CHAPTER 2.4

Generation of recombinant antibodies by display technologies for diagnostic applications

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Introduction

The efficient management of diseases is increasingly dependent on high quality diagnoses resulting from diagnostic testing in clinics. For decades, the most robust and routinely applied diagnostics are based on immunoassays, such as radioimmunoassay and enzyme-linked song have facilitated the introduction of new diagnostic platforms. However, these platforms still depend on immunological agents, in most cases antibodies with recognition properties and binding specificity for the target to be detected or measured [1]. As new diseases are coming to the fore, newer biomarkers that require new antibodies are being discovered regularly.

Initially, polyclonal antibodies from the serum of immunized animals were used for diagnostic applications. Polyclonal antibodies represent a collection of antibodies that result from an immune response to a single specific antigen. The obvious disadvantage of polyclonal antibodies is their lower specificity to the antigen compared with monoclonal antibodies, but this is compensated for by having multiple antibodies targeting multiple epitopes on an antigen. The generation of monoclonal antibodies (MABs) became possible following the development of hybridoma technology by Köhler and Milstein in 1975. Hybridomas are made by fusing antibody-producing B-cells from immunized mice with immortal myeloma cells [2]. The resulting hybridoma will inherit features from both cell lines, i.e., the specific antibody production of the B-cells and the immortal characteristics of the myeloma cells [3].

More recent innovations for diagnostic purposes involve the use of large collections of recombinant forms of miniaturized antibodies such as single-chain variable fragment (scFv) and fragment antigen binding (Fab), or antibody analogues, such as peptide aptamers and other protein scaffolds. These approaches require the ability to present large binder libraries on the surface of various available display systems, which permit the selection of peptides or proteins with high affinity and specificity for virtually any target [4]. Display technologies are highly suited to high-throughput, rapid, and robust generation of binders, making research and diagnosis cost effective [5].
Processes involved in antibody generation

Antigen-driven selection directs *in vivo* antibody production. There are three key stages in the generation of antibodies by the immune system. The first is the rearrangement of antibody gene segments to produce millions of different antibody genes. The second stage is the expression of the rearranged gene library on the surface of B-cells/lymphocytes, and the third stage is the proliferation and differentiation of antigen-driven clones of B-lymphocytes into antibody-producing plasma cells. Subsequent somatic mutation in the antibody V-genes and further rounds of selection by antigens generates high-affinity antibodies for the antigen.

Technical advances have made it possible to mimic *in vivo* antigen-driven immune selection in the laboratory by applying one of a number of display technologies. Mimicry of the *in vivo* sieving process is carried out via an affinity-based selection process to isolate specific binders from a highly diverse collection. Many of these selection platforms share several important steps with the protocol to produce antibodies in the immune system *in vivo*. The first step is the generation or cloning of genotypic diversity, which leads to a diverse repertoire of recombinant antibody or peptide genes like those from the B-lymphocytes of immunized individuals. The second step is the coupling of genotype to phenotype in which the collection of antibodies or peptide genes obtained previously is cloned to provide the physical link between each antibody or peptide’s antigen-binding properties and the encoding genes. The third step is the application of selection pressure to enrich the antibody or peptide repertoires by several rounds of selection with target antigens followed by amplification. Finally, the individual clones are screened for antigen binding.
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Figure 1. General scheme for the preparation and selection of a naïve recombinant antibody library. This figure indicates the general steps involved in the construction and selection of a recombinant antibody from a phage display library. The B-lymphocytes are first isolated from the blood of the donors. The total mRNA is extracted from the isolated lymphocyte cells and reverse-transcribed to complementary DNA (cDNA). The variable heavy (VH) and variable light (VL) chain antibody genes are amplified by polymerase chain reaction with gene-specific primers and assembled before cloning into a phage display vector. The resulting phages display antibody fragments on their surfaces. During selection, filamentous phage display libraries are incubated with antigens immobilized on a solid phase. The antigen-binding phages remains attached to the antigen, whereas the unbound phages are washed away. The bound phages are then eluted and amplified by infection with bacterial cells. By changing the expression host to a non-suppressor bacterial strain, the soluble antibody fragment can be expressed and secreted to the culture medium.

Several procedures have been described for antibody selection from phage and ribosome display repertoires with high affinity for target antigens. For instance, by incubation of the target antigen followed by extensive washing steps, improved off-rate library clones can be selected. Similarly, by using low and restricted amounts of antigen, library clones with lower dissociation constants (Kds) are obtained [6]. In a yeast display, the antibody/peptide–antigen complex dissociation time and antigen concentration optimization allow cell sorting by flow cytometry [7]. Antibodies or other alternative binders can also be selected for or with a particular functional assay, for example gene transfer [8], signaling [9], or receptor cross-linking [10].
Display technologies for the generation of recombinant antibodies

Several display methods can be exploited for generating binders, such as phage display, ribosome display, and yeast display. In general, the power of the various display technologies lies in the physical linkage between the displayed phenotype (protein or peptide) and its corresponding genotype (the DNA sequence encoding it) [11]. This presents the possibility of handling vast numbers of different constructs in a “single pot”, and isolating specific binders by an affinity-driven selection process. Further, handling large collections of binders in relatively small volumes allows for high-throughput/parallel screening against whole arrays of target molecules [11]. These features allow the in vitro evolution of the selected binders into optimized molecules for diagnostic and therapeutic applications [12].

Phage display

In nature, antibody receptors are bound to the surface of B-cells. After the antibody receptor binds to a target antigen, B-cell proliferation is induced resulting in the secretion of antibody molecules specific to the target antigen. The acquired immune system is able to register a repertoire of unique antibodies with different affinities to varying targets. This is possible owing to the high diversity of antibody genes generated through V(D)J recombination processes. To replicate this in vitro, a selection system that imitates all these features is necessary. This is emulated in vitro by the generation of libraries with high diversities via a combinatorial mix of both heavy and light chains. The generated repertoire is introduced into microorganisms that express and display the antibodies on their surfaces. This facilitates the simultaneous screening of millions or billions of microorganisms for binding to the immobilized antigens followed by enrichment of the selected microorganism [13].

The most widespread and successful surface display and selection system developed so far uses filamentous bacteriophage M13 and fd [6]. M13 contains a single-stranded DNA genome that consists of 11 genes in which the circular single-stranded DNA genome is encapsulated by the major coat protein (pVIII) and four minor proteins, two of which differ from each side of the tips [11]. Bacteriophages are viruses that infect only F’ bacteria, i.e., those Escherichia coli cells that bear the F-pilus. Infection is mediated by the attachment of the phage coat protein III (pIII) to the F-pilus of the male E. coli [14]. In contrast to the lytic bacteriophages, the filamentous phage does not produce lytic infection in infected E. coli, but instead, the phage particles are able to replicate and assemble after infection, whereas the infected bacteria continue to grow and divide [15]. The fusion foreign protein is directed to the infected bacteria cell membrane by a leader signal peptide encoded by the phagemid vector [16].

Phage display techniques involve the fusion or insertion of exogenous DNA sequences into a location adjacent to the phage protein DNA sequence on the bacteriophage genome, such that the encoded exogenous proteins are expressed or displayed on the surface of the filamentous phage as a fusion to one of the phage coat proteins [12]. Although all five coat
proteins have been used in display applications, the major capsid gene VIII protein and the minor capsid gene III protein are the most commonly used [17].

The first report of phage display was by G. Smith in 1985, and was based on the ability to express foreign peptides as a fusion to a coat protein on the surface of a bacteriophage [18]. Several applications of phage display technology have evolved, such as the identification of peptide agonists and antagonists for receptors [19], the use of random peptide libraries to identify peptide mimics of, e.g., antigenic epitopes [20], the identification of peptide drug candidates [21], the display of antibodies [22], and the development of vaccines [23]. With an array of applications, phage display techniques have proved to be useful and valuable research tools in both basic and applied science [24].

Phage display libraries can be made using phage vectors, which are based on the natural Ff phage sequence, or by using a phagemid. Phagemids are hybrids of a bacteriophage and plasmid vectors with a size of ~4.6 kb [15]. Such phagemids are designed to contain the origins of replications (ori) for both the bacteriophage and E. coli, gene III and/or gene VIII for fusion purposes including an appropriate secretory leader signal peptide sequence, a multiple cloning site, and an antibiotic resistance gene [12]. These phagemid vectors are relatively small, which gives them higher transformation efficacy than a phage vector, thereby facilitating the construction of large repertoires or libraries of protein or peptide binders. However, phagemids alone cannot produce infective phage particles [14] because they lack all the other phage genes that encode the structural and non-structural proteins required to produce a complete phage particle [11]. Therefore, a helper phage such as M13KO7 or VCSM13 that packages its own DNA less efficiently than the phagemid DNA is required [13].
Helper phages contain a slightly defective origin of replication and provide all the important genes for generating a complete phage in a process called “phage rescue” [12].

Phage particles are very durable in that they are able to withstand harsh conditions such as low pH (pH 2.2) and 4–6 mol/L urea whilst still retaining their infectivity to bacteria. It is also a convenient feature that bound phages do not need to be eluted from a surface for infection to occur. These features facilitate the selection and enrichment process for binders in a process called “bio-panning” [25]. The proteins or peptides displayed on the selected phages can be further identified by sequencing the coding gene for the displayed proteins or peptides, which are located in the single-stranded DNA within the selected phage. The specific selected phages and the soluble proteins or peptides released from the phage coat can be used as affinity reagents in diagnostic tests. Moreover, antibody phage display allows customization in panning protocols for tailored selections and the use of untapped specificities, which are unlikely to be achieved with conventional mice hybridoma-based approaches. The phage display method also enables the easy engineering of antibodies such as by adding tags, optimization, and format switching between Fab, scFv, and entire immunoglobulins [26].

As high-throughput screening platforms, antibody phage display libraries allow miniaturization and parallelization because they are effective at low concentrations [26], and they have a high impact on the screening and development of novel and patentable molecular entities [24]. The ability to transfer the panning process to magnetic particles has facilitated

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**Figure 3.** Schematic representation of the phage display bio-panning process. Antibody-presenting phages (top) are incubated with the surface-immobilized analyte of interest (right). Unbound phage particles are washed away (bottom), and analyte-bound phages are eluted (bottom) and amplified by re-infection of *Escherichia coli* (left). The amplified phages are rescued for the following round of panning. In the final round of panning, the antibody may be cloned out of the phage for retrieval.
a higher throughput for phage-derived antibody production [27]. This is evident in therapeutic and diagnostic applications, in which approximately 30% of all human antibodies currently in clinical development are actually provided by the phage antibody display [26].

**Microbial cell surface display**

In combination with antibody fragments, the most prominent microbial cell surface display system uses the yeast *Saccharomyces cerevisiae* whereby the protein or peptide is displayed on the yeast cell surface via fusion to the yeast mating factor, α-agglutinin yeast adhesion receptor [28], which is located on the yeast cell wall [6]. The α-agglutinin yeast adhesion receptor acts as an adhesion molecule to mediate cell–cell interactions during the mating of the “a” and α haploid yeast cells. The receptor consists of proteins Aga1 and Aga2. The yeast display platform depends on the successful association of Aga1 and Aga2 proteins to display a protein or peptide on the yeast cell surface. Aga1 is secreted from the yeast cell and becomes covalently linked to a β-glucan in the yeast cell wall, whereas Aga2 binds to Aga1 via two disulfide bonds, and after secretion remains attached to the cell through Aga1 [28].

Figure 4. The figure shows the concept of yeast display. The scFv is displayed as a fusion protein to Aga2 on the surface of the yeast cell. The expression of the scFv can be monitored by tagging the antibody fragment with e.g., a fluorescently-labeled anti-myc antibody. The interaction between the antibody fragment and the target antigen can be detected using the same principle. Here, for example, the antigen is biotinylated and detected with fluorescently labeled avidin.

The yeast display platform is similar to the phage display platform because it also physically links the phenotype with its genotype. Yeast display vectors are designed to encode the Aga2 gene for fusion protein surface expression and CEN/ARS origin for selection and maintenance.
in *S. cerevisiae*. The gene of interest is cloned into the yeast display vector (pYD1 or its derivative) and fused with the Aga2 gene. The resulting construct is then transformed into *S. cerevisiae* strain EBY100, which contains the Aga1 gene. The expression of the Aga2 fusion protein in the yeast display vectors and the expression of the Aga1 protein in the EBY100 host strain are tightly regulated by the GAL1 promoter, which does not allow any protein or peptide expression in the absence of galactose [29]. Affinity tags (HA-tag, His-tag, myc-tag, or FLAG-tag) can be introduced into the yeast display vectors to monitor the expression of protein, especially scFv antibody expression on the yeast cell surface, using flow cytometry with fluorescently labeled antibodies [30].

It has been suggested that *S. cerevisiae*, which is a eukaryote, offers an advantage for the post-translational modification and processing of mammalian proteins compared with a prokaryotic host, such as *E. coli*. Therefore, *S. cerevisiae* is believed to provide better expression and secretion functions for human-derived antibody fragments than a host such as *E. coli* [28]. Although *S. cerevisiae* is able to provide post-translational modifications, it has several weaknesses. The yeast cell surface display faces a bottleneck during the most important part of recombinant binder generation: library production. Yeast cells have very low transformation efficiencies compared with *E. coli*, which makes the process of cloning to generate highly diverse libraries cumbersome. This primarily results in low-affinity antibodies, because small library sizes are believed to correspond to low diversity. Although a yeast display facilitates the use of flow cytometry to conduct panning experiments, the speed of cell sorting and the requirement of sorting expertise and expensive equipment make it unpractical for most laboratories [6].

**Ribosome display**

In contrast to phage and yeast display systems, ribosome display is a cell-free system for the completely *in vitro* selection of proteins or peptides from large repertoires [31]. It is based on the generation of stable protein–ribosome–mRNA (PRM) complexes by ribosome stalling [32]. The production of PRM complexes by ribosome stalling has been carried out using two strategies. First, by the addition of antibiotics to prokaryotic and eukaryotic ribosomes to stop translation randomly [33], and second, by the deletion of the stop codon, which induces detachment of the nascent protein [34] to stall the ribosome at the 3’ end of the mRNA [35]. Hence, the nascent proteins (phenotypes) are coupled to their corresponding mRNA (genotypes) [34], because the translation process is made to stop without releasing the proteins, thereby maintaining the linkage between genotype and phenotype. This permits the simultaneous selection of a functional nascent protein together with the encoding mRNA through affinity segregation of the nascent protein for a ligand. The mRNA can then be converted and amplified as DNA by reverse transcription polymerase chain reaction (RT-PCR) for further manipulation, such as mutation and protein expression [31].
The first published description of a ribosome display was for peptide selection. It used a coupled *E. coli* S30 prokaryotic ribosome system and was termed a “polysome display” [32]. At the same time, a eukaryotic ribosome display system was also introduced by using a coupled rabbit reticulocyte lysate system for the selection of functional single-chain antibody fragments. This system was termed an antibody–ribosome–mRNA (ARM) display [36].

Generally, the DNA construct for the ribosome display should be designed to include the following elements: a promoter such as T7 or T3; a translation initiation signal such as the Shine-Dalgarno sequence for prokaryotic systems, or the Kozak sequence for eukaryotic systems [37]; a consensus sequence for protein initiation in both eukaryotic and prokaryotic systems [38]; and a spacer domain of at least 23–30 amino acids at the C-terminus to create a physical link to the ribosome and to enable the displayed protein to exit completely from the ribosome tunnel [39].

Cell-based display methods, such as phage display and yeast display, have a common restriction on their limited repertoire diversity. This is because the diversity is directly proportional to the cell transformation efficiency during the production of the “single pot”
library. However, cell-free systems such as ribosome display bypass the transformation step by producing PRM complexes via stalling of the ribosome at the end of translation. This in turn allows a very large binder library to be generated and screened, because PCR fragments are directly used as templates without the need of cloning by transformation [40]. However, certain formats are not amenable to ribosome display, such as Fab’s, because the light and the heavy chains are expressed from different mRNAs, and would therefore not assemble appropriately.

**Others**

The technology to display and screen expressed proteins has become an attractive tool, especially in protein engineering and high-throughput screening. As well as the common display methods, other feature-like methods are applicable for antibody production. Recently developed display systems include retroviral display, which exploits retroviruses for the expression of correctly folded and post-translationally modified proteins [41]. Baculovirus display, which expresses proteins or peptides on the surface of a baculovirus without affecting insect cell replication, is useful both in vivo and in vitro [42]. Protein-fragment complementation assay (PCA) is another example of a novel display system that fuses two proteins of interest to complementary fragments of a reporter protein such as an enzyme and a fluorescent protein [43]. Display systems based on protein–DNA linkage [44], single-molecule sorting [45], microbead display [46], and many other newly established systems have also been reported. There is no doubt that each of these technologies has specific advantages. However, their validation with whole libraries has been limited, and their benefits over more established systems such as phage display require verification [6].

**Antibody fragments for diagnostics**

With regard to diagnostic applications, antibodies are currently still the most important tools for the specific detection of proteins or other molecules. The generation of antibodies as an important class of binding molecules, either by the immune system or by synthetic immunoglobulin libraries, allows the isolation of a large variety of reagents with high specificities and high binding affinity for nearly any type of antigen [47].

Antibodies are tetramers that consist of two identical light and two identical heavy chains. Each light chain pairs with a heavy chain, and each heavy chain pairs additionally with another heavy chain. The chains are linked by covalent interchain disulfide bonds and non-covalent bonds. There are two main moieties in an antibody: the variable region, which mediates molecular recognition; and the constant region, which mediates the biological function of the antibody.

Each chain consists of a variable (V) and a constant (C) region. The heavy chain is assembled from variable (VH), diversity (D), joining (JH), and constant (CH) gene segments; whereas the light chain is assembled from variable (VL), joining (JL), and constant (CL) gene segments. The
amino acid and DNA sequences of the C regions are relatively conserved within a particular species, whereas the sequences of the V region are antigen-dependent. Pairing of the V-D-J regions of the heavy chain and the V-J regions of the light chain creates an antigen-binding site (the paratope) for the antibody, which recognizes a single antigenic determinant (the epitope) of the antigen. Each V region consists of an alternating framework (FW), which is more conserved, and three hyper-variable loops, i.e., the complementarity determining regions (CDRs). Variations in the amino acid sequences of the CDRs dictate the shape and ionic properties of the antigen-binding site. This results in the generation of different antibodies.

Figure 6. Schematic representation of IgG and antibody-derived fragments. This figure indicates the structure of a full-length immunoglobulin G (IgG) molecule and antibody-derived fragments: Fab (antibody fragment), Fv (fragment variable), and scFv (single chain fragment variable). The light chain is composed of a variable (VL) domain and one constant (CL) domain, whereas the heavy chain is composed of a variable (VH) domain and three constant (CH1-3) domains.

Fragment antigen binding (Fab) and single chain fragment variable (scFv) are among the functional recombinant antibodies that have been produced by display technology. The larger Fabs (50 kDa) consist of the entire light chain and a part of the heavy chain linked together by disulfide bonds. The scFv fragments (25–30 kDa) consist of the antibody-variable domains, which are artificially joined with a flexible peptide linker between the carboxyl terminal amino acid of one domain and the amino terminal of the paired domain. The peptide linker is usually a 15 amino acid linker with a (Gly4Ser)3 sequence [13]. The scFv fragments, which are only half the size of a Fab molecule, are preferred for clinical applications. Their small size facilitates faster blood clearance [48], lower retention times in non-target tissues [49], and better tumor penetration [50]. They are also suitable for the binding of cytosolic targets and intracellular expression in eukaryotic cells [51].
Even though antibodies are known to have high affinity and specificity, they have several disadvantages for diagnostic applications. It is usually costly to generate full length antibodies because they are produced by recombinant expression in mammalian cells such as CHO or NSO cells. The bottom half of the heavy chain constant region (CH2 and CH3), where the binding site of Proteins A or G resides, is made up of glycosylation sites. This modification is vital to allow proper folding and enhance the stability of the full antibody molecule. This requirement for glycosylation of the constant regions is often problematic for high-throughput expression, and the many natural binders of the constant region may lead to high levels of background signal in the diagnostic tests [52].

Miniaturization or down-sizing of conventional antibodies into smaller formats consisting only of the binding pocket of antibodies is of growing interest to retain the desirable characteristics (high affinity and specificity) of antibodies, while avoiding the drawbacks. Antibody fragments such as Fab, scFv, and VH are of particular interest as an alternative to the whole antibody molecule for in vivo clinical applications. With the removal of the CH2 and CH3 domains in these alternative formats, the effector functions are eliminated, and the half-life is lowered for rapid clearance from the body, making these formats ideal for in vivo diagnostics and imaging. Human phage antibody libraries can directly produce such Fab and scFv antibody fragments without the need to obtain them by conversion from whole antibodies.

Antibodies fragments also offer several advantages over full length antibodies for in vitro diagnostics. One main advantage of scFv and Fab is the significant reduction of background signal effects, because there is no Fc receptor binding or activation site available for non-specific binding. Another advantage is the ease of production of the antibody fragments, which can be rapidly obtained from prokaryotes including E. coli. If necessary, polyethylene glycol (PEG) molecules can be conjugated to scFv and Fab to increase the in vivo half-life of these antibody fragments [53].

**Conclusion**

Antibodies and their fragments such as Fab and scFv are still the most prominent class of binders used in diagnostic assay development. However, with developments in protein engineering, new alternative binders are being introduced besides immunoglobulin or antibody formats. These alternative binders can be based on peptides, proteins, ribonucleic acids, or single-stranded DNA [54]. Although these new molecules are being applied as binders in diagnostics, antibodies are still the molecules of choice. The advantage of recombinant antibodies for diagnostics is that their specificity and sensitivity can be altered via genetic modification, making them customizable for diagnostic platforms [55]. Display technologies also offer a more cost effective route than conventional methods, with the ability to upscale production by recombinant expression methods. The elimination of animal hosts is also beneficial because it speeds up the generation of antibodies [56]. Furthermore,
an obvious benefit of display technologies for antibody production is the ability to produce antibodies against toxic compounds or non-immunogenic targets otherwise hindered in host-dependent methods. As the influence of recombinant antibodies in diagnostics grows, antibody generation using display technologies is increasingly the preferred method of production.

References