Chapter 2

Recombinant Antibodies and In Vitro Selection Technologies

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Abstract

Over the past decade, the accumulation of detailed knowledge of antibody structure and function has enabled antibody phage display to emerge as a powerful in vitro alternative to hybridoma methods for creating antibodies. Many antibodies produced using phage display technology have unique properties that are not obtainable using traditional hybridoma technologies. In phage display, selections are performed under controlled, in vitro conditions that are tailored to suit demands of the antigen and the sequence encoding the antibody is immediately available. These features obviate many of the limitations of hybridoma methodology, and because the entire process relies on scalable molecular biology techniques, phage display is also suitable for high-throughput applications. Thus, antibody phage display technology is well suited for genome-scale biotechnology and therapeutic applications. This review describes the antibody phage display technology and highlights examples of antibodies with unique properties that cannot easily be obtained by other technologies.

Key words: In vitro selection, Phage display, Antibodies

1. Introduction

Methods for generating antibodies were initially developed more than a century ago with the production of polyclonal antibody preparations from animal immunizations (1). The advent of hybridoma technology in 1975 enabled the production of monoclonal antibodies through the fusion of myeloma cells with antibody producing B-cells (2). Hybridoma technology advanced our capacity for research and diagnostics by providing homogenous, purified antibody preparations that improved tracking, detection, and quantitation of target molecules in cells and serum. The hybridoma technology, however, is not without its limitations. With regard to generating human therapeutics, hybridoma antibodies are typically from murine sources, which limits their therapeutic

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applications due to human anti-mouse antibody reaction (3, 4). A variety of strategies have been developed to address this problem, including chimerization and humanization strategies (5–9), and transgenic animals with human immunoglobulin loci (10–14). Despite these efforts, the generation of antibodies by hybridoma technology is still costly and time-consuming. Further, since these antibodies are produced in animals, it is difficult to generate them against toxic and highly conserved antigens (15) as well as antigens that are not stable in animal systems.

Alongside the hybridoma technology, methods have been established to generate antibodies using in vitro display technologies. The first such method was antibody phage display, introduced 20 years ago (16–18), followed by yeast, ribosome, puromycin-based plasmid, and bacterial display systems (19–22). Selection platforms (23–25) and the design of antibody fragments (26, 27) for making libraries have been widely dealt with in previous reviews. The purpose of this review is to illustrate how in vitro selection, especially phage display, has yielded antibodies with remarkable properties that are difficult to obtain using traditional immunization methods.

Principles behind methods used in all in vitro display systems are similar whatever the display platform, and center on the coupling of genotype (gene) to phenotype (binding protein). In practice, this comprises the creation of DNA libraries encoding binding molecules such as antibodies, the display of the encoded proteins, the application of selective pressure based on the binding properties of the encoded proteins, followed by growth and screening of individual clones. Since in vitro display methods are performed in bacterial or yeast systems, the turnaround time for antibody generation is less, and the potential for high-throughput generation of binders is greater (28). The power of in vitro antibody selection is further enhanced by the ability to precisely control selection conditions. In contrast to animal immunization, where there is little control over the nature of antibodies produced, manipulation of selection conditions can be carried out in vitro, for example, by presentation of specific conformations of the target antigen or by including competitors to direct selection towards targets or epitopes of interest. In vitro selection methods also overcome the problem of tolerance, which limits the potential for making anti-self antibodies. As tolerance is applied to specific variable heavy (VH) and light (VL) domain combinations that recognize self-antigens, when in vitro libraries are created from natural sources new combinations with the capacity to recognize self-antigens can be created. This has been proven for example by the selection of hundreds of human antibodies from naive libraries to human targets (29–32). For libraries that are constructed using synthetic diversity, the concept of tolerance does not apply. This enables the selection of antibodies against highly conserved targets such as ubiquitin (33, 34), histones (35), hemoglobins (36), and posttranslational modifications (37–39).

Another advantage of in vitro selected antibodies is that the gene encoding the antibody is cloned simultaneously with selection. This is perhaps the most crucial difference between hybridoma and in vitro selection technologies and provides many advantages for engineering selected antibodies. For example, affinity maturation of selected antibodies can be easily performed using in vitro selection technologies. Maturation of antibody affinity to the picomolar range (40–44) has become relatively routine and, at least in one case, femtomolar affinity has been achieved (45). These affinities are far higher than those that can be obtained by immunization, which are limited to ~100 pM by the physiological mechanisms of B-cell activation (46–48). In addition, antibody specificities can be broadened or narrowed by appropriate selection conditions. As a result, in vitro selection has yielded antibodies with remarkable properties that are either a direct result of the flexibility and control that can be applied to all aspects of the selection processes, or novel properties developed as a direct result of the recombinant nature of selected proteins. Finally, the availability of the antibody gene allows the creation of a large variety of antibody derivatives with added functions by simple subcloning.

2. Phage-Displayed Antibody Libraries

Antibody libraries for in vitro selections can be generated from immunized repertoires, natural naïve repertoires, or designed "synthetic" repertoires. Strategies to generate antibody libraries have been described extensively (49, 50) and are discussed briefly here. Immune antibody libraries are generated by cloning antibody fragments, either single-chain variable fragments (scFvs) or antigen binding fragments (Fabs), from IgG mRNAs obtained from activated B-cells (49, 50). These libraries are biased for members that bind a specific antigen and require that a new library be generated for each antigen of interest. Immune antibody libraries have been generated against a number of different species (51, 52). Human immune libraries have been constructed from virus-infected patients to generate neutralizing antibodies and from cancer patients to isolate tumor specific antibodies (53–57).

Although natural naïve antibody libraries have been generated using IgM or IgG mRNAs from resting B-cells, initial results indicated that libraries based on IgM mRNA yield more binders (17), probably because the IgG fraction is biased towards recent immune responses. Naturally rearranged variable region genes have been used to construct large antibody fragment libraries (32, 58–61). In contrast to immune libraries, naïve libraries can be used to generate antibodies against a variety of antigens; however; they generally bind with lower affinity and may need to be affinity matured.

In synthetic antibody libraries, antibody diversity is designed and synthesized in a controlled manner. In these libraries, the composition of complementarity determining regions (CDRs) can be precisely defined. A number of approaches have been used to design synthetic libraries and they vary in the number of variable framework regions used, the design of CDR diversity, and the library construction method. Synthetic libraries have been constructed using a variety of different variable framework genes (62–65), with diversity introduced into the CDRs, primarily in CDRH3 and CDRL3. Results from selections with these libraries have shown that larger libraries yield antibodies with higher affinity and greater specificity (50), and that specific variable framework regions are over represented in the selected antibody fragments. This observation led to the development of libraries using a single VH and VL combination (66–68). A number of different libraries have been devised that differ in the variable gene and the diversification strategy. Most libraries use a common VH domain (VH3–23) as it is stable, expressed well in bacteria and on phage, and pairs with most VL domains. Synthetic libraries are constructed by cloning oligonucleotides into the CDRs of defined antibody fragments, and thus, these libraries are not limited to the diversity present in natural repertoires. However, libraries have been created by grafting natural CDRs into single frameworks (69), as well as by using designed oligonucleotides that mimic the CDR diversity observed in natural repertoires (68, 70). Libraries have also been designed with restricted diversity in the CDRs, based on the observation that tyrosine and serine are enriched in the antigen-binding sites of antibodies (71–73).

In addition to variable domain and CDR design, antibody fragments must be fused to the phage coat protein in order to establish the genotype/phenotype connection. Phage display requires that antibody fragments be displayed rather than IgGs. The two most popular antibody fragments used to display the variable antigen binding domains are the Fab and the scFv. The Fab is a heterodimer consisting of the variable and first constant domains of heavy and light chains. The scFv consists of the variable domains from the light and heavy chains joined by a peptide linker. These antibody fragments are most commonly displayed on phage by fusing them to pIII or pVIII coat proteins. The pVIII coat protein can potentially enable the display of multiple polypeptides on the phage surface, as approximately 2,500 copies of pVIII are present on each phage particle (74). However, large proteins are not well tolerated as pVIII fusions, which limits their use for displaying antibody fragments (75), and in direct comparisons, pIII display appears to be more efficient than pVIII (76). Thus, antibody fragments are more commonly fused to the pIII coat protein. There are approximately five pIII coat proteins on one tip of the phage particle. Fusion to the pIII coat protein results in low-level display of antibody fragments using phagemid systems (49), and display

levels can easily be switched between monovalent and oligovalent display (77), which allows selections from large libraries to be optimized for obtaining higher affinity antibody fragments by avoiding avidity effects.

2.1. Diverse Applications of Antibody Phage Display

Since the invention of antibody phage display, intellectual property issues have delayed its broad use and have limited the number of synthetic antibodies in the clinic during the 1990s. Nonetheless, as of 2010, phage display technology has been used to generate at least 35 human antibodies that are in clinical development (78). The FDA has approved two of these antibodies (adalimumab and belimumab) and one is under review (raxibacumab). The success of phage-derived antibodies in clinical trials is similar to monoclonal antibodies derived from other technologies (78). The number of antibodies generated using phage display is rapidly expanding and a comprehensive catalog is beyond the scope of this review. Below, we highlight some examples of phage-derived antibodies generated against extracellular targets as well as some of the unique features of antibodies that can be generated using phage display technologies.

2.2. Antibodies Against Extracellular Targets

Phage display technologies are extremely powerful for generating functional antibodies that disrupt normal or pathological extracellular signaling. Phage display selects for antibodies that bind their target with high affinity, however this does not guarantee that they will have the desired function. Phage display, however, can produce many antibodies that bind a given target, increasing the chance that some of the antibodies will possess the desired properties. One such example was the use of phage display to generate more than 1,200 antibodies against the B-lymphocyte stimulator (Blys) (79), a potent cytokine for B-cell proliferation and differentiation. Biochemical and cellular assays were used to subsequently identify antibodies, many with subnanomolar affinities, which blocked B-cell activation by inhibiting the interaction between Blys and its receptor. One of these antibodies, which showed specificity for secreted Blys, was affinity matured and shown to be a potent inhibitor of Blys signaling (80). This antibody, belimumab, has been approved by the FDA in March 2011 for use in treatment of systemic lupus erythematosus.

A second example of an antibody isolated by phage display against a cytokine target is the tumor necrosis factor alpha (TNFα) blocking antibody, adalimumab. TNFα is a proinflammatory mediator implicated in autoimmune conditions. Adalimumab has been approved for the treatment of several conditions including rheumatoid arthritis, ankylosing spondylitis, chronic plaque psoriasis, and Crohn's disease, which was the first fully human antibody approved by the FDA in 2002. A number of other antibodies against soluble ligands have been generated by phage display and are in advanced clinical trials (81).

Another strategy to block receptor signaling is to target receptor sites that prevent ligand binding. A recent series of studies highlights the use of phage-derived antibodies to block Insulin-like Growth Factor 1 Receptor (IGF-1R) signaling (82–84). Phage display was used to generate antibodies against two unique epitopes of IGF-1R. Both antibodies blocked binding of Insulin-like Growth Factor 1 (IGF-1) and Insulin-like Growth Factor 2 (IGF-2), but they did so by either directly competing for ligand binding or by an allosteric mechanism, which decreased the affinity of ligand binding (84). Interestingly, cotreatment with both antibodies improved both the potency and extent of IGF-1 and IGF-2 blockade compared to treatment with either antibody alone. Similar results have been observed with Her2 (Human Epidermal growth factor Receptor 2), where combinations of antibodies that bind unique epitopes have greater activity than either antibody alone (85).

Antibodies have also been generated to block ligand-induced conformational changes in Notch receptors (86). The ectodomain of the Notch receptor contains multiple epidermal growth factor (EGF) repeats and ligand binding induces a conformational change at the juxtamembrane negative regulatory region, which causes a protease cleavage site to be exposed. Subsequent proteolysis causes the intracellular domain to be translocated to the nucleus. Phage display was used to generate antibodies that target the juxtamembrane negative regulatory regions of Notch-1 and Notch-2 (86). These antibodies bind and stabilize the "closed" conformation of the Notch receptor, preventing proteolytic cleavage.

Ligands often act either by causing dimerization of their target receptors or by inducing conformational changes in preexisting dimers. Antibodies targeting the ligand-binding domain can, in some instances, mimic the effect of the natural ligand and cause receptor activation rather than inhibition. For example, phage display was used to generate antibodies that bind to Muscle Specific Kinase (MuSK) (87) or CD40 (88) and function as agonists for receptor activation (87). In another recent study, over 500 distinct antibodies were generated against TRAIL receptor-1 (TRAIL-R1) and TRAIL receptor-2 (TRAIL-R2) (89). TRAIL is a homotrimeric ligand that causes multimerization of TRAIL receptors, which in turn leads to apoptosis, particularly in tumor cells overexpressing the receptors. Ten agonistic antibodies specific for TRAIL-R1 and six antibodies acting only on TRAIL-R2 were identified. As expected, these antibodies competed for binding with TRAIL, but surprisingly, they were active agonists as monovalent antibodies in either scFv or Fab formats, and activity was not enhanced upon conversion to IgG. The mechanism of action for this unusual agonistic activity is still unclear.

In addition to selecting antibodies that bind to purified proteins, phage display can be used to select antibodies that recognize targets expressed on the surfaces of cells. A number of selection

protocols have been developed to select antibodies that bind cell surface proteins. These include strategies that incorporate negative selections or preabsorption steps (90–94), strategies to remove unbound phage (95, 96), and the pathfinder approach (97, 98). In vitro selection schemes have also been devised to select for antibodies that mediate receptor internalization (99–101). In these selections, phage libraries are incubated with target cells and then phage that bind the cell surface are removed and phage antibodies inside the cell are isolated. This strategy is useful for generating antibodies to deliver drugs to specific cells (102, 103). The ability to perform selections directly on cells with negative selections has proven to be a powerful trait of phage display technology.

2.3. Antibodies Against Infectious Disease Targets

Phage display has been used to select antibodies against a variety of infectious agents. For example, antibodies have been isolated that discriminate between strains of Hanta (104), Dengue (105, 106), Influenza (107, 108), Ebola (109), and Venezuelan equine encephalitis virus (110). Further, phage display selections do not require purified virus. For the Venezuelan equine encephalitis virus selection, the use of competitive binding conditions allowed antibodies to be generated against impure virus preparations (110). In these selections, cell extracts from uninfected cells were added to the binding buffer, which eliminated the isolation of antibodies against components of the host cell and allowed antibodies to be generated against the viral envelope. Human antibodies have also been selected against a number of bacterial bio-threat targets, including *Brucella melitensis* (111), *Burkholderia mallei*, *Burkholderia pseudomallei* (112), and anthrax toxins (113–117) and spores (118).

In one study, antibodies were used to block protein interactions associated with influenza entry into target cells. Phage display was used to generate antibodies that recognize the H5 hemagglutinin influenza ectodomain (119, 120). Structural characterization of one of these antibodies bound to H5 showed that it binds to hemagglutinin by inserting its heavy chain into a highly conserved pocket in the stem region, which prevents structural reorganizations required for membrane fusion. This conserved epitope is found in many different influenza viruses and this antibody was shown to neutralize H5N1, H1N1, H2N2, H6N1, H6N2, H8N4, and H9N2 viruses. Although antibodies have not been generated against this epitope by traditional immunization, and antibodies with this specificity do not normally arise during infection, antibodies with similar VH gene usage and neutralizing activity have been selected from phage antibody libraries created from human IgM + memory B-cells from recently infected individuals (121).

2.4. Antibodies with Ultra-High Specificity

With protein targets, antibodies have been selected that display high specificity for a chosen target. For example, antibodies have been generated that differentiate between chicken and quail

lysozyme, which differ by only four amino acids (122). Another example is the isolation of antibodies that distinguish between the SH2 domains of ABL1 and ABL2 tyrosine kinase (29, 123), which differ by only 11%, and for which it has not been possible to obtain specific antibodies by immunization. In such studies, negative selection steps have been incorporated into phage display selections to generate antibodies with desired specificity. For example, antibodies were generated that recognize only fetal and not adult hemoglobin (124). In this study, antibodies that recognize adult hemoglobin were depleted by preincubating the antibody phage library with adult hemoglobin prior to each round of selection against fetal hemoglobin. Phage display has also been used to generate antibodies that recognize specific protein complexes, for example the generation of antibodies that recognize unique peptides in the context of specific MHC molecules (125–128).

The technology has also been applied to selectively target alternatively spliced fibronectin variants associated with tumor neovasculature. Extra-domain A (EDA) and B (EDB) are fibronectin variants each of which contain an additional domain, both being highly conserved between human and mouse. Using a synthetic antibody library, it was possible to select human/mouse cross-reactive scFvs against each of the recombinantly purified extra domains (129, 130), and these antibodies were effective for immunohistochemical analysis in vitro and for biodistribution studies in vivo. In the case of the anti-EDB antibody (130), the modular nature of the scFv was exploited to engineer numerous fusion proteins with potential for cancer therapy (131), and three of these derivatives are now in clinical trials.

2.5. Antibodies Against Specific Protein Conformations

Phage display has been used to generate antibodies that recognize specific protein conformations. Many signaling proteins exist in specific conformational states that mediate distinct cellular responses. Antibodies that recognize specific protein conformations provide a unique resource for characterizing signaling pathways. These types of antibodies are difficult to generate via immunization strategies, as protein conformations are often unstable in an immunized animal. In contrast, in vitro selection technologies are ideally suited for these applications because selection conditions can be precisely controlled to favor particular conformations. Negative selections can be used to deplete nonspecific binders and affinity maturation strategies can be employed to fine-tune specificity.

Phage display has been used to generate antibody fragments that specifically recognize the GTP-bound form of Rab6 (132) and active and inactive forms of Caspase-1 (133). scFvs specific to the GTP-bound form of the small guanosine triphosphatase (GTPase) Rab6 were generated by performing selections against a

GTP-locked mutant (132). Fabs specific to on and off states of caspase-1 were generated by selecting libraries against capase-1 complexed to small molecules that lock it in the on or off state (133). Fabs were converted into full-length IgGs to produce highly sensitive affinity reagents that could be used to probe the localization of active caspase-1 in cells (133). Conformation-specific antibodies have also been generated against active cell membrane receptors by performing phage selections on whole cells (134).

2.6. Antibodies Against Integral Membrane Proteins

Integral membrane proteins absolutely require a membrane or detergent environment to maintain their native conformation. Consequently, the generation of conformation-specific antibodies against membrane proteins by animal immunization is severely limited by the denaturing effects of the serum environment. In contrast, the ability to control selection conditions makes in vitro techniques much more amenable to this task. By performing selections in the presence of detergent, high affinity Fabs were isolated against the citrate transporter CitS from *Klebsiella pneumoniae* (135), as well as against the potassium channel KcsA from *Streptomyces lividans* (136). In the latter case, the Fabs were used as crystallization chaperones that enabled the elucidation of the crystal structure of the full-length potassium channel.

2.7. Antibodies Against RNA

Phage display has been used to generate antibodies that recognize structured RNA molecules (137), which have proven to be essentially nonimmunogenic for hybridoma methods. Using a nuclease free selection buffer, high affinity Fabs were isolated against a structured domain from the *Tetrahymena* group I intron. The structure of the Fab/RNA complex was solved to high resolution, highlighting the use of antibody fragments as chaperones for RNA crystallization. Fabs were also obtained against a class I ligase ribozyme and were used as chaperones to obtain the crystal structure (138). One Fab recognized a small, discrete sequence in the ribozyme and retained binding capacity when this sequence was transferred to other RNA structures, providing a novel RNA crystallization chaperone system.

2.8. Antibodies Against Posttranslational Modifications

Phage display has been useful for detecting posttranslational modifications that have proven intractable to immunization. For example, sulfotyrosine is a posttranslational modification predicted to occur in 30% of all secretory and membrane proteins (139). Perhaps because of its ubiquitous nature, traditional immunizations have consistently failed to produce anti-sulfotyrosine antibodies. However, using phage display, antibodies were readily generated to recognize proteins containing sulfotyrosine (but not tyrosine or tyrosine phosphate) independently of protein context or sequence (39, 140).

3. Exploiting the Recombinant Nature of In Vitro Antibodies

Since phage display is an in vitro selection method, it offers many advantages for engineering antibodies. The phage display system provides the antibody gene and sequence following selection against a particular target. This allows antibodies to be easily further evolved and engineered to improve binding, to narrow or broaden specificity, or to improve expression as IgGs or as fusions to functional moieties.

Antibody fragments isolated from an initial phage display selection can be used directly as an affinity reagent, or they can be used as leads for further improving binding. Since the sequence of isolated antibody fragments are rapidly determined by sequencing, it is straightforward to make second-generation libraries by introducing mutations into antibody fragments. Affinity maturation strategies have been used to generate antibodies with affinities that exceed those of natural antibodies, which are limited to a ceiling of $K_d > 0.1$ nM by the nature of the B cell response (46–48). There are many different approaches for introducing diversity into antibody fragments to improve the affinities obtained from combinatorial libraries. With in vitro affinity maturation selections, randomized antibody fragments undergo selection with increased pressure to identify variants with enhanced affinity (141). In general, there are two approaches for generating diversification: targeted and nontargeted. There are many examples of in vitro affinity maturation, and here we highlight some key studies that demonstrate the power of the process.

Error prone PCR is the most common method for introducing nontargeted mutations (142). In this method, sequence diversity is randomly introduced into the antibody fragment gene by mutagenic PCR strategies (50). The down side of this approach is that deleterious mutations can be introduced into the conserved framework region, which reduces the number of functional antibody fragments in the library. DNA shuffling is another method for introducing nontargeted mutations (143). In this method, a group of closely related sequence are randomly fragmented and then reassembled by PCR, which leads to a shuffling of DNA fragments. The approach can be combined with PCR mutagenesis to further enhance diversity. This method was used to increase the affinity of an scFv for fluorescein by 1,000-fold, resulting in subpicomolar affinity (45).

As an alternative to random PCR mutagenesis, knowledge of the antibody sequence enables precise targeting of mutations for affinity maturation. Targeted mutation strategies have the advantage of focusing mutations to CDR loops, which are most likely to enhance affinity without introducing deleterious mutations in regions that may affect protein folding and stability. Targeted mutagenesis can be

performed using degenerate oligonucleotides, which allows for precise control over the locations where diversity is introduced. Further, CDRs can be targeted in either a parallel or sequential fashion. By targeting CDR loops in phage-displayed antibody libraries, an anti-HIV-1 antibody (44) and an anti-c-erbB-2 (43) antibody were affinity matured to the low picomolar range.

In addition to affinity, the specificity of antibodies can be altered by phage display. While absolute specificity for a single antigen is generally the goal of antibody design, cross-reactivity is desirable for certain applications. For example, in the case of antibody therapeutics, species cross-reactivity enables assessment of therapeutic efficacy and toxicity in animal models. Cross-reactive antibodies are often difficult to obtain by hybridoma methods because of the conservation of functional sites on proteins across species. In contrast, in vitro phage antibody libraries are not affected by immune tolerance, and generation of antibodies that target conserved sites across species orthologues has proven to be the rule rather than the exception. For example, antibodies that cross-react with human and mouse VEGF were obtained directly from phage libraries without further selections to broaden specificity (144, 145). For BAFF/BLys receptor 3 (BR3), antibodies generated against human BR3 showed weak cross-reactivity with mouse BR3. In this case, phage display was used to select cross-reactive antibodies from secondary libraries (146). This strategy has also been used to generate antibodies with cross-reactivity towards CXCL10 and CXCL9 homologues (147). In an extreme example, this approach has been used to broaden the specificity of trastuzumab so that it cross-reacts with Erb-B2 and VEGF, two proteins that share no sequence or structural homology (148). In this case, secondary libraries were created by diversifying the light chain, which plays a minor role in Erb-B2 recognition. Extensive affinity maturation produced antibodies with low nanomolar affinity for both ErbB2 and VEGF (148).

The ability to improve affinity and broaden specificity also has major implications for the development of antibodies against infectious disease agents. For the effective inhibition of viral infection and bacterial toxins, antibodies must be of very high affinity, and at the same time, they should be cross-reactive with a variety of antigen subtypes to afford broad protection against pathogen variants. A powerful example of using affinity and specificity selection cycles was demonstrated for an antibody with broad specificity for different subtypes of Botulinum toxins. Remarkably, this antibody is able to recognize Botulinum toxins A, B, E, and F, all the serotypes that afflict humans (149, 150).

The ability to rapidly obtain the gene for a selected antibody fragment allows the antibody to be easily engineered by simple subcloning strategies. Antibody fragments produced from phage display selections can be subcloned into IgG expression systems to

produce antibodies in mammalian tissue culture systems (151). Antibody fragments have also been engineered with other functions by fusing them to peptides and proteins that induce dimerization (152) and multimerization (153–155) to facilitate detection and purification, or that provide them with fluorescent (156–158) or enzymatic (159) properties. In vivo peptide biotinylation tags have been fused to the C-termini of antibody fragments to enable antibodies to be immobilized or multimerized (153, 160–163). Antibody fragments have been fused to the dimeric enzyme alkaline phosphatase, which provides both dimerization and alkaline phosphatase activity that greatly enhances functionality and simplifies screening (30, 159). scFvs have also been fused to Fc domains, converting them into antibody-like molecules with properties similar to IgGs (164–167).

Recombinant technologies have enabled the generation of a large variety of bispecific antibodies that recognize two different targets (ref. 168 and see also Chapter 16). This can be accomplished by engineering two different Fc domains to allow heterologous pairing (169, 170). Alternatively, scFvs can be fused recombinantly to IgGs to impart bifunctionality (171). This strategy has recently been used to generate bispecific antibodies against IGF-1R, which blocked ligand binding better that either monospecific IgG (172). The bispecific antibodies also showed an improved ability to reduce the growth of multiple tumor cell lines, to inhibit ligand-induced IGF-1R signaling in tumor cells, and to block in vivo tumor growth (172). Bispecific antibody fragments have also been generated by varying the peptide linker length that connects VH and VL domains in scFvs. This strategy has been used to generate dimers (173–175), trimers (174, 176, 177), and tetramers (178). Various other bispecific antibody designs have also been created (see ref. 179 for a review).

Within the context of improved antibody therapeutics, fusion proteins that can extend the capabilities of natural IgGs have been constructed. By exchanging or engineering the Fc region, antibodies with designed pharmacokinetics and improved effector functions have been obtained (for reviews see refs. 180, 181).

4. High-Throughput Antibody Selections and Next-Generation Sequencing

The ease and speed with which antibody fragments can be selected using phage display, usually between 1 and 2 weeks, allows the technology to be implemented in a high-throughput manner (29, 30, 123, 182, 183) and see also Chapters 3–6. Initial experiments using a limited number of targets showed that antibodies could be generated from semiautomated selections using phage antibody

libraries (182–184). More recent studies have shown that phage display selections can be scaled up to target a larger number of antigens (29, 30, 123, 182, 183). For example, phage antibody selections were carried out on over 400 different antigens representing 292 proteins. In total, 25% of antibodies screened were positive, and 80% of these were specific when screened against irrelevant antigens (30). The practicality of generating antibodies against a broad array of different targets was further demonstrated in a recent multinational study in which antibodies against 20 different SH2 domains were generated via immunization and hybridoma technology or by phage display technology (31). The phage display selections were successful against all 20 targets and yielded at least ten unique binders for each target. Both phage display and hybridoma technologies produced many binders with low-nanomolar affinities. Antibodies were validated using a number of different assays, including microarrays, immunoblots, immunofluorescence, and immunoprecipitation. Overall, this study shows that antibodies with high affinity and specificity can be efficiently generated using high-throughput phage technologies.

Next-generation sequencing (NGS) technologies have been used to improve the characterization of mouse immunizations and phage display selections and to speed up the identification of antibodies. Several studies have utilized NGS for high-resolution analysis of natural (185) and synthetic (186) antibody repertoires. The 454 sequencing platform, which provides sequencing reads between 250 and 400 bases, was used to provide information on the diversity of CDRs and on VH and VL pairings (185, 187). The Illumina sequencing platform, which provides a higher number of shorter reads (~100 bases), was used to analyze the CDRH3 region of an scFv library (186). NGS platforms provide information on V-gene family frequency, CDR length and diversity, and a comparison of the theoretical and actual properties of the library (188). NGS has also been used to characterize how the immunoglobulin repertoire changes after immunization, where NGS was used to monitor enrichment in antigen specific V-genes (189).

For phage display selections, NGS was used to monitor enrichment of antibody sequences during successive rounds of selection (186). The information generated by NGS can be used to bypass antibody screening, which is time-consuming and expensive. For antibodies generated by mouse immunization, NGS was used to indentify heavy and light chains and pairings between them were inferred based on their frequencies in the repertoire (189). A similar strategy was used to identify antibodies from phage display selections (186). High frequency antibody fragments were identified following rounds of selection and desired antibody fragments were recovered by PCR (186). Identification of antibody sequences following selection eliminates the characterization of redundant clones. Further, it reduces the amount of target protein

required to perform and characterize antibodies from immunizations or phage selections. Lastly, NGS was used to characterize an antibody phage display selection against a protein target IL-6, expressed on the surface of *E. coli* (190). This study highlighted the potential for using NGS to characterize phage display selections against complex targets.

5. Conclusions and Future Perspectives

It is now well accepted in the scientific community that there is an urgent need to improve antibody quality in general, as an alarmingly high proportion of commercial antibodies either show poor specificity, or fail to even recognize their targets (191–194). At the same time, high-throughput genomics and proteomics technologies have vastly expanded the scope of proteins and pathways that now await detailed analysis at the cell biology level. To deal with the thousands of new proteins revealed by genomics and proteomics projects, there is an urgent need for high quality antibodies, and it is clear that the current hybridoma methods are not suitable for this task.

In this landscape, the emergence of high quality in vitro antibody libraries is both timely and opportune. Numerous studies have reported in vitro repertoires that routinely yield antibodies that rival or surpass hybridoma antibodies in terms of functionality. Moreover, while further improvements in hybridoma technology are likely to be slight, in vitro repertoires and selection methods continue to improve. Universal in vitro libraries that can provide antibodies against virtually any antigen are now a reality, and it is hoped that the technology can be broadly disseminated in the near future.

The recombinant nature of in vitro repertoires is a fundamental advantage that extends the technology beyond the scope of hybridoma technology. With synthetic antibodies in particular, frameworks can be chosen for favorable traits such as low immunogenicity or high stability, initial clones can be rapidly affinity matured and reformatted, and antibodies can be shared and distributed in the form of synthetic DNA. Furthermore, precise control over selection conditions allows for high precision engineering of specificity and affinity. Further standardization of libraries and selection methods will enable the adaptation of the technology to high-throughput pipelines to enable antibody generation on a proteome scale, and the ability to select directly against cells and tissues will further expand the scope of the technology. Clearly, in vitro antibody libraries are ideally suited for addressing the challenges of cell biology in the genomics era, and the technology is poised to play an ever-expanding role in the future of biological research.

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