII). The vector into which most antibody libraries are cloned is a phagemid vector that requires a helper phage for the production of phage particles. Use of a phagemid vector makes propagation in bacteria much easier to accomplish than would be possible with a phage vector (Hust & Dubel, 2005). A general scheme for the isolation of antibody fragments by phage display is shown in figure 2. Libraries with $10^{6\text{-}11}$ individual clones can be made using recombinant-based protocols. Due to limitations of the *E. coli* folding machinery, complete IgG molecules are very difficult to express in *E. coil* and display on the surface of phage. Therefore, smaller antibody fragments such as Fab, scFv and sdAb are primarily used for antibody phage display.

4.2 Yeast surface display

Yeast surface display is a powerful method for isolating and engineering antibody fragments (Fab, scFv) from immune and non-immune libraries, and has been used to isolate recombinant antibodies with binding specificity to variety of proteins, peptides, and small

molecules (Boder & Wittrup, 2000; Chao et al., 2006). In this system, antibodies are displayed on the surface of yeast *Saccharomyces cerevisiae* via fusion to an α-agglutinin yeast adhesion receptor, which is located in the yeast cell wall.

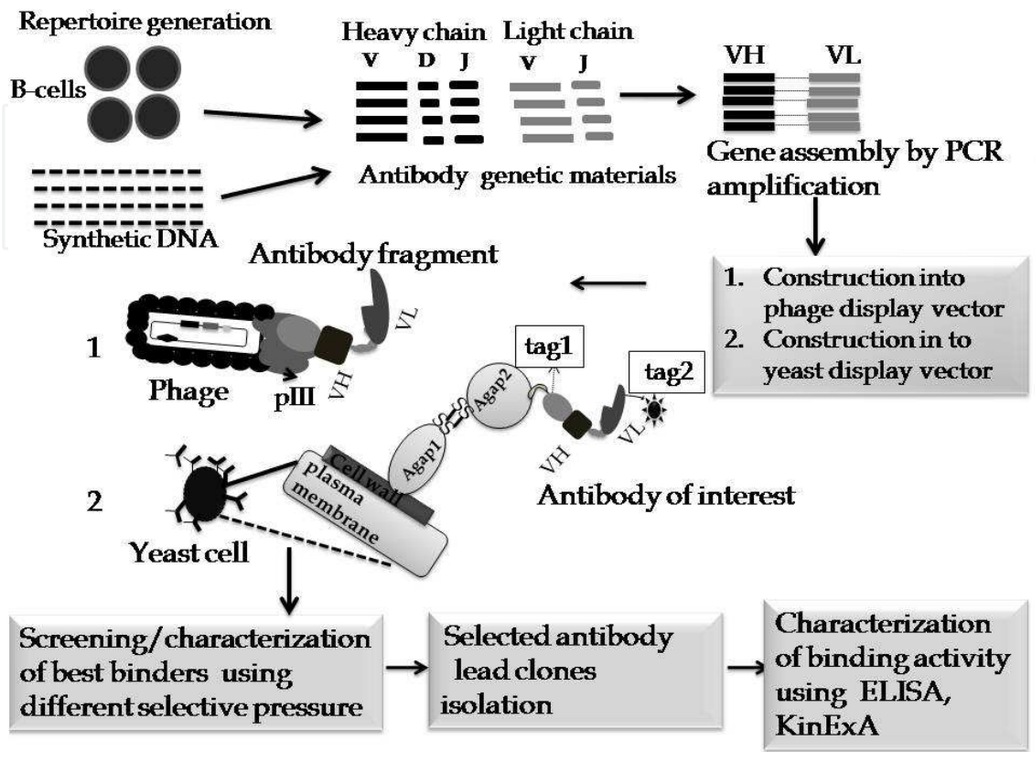

Fig. 2. Schematic diagram for construction of antibody libraries and *in vitro* display system; phage and yeast display.

Like phage display, yeast display provides a direct connection between genotype and phenotype; a plasmid containing the gene of interest is contained within yeast cells, while the encoded antibody is expressed on the surface. The display level of each yeast cell is variable, with each cell displaying $1x10^4$ to $1x10^5$ copies of the scFv. Variation of surface expression and avidity can be quantified using fluorescence activated cell sorting (FACS), which measures both antigen binding and antibody expression on the yeast cell surface (Feldhaus et al., 2003). The main advantage of yeast surface display over other display technologies is the eukaryotic expression bias of yeast, which contains post-translational modification and processing machinery similar to that of mammalian cells. Thus, yeast may be better suited for the expression of antibodies as compared to prokaryotes such as *E. coli*. Yeast display libraries have been used during the affinity maturation of scFvs from mutagenic libraries (Boder et al., 2000; Lou et al., 2010; Orcutt et al., 2011). Limiting factors of yeast display include a more limited transforming efficacy of yeast as compared to bacteria, which can lead to a smaller functional library size (about 10^7-10^9) than is possible with other display technologies.

4.3 Ribosomal display

Ribosomal display is an *in vitro* selection and evolution technology for proteins and peptides from large libraries (Dreier & Pluckthun, 2011; Hanes & Pluckthun, 1997). The general

scheme of ribosomal display is shown in figure 3. This display system was developed from a peptide-display approach that was extended to screen scFv and scaffold proteins having very high affinity for antigen (K_ds as low as 10^{-11} M) from very large libraries (Binz et al., 2004; Zahnd et al., 2007). The DNA library coding for proteins such as antibodies and scaffolds are transcribed *in vitro*. The mRNA has been engineered without a stop codon; therefore, the translated protein remains attached to the peptidyl tRNA and occupies the ribosomal tunnel. This allows the protein of interest to protrude out of the ribosome and fold. Ribosomal display is performed entirely *in vitro*, and it has two advantages over other selection technologies. First, the diversity of the libraries is not limited by the transformation efficiency of bacterial cells (~$1x10^{11}$ to $1x10^{13}$), but only by the number of ribosomes and different mRNA molecules present in the test tube. Second, random mutations can be introduced easily after each selection round, as no cells must be transformed after any diversification step. In ribosomal display, the physical link between the genotype and the corresponding phenotype is accomplished by a complex consisting of mRNA, ribosome and protein.

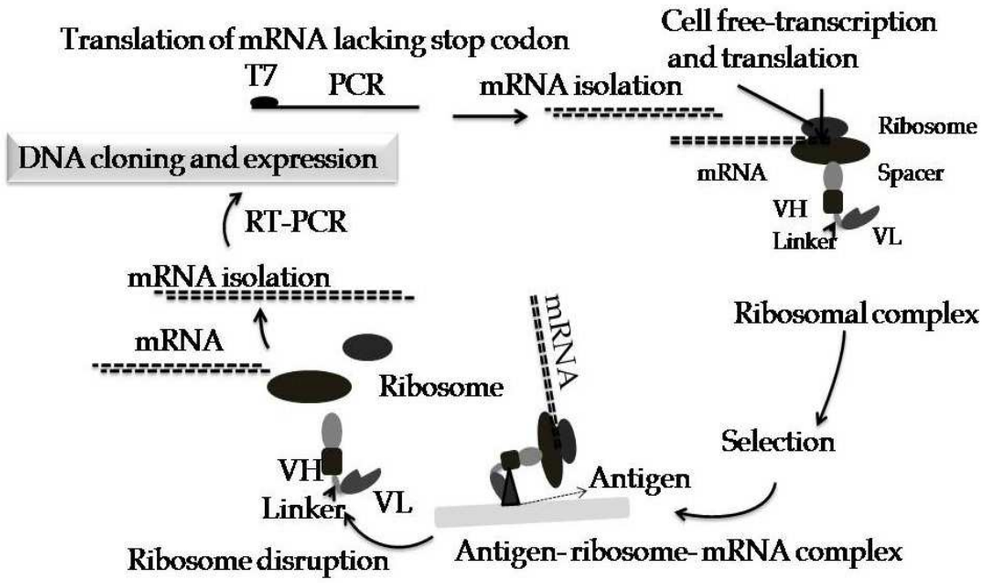

Fig. 3. Schematic diagram for isolation of specific antibody fragment from ribosomal display

Ribosomal display has been used to isolate antibodies that bind to haptens with nanomolar affinities (Yau et al., 2003). A summary of the *in vitro* display systems available to researchers is shown in table 1.

5. Applications of rAbs against low molecular ligands

A large number of rAbs have been used successfully to develop diagnostic kits, therapeutics and biosensors (Holliger et al., 2005; Huang et al., 2010; Kramer & Hock, 2003). The majority of the targets were large molecular weight analytes such as proteins and peptides. Prior to 1990, there were few reports of the isolation of rAbs against low molecular weight molecules (haptens) such as drugs of abuse, vitamins, hormones, metabolites, food toxins and environmental pollutants, including heavy metals and pesticides. Hapten-specific antibodies

Name	Display	Library size	Main applications	Advantages	Disadvantages
Phage display	Monovalent Multivalent	10^{10} to 10^{11}	Abs from natural & synthetic libraries; Affinity maturation & stability increase	Easy and versatile for large rAbs panels	Laborious to make large libraries; Not truly monovalent
Yeast surface display	Multivalent	10^{7}	Abs from natural & synthetic libraries; Affinity maturation & stability increase	Rapid when used in combination with random mutagenesis	Small rAb panels, FACS expertise required
Ribosome display	Monovalent	10^{12} to 10^{13}	Abs from natural & synthetic libraries; Affinity maturation & stability increase	Intrinsic mutagenesis, fastest of all systems	Small rAb panels, limited selection scope and technically sensitive

Table 1. Comparing the main *in vitro* selection platforms for isolation of rAbs

are necessary reagents for the development of immunoassays, immunosensor technologies (Charlton et al., 2001), and immunoaffinity chromatography purification columns (Sheedy & Hall, 2001). Commercial immunoassays for haptens such as small environmental contaminants still rely mostly on polyclonal antibodies rather than monoclonal or recombinant antibodies fragments (Sheedy et al., 2007). The complexity and costs associated with the production of anti-hapten antibodies by hybridoma technology and the preferential selection of antibodies that recognize the conjugated form of the haptens over antibodies that specifically recognize free haptens are two of the most important problems that have limited the development and application of antibodies that recognize haptens and other low molecules ligands. Moreover, some small molecular weight ligands will not trigger the animal immune system even when conjugated to a carrier protein, thereby making the production of antibodies against that such analytes very difficult.

In recent years, the production of recombinant antibodies to low molecular weight ligands has increased significantly, as shown in table 2. A single methyl or hydroxyl group can have a considerable effect on the biological properties of a steroid hormone. Similarly, protein phosphorylation, acetylation and sulfation, all of which are relatively simple post-translational modifications in chemical terms, can dramatically affect signal transduction (Bikker et al., 2007; Hoffhines et al., 2006; Kehoe et al., 2006). Antibodies capable of discerning such relatively simple chemical modification are of great values in studying these effects. The display methods to tailor both affinity and specificity have generated antibodies capable of discerning minor difference between related small molecules far better than those obtained by immunization.

6. Improving the specificity and affinity of rAbs to low molecular weight ligands

Although recombinant antibody technology has been able to open the bottleneck in the isolation of antibodies against virtually any antigen, it remains difficult to obtain high-

affinity antibodies against small molecules using immune and naïve libraries. Various approaches have been utilized, including identifying the key binding residues, developing more effective procedures for selection of the most specific binders and avoiding interfacial effects that can compromise the yield and stability of rAbs.

Target Hapten	Ab format	Antibody library	*In vitro* display	Reference
Aflatoxin B1	scFv	Naïve	Phage	(Moghaddam et al., 2001)
Digoxigenin	scFv	Naïve	Phage	(Dorsam et al., 1997)
Doxorubicin	scFv	Naïve	Phage	(Vaughan et al., 1996)
Estradiol	scFv	Naïve	Phage	(Dorsam et al., 1997)
Indole-3-acetic acid	VHH	Naïve	Phage	(Sheedy et al., 2006)
Fluorescein	scFv	Naïve	Phage	(Vaughan et al., 1996)
Phenyloxazolone	scFv	Naïve	Phage	(de Haard et al., 1999)
Picloram	VHH	Naïve	Ribosome	(Yau et al., 2003)
Progesterone	scFv	Naïve	Ribosome	(He et al., 1999)
Fumosinin B1	scFv	Naïve	Phage	(Lauer et al., 2005)
Atrazine	scFv	Immune	Phage	(Li et al., 2000)
Azo-dye RR1	VHH	Immune	Phage	(Spinelli et al., 2000)
Cortisol	scFv	Immune	Phage	(Chames & Baty, 1998)
Digoxin & analogues	scFv	Immune	Phage	(Short et al., 1995)
Isoproturon	scFv	Immune	Phage	(Li et al., 2000)
Mecoprop	scFv	Immune	Phage	(Li et al., 2000)
Simazine	scFv	Immune	Phage	(Li et al., 2000)
Triazine	scFv	Immune	Phage	(Kramer, 2002)
4-Hydroxy-3-iodo-5-nitrophenol	scFv	Semi-synthetic	Phage	(van Wyngaardt et al., 2004)
Fluorescein	scFv	Semi-synthetic	Phage	(van Wyngaardt et al., 2004)
Microcystin LR	scFv	Semi-synthetic	Phage	(Strachan et al., 2002)
Phtalic acid	scFv	Semi-synthetic	Phage	(Strachan et al., 2002)
Trichlocarbon	VHH	Naïve	Phage	(Tabares-da Rosa et al., 2011)
6-Monoacetylmorphine but not morphine	scFv	Naïve	Phage	(Moghaddam et al., 2003)
Metallic gold	Fv	Naïve	Phage	(Watanabe et al.,, 2008)
Anti-Aluminum	VHH	Semi-synthetic	Phage	(Hattori et al., 2010)
Anti-Cobalt	VHH	Semi-synthetic	Phage	(Hattori et al., 2010)
Anti-Uranium	scFv	Immune	Phage	(Zhu et al., 2011)
Domoic acid	scFv	Immune	Phage	(Shaw et al., 2008)
Azoxystrobin	VHH	Immune	Phage	(Makvandi-Nejad et al., 2011)
Methamidophos	scFv	Immune	Phage	(Li et al., 2006)

Table 2. List of small molecule-specific recombinant antibodies

There are, however, unique challenges to the development of antibodies that will perform well in assays for low molecular weight ligands. Antigen binding sites are generated by the cooperation between the variable domains of the HC and LC (VH/VL). The amino acids of FRs compose rigid scaffolds that position the amino acids in the CDRs in loops that extend outward from scaffold. These loops play important roles in making contact with the antigen. Hapten antigens have remained a great challenge for immunodiagnostics, because the hapten portion of the antigen often ends up almost buried inside the concave–shaped antigen binding pocket. The extended shape of this binding pocket then facilitates additional interactions between amino acid residues in binding site and portions of the hapten-protein conjugate present in the bridge between the hapten and the protein carrier. These additional interactions mean that the antibody often binds to the much more tightly to the hapten-protein conjugate than to the soluble hapten. In our laboratory, we have studied this phenomenon with 10 different anti-hapten antibodies. In this study, the antibody always bound more tightly to the protein conjugate than to the soluble antigen. The differences in affinity ranged from 1.5 to 1600 fold, depending upon the antibody being analyzed (Blake et al., 1996, Melton, 2010). Thus, when given a choice, anti-hapten antibodies almost always prefer binding to the hapten-protein conjugate, and additional soluble hapten is required to inhibit this interaction, thus reducing assay sensitivity. Selective panning and affinity maturation are methods available in recombinant technology for reducing selective binding of hapten antibodies to the hapten-protein conjugate.

6.1 Panning optimization

A variety of selection strategies have been reported for the isolation of high affinity rAbs against chelated metals and other haptens (Sheedy et al., 2007; Zhu et al., 2011). The most successful strategies employed first loose and then increasingly stringent panning conditions to enrich the population of phage antibodies as follow: (i) the concentration of coating antigen was gradually decreased during successive rounds of panning (Strachan et al., 2002; Zhu et al., 2011); (ii) soluble hapten was used to elute ligand-specific antibodies in place of the triethylamine more commonly used for elution; (iii) during the panning of immune scFv libraries, the conjugate carrier protein and/or other linker peptides were included for several intermediate incubation steps at high concentration and subsequently decreased to remove phage antibodies that bound to the protein conjugate rather than the soluble hapten; (iv) the phage antibodies were incubated with structural analogues of the hapten prior to incubation with the immobilized target hapten to eliminate phage antibodies with unwanted cross reactivities (Charlton et al., 2001; Zhu et al., 2011). Such panning optimization strategies have led to the isolation of antibodies with higher affinity and specificity and lower levels of cross-reactivity. For an example from the isolation of antibodies to metal-chelate complexes, such subtractive panning strategies were employed to isolate an antibody that bound tightly to uranium in complex with 2,9-dicarboxyl-1,10-phenanthroline, (DCP), but weakly to metal-free DCP. In successive rounds of panning, the phage antibody population was incubated with a high concentration of carrier protein (BSA) and increasing concentrations of soluble DCP in immunotubes coated with decreasing concentrations of a UO_2^{2+}-DCP-BSA conjugate as shown in table 3.

Round of Selection	Percent of maximum conjugate coated onto immunotubes	Percent of maximum metal-free chelator added to the phage binding buffer	Percent of maximum carrier protein added to the phage binding buffer
1	100	10	100
2	100	10	100
3	10	20	100
4	10	20	100
5	1	100	100

Table 3. General selection strategy for the isolation of scFvs that bind to a metal-loaded but not a metal-free chelator (Zhu et al., 2011).

6.2 *In vitro* antibody affinity maturation

The affinity maturation procedure contains two stages: (a) making a modified antibody library with a larger diversity than the original library (b) selecting desired antibodies molecules from the library using the previously discussed *in vitro* display and panning methods. An antibody's affinity for its antigen is dependent on the identity and conformation of the amino acid sidechains in the CDRs of both the HC and LC. Improvement in the antigen-binding affinity can be attained using a number of strategies. The mostly common used are random mutagenesis, site-direct mutagenesis and chain shuffling. These processes are often referred to as *in vitro* affinity maturation, to distinguish the process from the affinity maturation that takes place in the animal. Although a considerable number of successful affinity maturation processes have been reported for antibodies against macromolecule antigens like proteins, affinity maturation for low weight molecules like haptens and metals is obviously more difficult, and consequently, only a limited number of successful studies have thus far been reported.

6.2.1 Random mutagenesis (Error Prone PCR; E-p PCR)

Random mutagenesis is the process that most closely mimics the *in vivo* process of somatic hypermutation. This process makes no assumptions as to which sites are the best to mutate in order to increase affinity, and it is also technically rather simple to execute. Error prone PCR uses low fidelity polymerization conditions to introduce a low level of point mutations randomly throughout a wide region of a target gene (e.g. , the entire VH and VL). Error prone PCR has been used to demonstrate the effect of mutation frequency on the affinity maturation of antibodies against both proteins and small ligands (Daugherty et al., 2000). When wild type antibodies to the hapten, diogoxin, were subjected to E-p PCR, the higher affinity clones isolated from libraries all contained aromatic residues substitutions in the antibody binding site. These resides were thought to be important for hydrophobic interaction with the planer aromatic structure of digoxin (Short et al., 1995). A disadvantage of E-p PCR is that surface-selection often enriches binders with increased tendency for dimerization, especially when using the scFv format. In addition, most of the mutants lacked detectable expression or lost antigen-binding affinity. A few mutants lost specificity and showed increased cross-reactivity to analogs (Fuji, 2004; Sheedy et al., 2007). Point mutation can cause profound effects on the binding affinity and specificity of an antibody for its small ligands. The affinity maturation processes reported for anti-hapten scFvs are listed in table 4.

6.2.2 Site-directed mutagenesis

In site-directed mutagenesis, the investigator changes specific amino acid residues. Site-directed mutagenesis is often used in combination with in silico modeling, crystallographic data, and ligand docking programs, which allow the investigator to hypothesize about the roles that individual binding site amino acid residues have in antigen binding. The CDRs of VH and VL are usually targeted for both haptens and protein antigens (Siegel, et al., 2008), and mutations in CDRs as opposed to within the framework residues generally contribute more to increases in affinity (Orcutt et al., 2011; Short et al., 2002). In one study, the most significant increase in affinity was correlated with mutations in the light chain CDR1 even though this CDR1 was not contacting the hapten directly (Valjakka et al., 2002). Hapten-specific antibodies whose affinity has been increased by site directed mutagenesis are listed in table 4.

Target hapten	Fold increase in affinity	Ab format/ *in vitro* display	Affinity maturation	Reference
phOx-GABA	290	scFv/Phage	Chain shuffling	(Marks et al., 1992)
Cortisol	7.9	scFv/Phage	Site-directed	(Chames et al., 1998)
Estradiol-17 β	12	Fab/Phage	Site-directed	(Kobayashi et al., 2010)
Fluorescein	2600	scFv/Yeast	Ep-PCR /DNA -shuffling	(Boder et al., 2000)
Testosterone	35	Fab /Phage	Site-directed	(Valjakka et al., 2002)
Tacrolimus	15	scFv/Yeast	Site-directed	(Siegel et al., 2008)
DOTA-chelate	1000	scFv/Yeast	Site-directed	(Orcutt et al., 2011)

Table 4. List of successful affinity maturations of anti-hapten antibodies

6.3 Shuffling of antibody genes

Shuffling of antibody genes to create new antibody libraries can be accomplished in several ways: chain shuffling, DNA shuffling, and staggered extension processes.

6.3.1 Chain shuffling

In this procedure, one of the two chains (VH of VL) is fixed and combined with a repertoire of partner chains to yield a secondary library that can be searched for superior pairings against antigens. This approach takes advantage of "random" mutations that have been introduced into VH and VL germline genes *in vivo*. Phage display and yeast display are often used to facilitate the selection of improved binders from these secondary libraries (Lou et al., 2010; Marks, 2004; Persson et al., 2006). This procedure has also been used to increase the affinity of anti-hapten antibodies and the results are reviewed in table 4. Chain shuffling is only a suitable mutagenesis strategy when VH and VL sequences are available from immune libraries. Chain shuffling is, therefore, not useful with naïve libraries since heavy and light chains available in these libraries have not been exposed to the antigen of interest.

6.3.2 DNA shuffling by random fragmentation and reassembly

DNA shuffling is based on repeated cycles of point mutagenesis, recombination and selection, which allows *in vitro* molecular evolution of protein (Stemmer, 1994). The process

mimics somewhat the natural mechanism of molecular evolution (Ness et al., 1999). This shuffling technique involves the digestion of a large antibody gene with DNase I to create a pool of random DNA fragments. These fragments can then be reassembled into full-length genes by repeated cycles of annealing in the presence of DNA polymerase. DNA shuffling offers several advantages over more traditional mutagenesis strategies. It uses longer DNA sequences and also permits the selection of clones with mutations outside of the antibody binding site.

7. Modification of rAbs fused with signal enhancer proteins

Antibody engineering enables the preparation of fusion proteins combining scFvs and enzymes via the expression of a single scFv-enzyme fusion gene. Such recombinant scFv-fusion proteins have been reported for numerous applications, including the detection of a plant virus (Griep et al., 1999), the human pathogen hantaviruses (Velappan et al., 2007), other protein targets such as *Bacillus anthraces* (Wang et al., 2006), cholera and ricin toxins (Swain et al., 2011), and the haptens morphine (Brennan et al., 2003) and 11-deoxycortisol (11-DC) (Kobayashi et al., 2006). These fusion proteins provide a much higher signal/noise ratio in the ELISA format than conventional enzyme-labeled antibodies because the fusion proteins can be obtained as a single molecule species having a 1:1 rAb/enzyme ratio, and thus are uncontaminated by unconjugated enzyme and rAb molecules. As an example, the sensitivity of a competitive immunoassay for 11-deoxycortisol was 10,000-fold higher when an scFv-alkaline phosphatase fusion protein replaced the standard enzyme-labeled secondary antibody (Kobayashi et al., 2006; Martin et al., 2006).

8. Beyond antibody fragments (Scaffold protein)

Conventional diagnostic immunoassays are limited to the analysis of a few hundred assays per day, whereas with antibody microarrays using individually addressable electrodes, thousands of assays can be run in parallel (Dill et al., 2004). Antibody fragments are providing valuable alternatives to full length mAbs for new biosensing devices because they provide small, stable, highly specific reagents against the target antigens. In addition, because the recombinant antibody is smaller than the intact IgG, the density of binding sites that can be immobilized on the surface of these sensors can be increased. The stability of surface-immobilized ligands is also crucial in immunoassay format. Therefore, a great deal of interest has been focused on simplifying the antibody scaffold, and molecular engineering has pushed the concepts of antibody miniaturization to develop more stable binders that are less sterically hindered when immobilized on surfaces.

To overcome the limitation of antibodies, the several alternative protein frameworks have been developed. Design of these protein frameworks, collectively called “scaffolds” or “scaffold proteins”, usually involves the adaptation of structurally well-defined polypeptide frameworks by the introduction of novel functionality. The new functionality is added to those parts of the protein surface that are not considered important for protein folding or stability. The recent development of non-biological alternatives to antibodies, including both scaffold proteins and plastibodies, may create distinct opportunities for future improvements in immunoassay technology. This could be particularly relevant in applications where compatibility of the binding probe with organic solvents and the ability to withstand

thermal and mechanical stress are required. Currently, there are more than 60 non-antibody scaffolds suggested as affinity ligands, primarily for therapeutic and diagnostic purposes (Binz et al., 2005a; Caravella & Lugovskoy, 2010; Lofblom et al., 2010; Skerra, 2007). One of the current problems with bacterial expression of antibody fragments is that of disulfide bond formation, which occurs primarily in the periplasm of bacterial cells. Because of the intradomain disulfide bonds required for proper immunoglobulin folding, neither scFvs nor Fabs are compatible with intracellular expression and only very stable scFv fragments have been expressed in the cytoplasm of *E. coli* (Martineau & Betton, 1999; Ohage et al., 1999). The ideal scaffold therefore should be stable without disulfide bonds, expressed in high amounts in *E. coli* and compatible with current display techniques. The scaffold should contain loops or other structures on its surface that can be modified to form the binding site. This can be a natural binding site or created *de novo*. Randomizations made to the binding region should be able to generate binders with high specificity and affinity (Binz & Pluckthun, 2005b; Gebauer et al., 2009; Gronwall & Stahl, 2009; Kim et al., 2009; Lofblom et al., 2010). All of the scaffolds reported to date, including affibodies, anticalins, and designed ankyrin repeats (DARPin), can be engineered for interaction with analytes by mimicking the way the immune system shuffles sequences to create diversity in loop structures. The goal is to randomize the loops without affecting the overall structure and stability of protein. Thus, it is possible to engineer binding properties that are totally independent of their original biological function. An example of this strategy is the recently developed anticalin with picomolar affinity for DTPA-chelated lanthanides, especially Y^{III} (Kim et al., 2009). This anticalin forms a tight non-covalent complex (with slow dissociation kinetics) under physiological conditions in the presence of the chelated metal ion and, after fusion with an appropriate targeting domain; it may provide an ideal tool for applications in 'pretargeting' radioimmunotherapy. Notably, the only established non-Ig scaffold that intrinsically provides pockets and thus allows tight and specific complexation of small molecules is the one of the lipocalins.

9. Conclusion

Immunoassay techniques provide simple, powerful and inexpensive methods for the measurement of small ligands. However, the progress of the development of new immunoassays and related immunotechnologies is still limited by the availability of antibodies with the desired affinities and specificities for given applications. Advances in molecular biology have led to the ability to synthesize antibodies *in vitro,* completely without the use of animals. Recombinant molecular technology that can generate variability, combined with high-throughput screening methodologies, can be used to produce engineered antibody-like molecules and novel antibody-mimic domains on scaffold proteins. The rAbs fused with other functional proteins can enhance the sensitivity of antibody-based assays and reduce the cost and labor involved in chemically synthesizing conjugates. Antibody engineering had already matured into a technology available to the general scientific community. Further advances will lead to better binding proteins that will permit the development of novel, high-throughput sensing systems for low molecular weight ligands.

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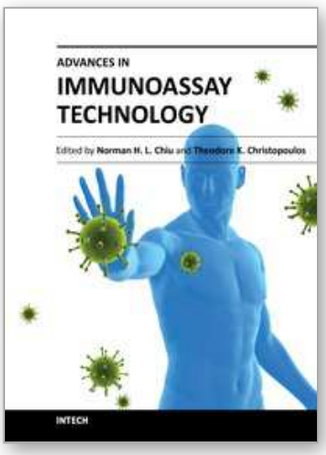

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From the basic in vitro study of a specific biomolecule to the diagnosis or prognosis of a specific disease, one of the most widely used technology is immunoassays. By using a specific antibody to recognize the biomolecule of interest, relatively high specificity can be achieved by immunoassays, such that complex biofluids (e.g. serum, urine, etc.) can be analyzed directly. In addition to the binding specificity, the other key features of immunoassays include relatively high sensitivity for the detection of antibody-antigen complexes, and a wide dynamic range for quantitation. Over the past decade, the development and applications of immunoassays have continued to grow exponentially. This book focuses on some of the latest technologies for the development of new immunoassays.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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