

Beyond natural antibodies: the power of *in vitro* display technologies

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***In vitro* display technologies, best exemplified by phage and yeast display, were first described for the selection of antibodies some 20 years ago. Since then, many antibodies have been selected and improved upon using these methods. Although it is not widely recognized, many of the antibodies derived using *in vitro* display methods have properties that would be extremely difficult, if not impossible, to obtain by immunizing animals. The first antibodies derived using *in vitro* display methods are now in the clinic, with many more waiting in the wings. Unlike immunization, *in vitro* display permits the use of defined selection conditions and provides immediate availability of the sequence encoding the antibody. The amenability of *in vitro* display to high-throughput applications broadens the prospects for their wider use in basic and applied research.**

For the past 35 years, hybridoma technology has enhanced our capacity for research and diagnostics by providing monoclonal antibody reagents to track, detect and quantify target molecules in cells and serum. Recently, several more advanced methods to harness the immune response^{1–3} have substantially increased the number of antibody-producing cells that can be screened. In addition to these improvements in more traditional methods of making monoclonal antibodies, researchers can also benefit from refinements in *in vitro* display technologies. Although their merits do not seem to be fully appreciated across the broader research community, display technologies permit more control over the nature of the derived antibodies than immunization. Since the conception⁴ and first implementation⁵ of phage display, perhaps the most notable landmarks in the evolution of display technologies have been the expression of antibody fragments in bacteria⁶ and PCR-mediated amplification of antibody genes and libraries^{7–11}. The most popular technologies, phage^{8,12,13} and yeast display^{14,15}, which are complementary in their properties, can be used with naive, immunized or synthetic repertoires.

The advent of high-throughput biology has dramatically increased the demand for renewable, high-quality affinity reagents for use in proteome-scale experiments. Whereas further advances in animal immunization technologies are expected to be slim, *in vitro* methods have the potential to substantially improve the parallelization,

automation and miniaturization of antibody screens. Furthermore, a raft of recent papers^{16–22} point to an alarmingly high proportion of commercial antibodies demonstrating poor specificity, or even failing to recognize their targets at all. Given that much of modern biological research relies on the fidelity of commercially supplied antibodies, this underscores the importance of robust approaches to improve antibody quality. The high-throughput potential of *in vitro* technologies make them ideal platforms for large-scale projects to derive antibodies for all human proteins. Once completed, these initiatives are likely to have impacts that potentially rival the completion of the human genome.

Our primary goal here is not to reiterate the ways in which libraries used in display technologies are made or used. Instead, we discuss the scope of *in vitro* display technologies and what they have enabled, in particular in the context of distinguishing subtle differences in protein sequences and conformations or even minor changes in the chemical structures of small molecules. We hope to illustrate how *in vitro* display methods have yielded antibodies with remarkable properties, some of which have never been obtained by immunization. Most of the examples we discuss relate to antibody fragments. However, display technologies have allowed the development of nonantibody scaffolds. These also provide affinity reagents with similar, or in some applications, superior properties to those described here. Selection platforms^{23–25} and different scaffold proteins^{26–28}, including antibody fragments²⁹, have been reviewed elsewhere.

Unique features of display technologies

By permitting control over selection and screening conditions, display technologies allow the generation of antibodies against defined antigen conformations or epitopes (**Fig. 1**). For instance, the inclusion of competitors can direct selection toward specific targets. The use of variable regions from immunized sources with display technologies can also enable selection of specificities not detectable by traditional immunological techniques³⁰. Because the gene encoding the antibody is cloned at the same time as the antibody is selected, simple subcloning steps after *in vitro* display permit the creation of constructs with added functionalities (**Fig. 1**). Libraries of mutagenized variants can be created and the same selection process repeated to yield variants with superior specificity and affinity. The improvement of antibody affinity to picomolar levels^{31–35} has become relatively routine, with one study describing an antibody in the femtomolar range³⁶. These affinities are far higher than those of antibodies obtained by immunization, which are limited to ~100 pM by the physiological mechanism of B-cell activation^{37,38}. In addition, antibody specificities can be broadened or narrowed by appropriate selection and screening.

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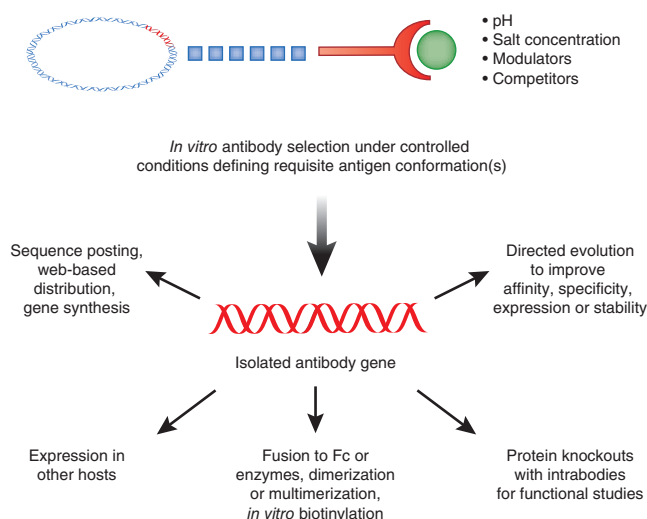
Figure 1 The unique capabilities of *in vitro* selection offer advantages over the immunization of animals for antibody generation. The direct coupling of the antibody and its encoding gene is characteristic of all display methodologies, including phage, yeast and ribosome display. Defined panning conditions with the desired buffer conditions, cofactors and competitors ensure that libraries can be screened using antigens with the desired conformation and biochemical properties to select for the requisite level of binder specificity and affinity. Binders can be selected sequentially, using different antigens to identify shared epitopes. The immediate availability of the antibody gene provides much additional value relative to antibodies obtained by immunizing animals.

As these *in vitro* methods are based on microbial systems, selection and screening are more amenable to automation than earlier hybridoma-based approaches. This provides the potential for high-throughput generation of binders^{39,40}. *In vitro* methods also overcome immunological tolerance, allowing the selection of affinity reagents that recognize highly conserved targets such as ubiquitin⁴¹, histones⁴², hemoglobins⁴³ and post-translational modifications^{44–46}. Several approaches are available for overcoming tolerance during immunization^{47,48}. However, none is required to select antibodies against conserved proteins using *in vitro* display methods. Remarkably, the selection of hundreds of different antibodies from naive human antibody repertoires against many different individual human targets has not been problematic^{39,49,50}.

Recognition of chemical modifications and small molecules

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. Binders capable of discerning such relatively simple chemical modifications are of great value in studying these effects. Monoclonal and polyclonal antibodies with specificities for small molecules have been obtained by traditional immunization^{51–55}. Even so, the ability of display methods to tailor both affinity and specificity has generated antibodies capable of discerning minor differences between related small molecules far better than those obtained by immunization (Table 1 and Fig. 2).

Although space constraints do not permit discussion of all of these studies, the use of phage display to study tyrosine sulfation warrants particular mention. The sulfation of tyrosine residues is a post-translational modification predicted to occur in 30% of all secretory and membrane



proteins⁵⁶. Despite decades of efforts involving immunization, it has proved impossible to generate antibodies recognizing sulfotyrosine using traditional means. This probably results from the innate tolerance of immune systems for such ubiquitous protein modifications, as well as the presence of the recognized target in the secretory pathway, resulting in retention and an inability to secrete the antibody. Using phage display, two groups recently selected antibodies recognizing proteins containing sulfotyrosine (but not tyrosine phosphate), independently of protein context or sequence^{45,46}. These antibodies recognized sulfotyrosinated proteins in western blot analysis, immunofluorescence, enzyme-linked immunosorbent assays (ELISA) and immunoprecipitation, and recognition could be abolished by sulfatase treatment or preincubation with free tyrosine sulfate. This represents an enormous advance in the analysis of this modification, which has traditionally required thin-layer chromatography of radiolabeled protein hydrolysates⁵⁷ or mass spectrometry⁵⁸, with the presence of sulfate groups often inferred, rather than proven. The ability to select antibodies with such subtle recognition properties is likely to be a boon in the development of novel materials, in which antibodies could be used to facilitate and detect chemical patterning.

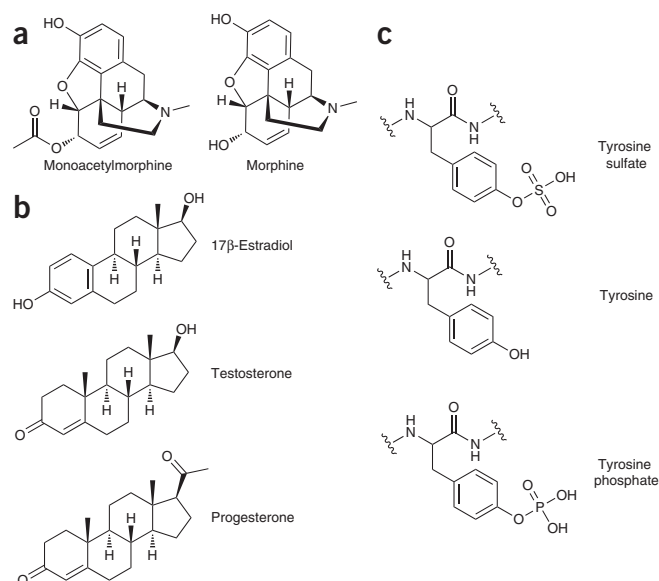
Recognition of subtle differences in proteins

In vitro display technologies allow the generation of antibodies against almost any target, including toxins, pathogens and antigens that are neither immunogenic nor highly conserved. With respect to protein targets, the exquisite specificity of the antibodies selected is exemplified by binders capable of differentiating, for example, as shown (see A.B. and collaborators⁵⁹), between chicken and quail lysozyme (which differ by a single surface amino acid) and the SH2 domains of ABL1 and ABL2 (refs. 60,61 and Table 2 (which have 89% sequence similarity)). Phage antibody libraries have been widely used to select antibodies against infectious agents. These include antibodies that discriminate between different strains of hantavirus⁶², dengue virus⁶³, influenza^{64,65}, Ebola⁶⁶ and Venezuelan equine encephalitis virus⁶⁷. Given that many of these viruses are classified serologically, the ability to select phage antibodies with similar specificities is not surprising. Nonetheless, unlike antibodies generated by immunization, these have the potential to be used therapeutically. Human antibodies, some of which are protective in animal models^{68–70}, have also been selected against a number of bacterial biothreat targets, including *Brucella melitensis*⁷¹, *Burkholderia mallei* and *Burkholderia pseudomallei*⁷², anthrax toxins^{68,73,74} and spores⁷⁵, and botulinum toxin^{31,76}.

Table 1 *In vitro* selected antibodies recognizing small molecules and modifications

Targets	Notes	Reference
6-monoacetylmorphine and morphine	Competition with morphine during panning to avoid cross-reactivity	154
Fluorescein	Affinity matured to 48 fM by yeast display	36
Testosterone, progesterone and 17 β -estradiol	Structurally similar steroid hormones with very different physiological effects	155–159
Sulfotyrosine as a post-translational modification	Antibodies recognize all sulfotyrosinated proteins and peptides	45,46
Sulfur mustard–modified keratin	Antibodies recognize skin affected by sulfur mustard	160
Fluorogenic dyes	Antibody binding increases dye fluorescence up to 15,000 times by limiting conformational movement	161
Metallic gold	Two-step selection strategy	162

Figure 2 *In vitro* selected antibodies can recognize minute differences in small molecules. (a) Antibodies against 6-monoacetylmorphine, the major heroin metabolite, do not recognize the closely related morphine¹⁵⁴. (b) Many different antibodies have been selected and subsequently had both affinity and specificity matured to specifically recognize 17 β -estradiol, testosterone and progesterone without cross-reacting with closely related steroids (Table 1). (c) Antibodies against proteins bearing sulfated tyrosine residues do not recognize proteins containing either tyrosine or tyrosine phosphate^{45,46}.



One library⁷⁰ was generated from military donors vaccinated against a plethora of different biothreat agents, reflecting the additional ability of display technologies to exploit antibodies generated during traditional immunization.

The *in vitro* nature of phage display technology has been exploited to target particular features of blood cells. In one study⁴³, antibodies recognizing fetal hemoglobin, but not adult hemoglobin, were selected by depleting high-affinity, cross-reactive antibodies followed by a selection against the fetal protein. Notably, the selected discriminatory antibody was of much lower affinity than cross-reactive antibodies, demonstrating the power of negative selections to favor clones with desirable binding specificities, even if their affinity is lower. Similar methods applied to cells have been used to select antibodies specifically recognizing fetal nucleated red blood cells⁷⁷.

Protein allostery is a common means for the regulation of protein function, and many signaling proteins exist in alternative conformational states that mediate different cellular responses. Antibodies that recognize specific protein conformers are powerful tools for probing the details of cell signaling (Table 2). However, the generation of such antibodies by immunization is complicated by the difficulty of maintaining a particular protein conformation in an immunized animal. Two ways in which *in vitro* selection technologies can address these limitations involve the use of negative selections to deplete nonspecific binders as well as those binders recognizing the undesired protein conformation, and then following these with affinity maturation strategies to fine-tune specificity. In one study, single-chain Fvs (scFvs) specific to the GTP-bound form of the small GTPase Rab6 were generated by performing selections against a GTP-locked mutant⁷⁸. In another study, one of our groups (S.S. and collaborators⁷⁹) used small molecules to covalently lock caspase-1 in either the active or inactive form and the locked antigens were used to select Fabs that

were highly selective for either the 'on' or 'off' form of the protease. The concept of using *in vitro* selections to generate conformation-specific antibodies has also been combined with selections on whole cells in a powerful strategy that enables the probing of cell surfaces for conformational changes in response to various stimuli⁸⁰.

Phage display has also generated antibodies able to recognize structured RNA molecules⁸¹, which are essentially nonimmunogenic, and not amenable to detection using simple nucleotide probes (Table 2). By ensuring a nuclease-free *in vitro* environment and selecting under conditions optimized for the structural stability of the RNA, high-affinity Fabs were isolated against a structured domain from the *Tetrahymena* group I intron. These results establish general methods applicable to the generation of antibodies against other structured RNAs and may be useful to decipher the biological roles of the vast numbers of noncoding RNAs found in metazoan transcriptomes.

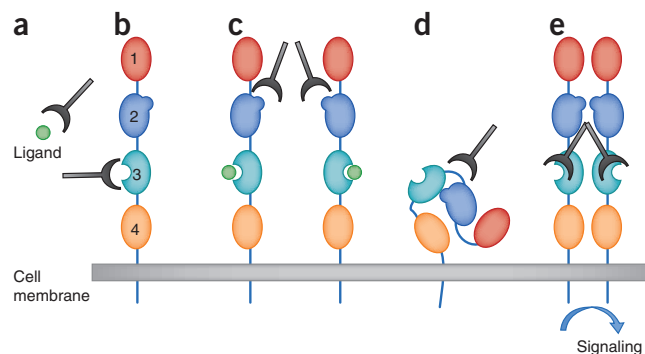
Recognition of cell surface receptors

Communication between cells is largely controlled by interactions between cell surface receptors and the ligands they recognize.

Table 2 *In vitro* selected antibodies recognizing protein sequences and conformations

Target	Notes	Reference
Peptide MHC complexes (similar to T-cell receptor recognition)	Similar antibodies obtained by immunization have lower affinities	163–168
Fibronectin splice variants, EDA and EDB	Selection directed toward recognition of both human and mouse variants, allowing same antibody to be used in both models and clinical studies	169–171
Fetal hemoglobin	Allow identification and potential purification of fetal red blood cells in maternal circulation	43
Fetal nucleated red cells		77
GTP-bound Rab6	Antibodies were used to track activated Rab6 in the cell as GFP fusions	78
Caspase 1	Antibodies recognize either the 'on' or 'off' forms	79
Integral membrane proteins	CitS from <i>Klebsiella pneumoniae</i> and KcsA from <i>Streptomyces lividans</i> . KcsA antibodies used as crystallization chaperones	172,173
RNA	Structured domain from <i>Tetrahymena</i> group I intron. Antibody used as crystallization chaperone	80
ABL1 versus ABL2	Differ by only 11%	60,61
Chicken versus quail lysozyme	Differ by only four amino acids, of which only one is surface exposed	59
MHC, major histocompatibility complex.		

Figure 3 Mechanisms for blocking or activating receptor signaling using antibodies. The EGF receptor, a single transmembrane domain with multiple extracellular domains (ovals) with different functional domains, is used to exemplify mechanisms by which antibodies can block signaling by different classes of receptor. In this example, binding of ligand (green circle) occurs at domain 3, receptor dimerization occurs through domain 2 and interactions between domains 2 and 4 stabilize the 'closed' conformation of the receptor. (a–d) Antibodies can block signaling by binding to the ligand and preventing interaction with receptor (a), binding the ligand-binding site of the receptor and preventing interaction with ligand (b), preventing dimerization by binding the dimerization domain or sterically blocking the interaction (c) or stabilizing the closed conformation of the receptor (d). (e) Activation can occur by binding the ligand-binding site typically with bivalent antibodies.



Antibodies can modify such interactions and many therapeutic antibodies exert their effects by interfering in communications at the cell surface using different mechanisms (Fig. 3 and Table 3). *In vitro* display technologies provide a powerful route to generating functional antibodies that interfere with normal or pathological extracellular signaling. Although it is usually difficult to select for function directly, display technologies have the ability to generate thousands of independent binders, each of which can then be screened for functional activity. For example, >1,200 different antibodies directed toward B-lymphocyte stimulator (BLyS) were generated by phage display⁸². This large panel was subsequently screened using biochemical and cellular assays to identify antibodies that bound to BLyS, preventing its interaction with the receptor (Fig. 3a) and thereby blocking B-cell activation. In some cases, blocking antibodies with subnanomolar affinities were isolated directly from the naive antibody–phage display library⁸². One of these antibodies, specific only for the secreted form of BLyS (Benlysta), was affinity matured⁸³ and is close to approval for treatment of systemic lupus erythematosus. Similar results have been reported for the selection of phage antibodies against a panel of 28 different, potentially therapeutic targets, with an average of 120 functionally active (that is, antagonistic or agonistic) antibodies selected per target⁵⁰.

An alternative strategy to block receptor signaling is to target the ligand-binding sites on the receptors, thereby blocking access to the natural ligand (Fig. 3b). This was used to select antibodies that prevent the interaction of insulin-like growth factor type 1 (IGF-1) with the IGF-1 receptor⁸⁴. Several groups of receptor binders were generated that competed with ligand binding and blocked cell growth *in vitro* and *in vivo*. These antibodies were also found to reduce receptor expression by internalization and catabolism.

Studies on a panel of therapeutic antibodies targeting the epidermal growth factor (EGF) receptor (ErbB1) have also shown competition with ligand binding. However, antibodies can also block receptor signaling by alternative mechanisms⁸⁵. The four extracellular domains of ErbB1 adopt a mainly closed conformation in the absence of ligand, and a more extended conformation (allowing dimerization and subsequent phosphorylation of the intracellular domain) in the presence of ligand. Structural studies have shown that whereas antibodies such as zalutumumab keep intracellular domains apart, preventing phosphorylation (Fig. 3c), cetuximab (Erbbitux) stabilizes the receptor in the closed conformation (Fig. 3d). Among the anti-ErbB2 antibodies, pertuzumab (Omnitarg) appears to work by preventing dimerization (Fig. 3c), whereas trastuzumab (Herceptin) prevents receptor shedding and

Table 3 *In vitro* selected antibodies recognizing cell surface receptors

Target	Notes	Reference
BLyS	Systemic lupus erythematosus	82
Tumor necrosis factor- α	Phage display was used to convert a murine monoclonal antibody into a human antibody by guided selection. Rheumatoid arthritis, ankylosing spondylitis, chronic plaque psoriasis and Crohn's disease, antibody developed by guided selection phage display	174,175
IGF-1 receptor	Blocking of ligand-binding site of receptor and receptor downregulation by endocytosis. Potential application in cancer	84
Notch	Prevent proteolysis of juxtamembrane NRR domain	86,87
Met	Dimeric antibodies are agonistic, monomeric ones are antagonistic and prospected for non-small cell lung cancer	88
MuSK	Agonistic antibodies demonstrate that MuSK activation is capable of triggering a key event in neuromuscular junction formation	176
CD40	Agonistic antibodies that activate normal human B cells suppress HIV-1 infection <i>in vitro</i>	177
Hemagglutinin	Antibodies recognize a previously unknown conserved conformational epitope. Isolated from both naive and immunized libraries	30,90,91
EphA2 and CD44	Selected from phage antibody library on yeast-displayed antigen, followed by selection for internalization on cells	94
CD166		178,179
ErbB2	Internalizing antibodies selected directly for internalization on cancer cells (CD166 on prostate, ErbB2 and transferrin	92
Transferrin receptor	receptor on breast, EGFR on A431). Antigen identified after selection. Potential utility for internalization of chemotherapeutics	92
EGFR		93
TRAIL-R	Over 500 different scFvs and Fabs isolated by phage display	89

NRR, negative regulatory region.

forms inactive tetramers⁸⁵. Although the original blocking antibodies in these examples were generated from mice, they demonstrate the therapeutic approaches that could benefit from human antibodies isolated directly from display technologies.

Antibodies that block Notch signaling reveal yet another mechanism of action. Following ligand binding, a conformational change occurs at the juxtamembrane negative regulatory region. This exposes a protease cleavage site, resulting in the release and translocation to the nucleus of the intracellular domain. In addition to generating antibodies that block the interaction with ligand, antibodies recognizing the negative regulatory region domains stabilized the 'closed' confirmation of the Notch receptor (Fig. 3d), preventing the proteolytic cleavage and translocation of the intracellular domain^{86,87}.

Dimeric antibodies targeting ligand-binding domains sometimes mimic the natural ligand, causing receptor activation rather than inhibition. This is the case for antibodies recognizing c-Met⁸⁸, with monomeric antibodies being antagonistic. Even so, in the case of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) (ref. 89), an analysis of >500 distinct selected antibodies revealed some that were agonistic even as monomeric scFvs or Fabs. This is difficult to reconcile with the mode of action of TRAIL, which is a homotrimeric ligand that causes multimerization of TRAIL receptors, leading to apoptosis particularly in tumor cells overexpressing the receptor.

Antibodies also have great potential in blocking protein interactions associated with viral entry into target cells, illustrated by antibodies selected from naive antibody libraries against recombinant hemagglutinin 5 (H5) influenza ectodomain^{90,91}. Structural analysis of one of the antibodies showed that it bound to hemagglutinin at a highly conserved, previously unrecognized pocket found in many different influenza viruses. Binding prevents the structural reorganization required for membrane fusion, rendering the antibody neutralizing. Although antibodies have not been generated against this epitope by traditional immunization or infection, antibodies with similar V_H gene usage and neutralizing activity have been selected from phage antibody libraries created from recently infected individuals³⁰, showing that phage display can access the diversity of immune responses in ways not possible by traditional immunological means.

In vitro selection schemes have also been devised that allow the direct selection of antibodies mediating internalization⁹². This was carried out by incubating phage libraries with target cells and isolating those phage antibodies found within the cell after removing phage antibodies bound to the cell surface. The recognized antigen is usually identified after selection. However, the use of mammalian cells transfected with the target of interest⁹³, or yeast displaying targets of interest on their surface⁹⁴, provides a means of carrying this out on predetermined targets. This approach is particularly suitable for the selection of antibodies used for specific targeting of chemotherapeutics^{95,96}.

In summary, antibodies and other binding molecules provide a means of modulating biological function by specifically interfering in protein interactions. *In vitro* display systems provide a means of presenting targets in appropriate conformations, including on cell surfaces. This facilitates rapid screening for potentially rare functional binders.

Table 4 Affinity and specificity maturation of antibodies by *in vitro* selection methods

Target	Notes	Reference
Affinity maturation		
HIV	CDRs targeted for mutation, 15pM affinity	35
c-erbB-2	CDRs targeted for mutation, 13pM affinity	34
Insulin	Ribosome display, random errors, 82pM affinity	33
Fluorescein	Affinity matured to 48 fM by yeast display	36
Modification of recognition specificities		
CXCL10 and CXCL9	Antibody selected against CXCL10 and evolved to also recognize CXCL9	102
VEGF and ErbB2	Antigens are completely unrelated, and antibody binds with 3 nM and 0.2 nM affinity to VEGF and ErbB2, respectively	103
Botulinum toxins A, B, E and F	One antibody able to recognize all botulinum types afflicting man was selected by yeast display	107

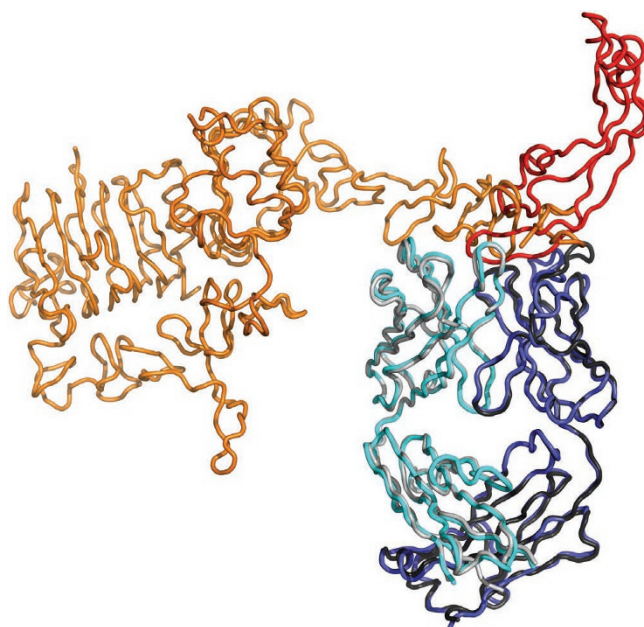
Improving antibody affinity and specificity

Although initial leads can be used directly as affinity reagents, a major advantage of *in vitro* methods is that it is possible to further improve function by constructing secondary libraries that introduce additional mutations. Secondary libraries are most commonly used to improve affinity, and all three major display formats (phage, yeast and ribosome) have been applied to develop affinities that exceed those possible with natural antibodies (Table 4). Both stepwise⁹⁷ and computational⁹⁸ methods generate similarly high-affinity antibodies, but they have not been used as widely as *in vitro* display methods. There are many examples of *in vitro* affinity maturation, and here we highlight some key studies. In ribosome display, each selection cycle involves a PCR-amplification step, which is ideal for introducing additional mutations by error-prone PCR. This strategy has been used to simultaneously select and affinity-mature anti-insulin antibodies with affinities in the subnanomolar range³³. Yeast-displayed libraries are smaller than phage and ribosome libraries, yet they allow quantitative and exhaustive screening by fluorescence-activated cell sorting. Coupled with sequential rounds of error-prone PCR, modest libraries of 10⁵–10⁷ unique clones were sufficient to affinity-mature an anti-fluorescein scFv to affinities <100 fM³⁶.

Antibody engineering efforts usually strive to obtain specificity for an antigen of interest. However, in certain applications, defined cross-reactivity is extremely useful. Species cross-reactivity allows better assessment of therapeutic efficacy and toxicity in animal models. Unfortunately, cross-reactive antibodies are often difficult to obtain by hybridoma methods owing to tolerance. In contrast, *in vitro* libraries are unaffected by immune tolerance and antibodies targeting conserved sites across species have proven to be more the rule than the exception. For highly conserved proteins, such as vascular endothelial growth factor (VEGF), human/mouse cross-reactive antibodies have been obtained directly from naive libraries^{99,100}. In the case of less conserved orthologs, such as BAFF/BLyS receptor 3 (BR3), initial anti-human antibodies with weak cross-reactivity to the mouse protein have been obtained from naive libraries and evolved to be highly cross-reactive¹⁰¹. Similar approaches have been used to generate antibodies recognizing two closely related chemokines (C-X-C motif ligand 10 and C-X-C motif 9)¹⁰², thereby permitting neutralization of two human chemokines with a single antibody.

Most attempts to engineer specificity involve improving on pre-existing weak recognition, due to homology between the recognized targets. In perhaps the most extreme case of engineered cross-reactivity, trastuzumab has been evolved to recognize a very different

Figure 4 An engineered dual specificity synthetic Fab. The bH1 Fab binds to both Her2 (orange, Protein Data Bank (PDB) ID: 3BDY) and VEGF (red, PDB ID: 3BE1). The heavy and light chains of the Fab are colored cyan/gray or blue/black respectively, with the different colors derived from structures of bH1 binding to either Her2 or BEGF.



protein, vascular endothelial growth factor (VEGF), as well as its original target, ErbB2 (ref. 103). After considerable evolution, the affinities for both targets were comparable to those of therapeutic antibodies ($K_d = 3/0.2$ nM for VEGF/ErbB2). The antibody inhibited both VEGF and ErbB2-mediated cell proliferation *in vitro* and tumor progression in mouse models. The structures of the bispecific Fab in complex with ErbB2 or VEGF revealed a common paratope, with the ErbB2 functional paratope located predominantly on V_H , and that for VEGF on V_L (Fig. 4). The ability to design antigen-binding sites with dual specificity against structurally unrelated antigens may be important in therapeutic strategies targeting two distinct signaling pathways with a single antibody.

The ability to improve affinity and broaden specificity also has major implications for the development of antibodies against pathogens. For the effective inhibition of viral infection and bacterial toxins, antibodies would ideally recognize a variety of antigen subtypes with high affinity, to afford broad protection against pathogen variants. Furthermore, several studies have shown that multiple antibodies targeting distinct epitopes provide synergistic effects necessary for effective neutralization of pathogens^{104,105}. *In vitro* antibody technologies provide an effective means for achieving these demanding criteria, as exemplified by a long-term study of neutralizing antibodies against the botulinum neurotoxin. Phage antibody libraries from immunized mice and humans resulted in the isolation of three antibodies recognizing nonoverlapping epitopes on botulinum neurotoxin¹⁰⁶. The use of these three antibodies together as an oligoclonal IgG provided strong synergy that dramatically increased toxin neutralization. A long series of affinity and specificity maturation cycles using yeast display resulted in the final development of a remarkable antibody able to recognize botulinum toxins A, B, E and F, the four serotypes afflicting humans^{107,108}.

Exploiting the recombinant nature of antibodies selected *in vitro*

All *in vitro* selection systems immediately provide the genes and corresponding sequences of antibodies selected against a particular target. This provides ready access to additional antibody formats by simple subcloning. Functions adopted using this 'gene-based' approach include dimerization¹⁰⁹, multimerization^{110,111} and fusions to enzymes¹¹², tags¹¹³ or fluorescent proteins¹¹⁴ (Fig. 1). Fusion to alkaline phosphatase is a particularly useful example of improved functionality. As this is a dimeric enzyme, fusing antibodies, either individually or as libraries, to alkaline phosphatase simultaneously provides dimerization and alkaline phosphatase activity, greatly facilitating screening by increasing the effective affinity and avoiding the need for secondary reagents^{39,112}. Short peptides acting as *in vivo* biotinylation tags¹¹³, placed at the C terminus of antibody fragments, allow stoichiometrically defined, site-specific antibody biotinylation, as well as straightforward multimerization¹¹⁵. Antibody fragments can also be transformed into full-length antibodies¹¹⁶, or scFv-Fc fusions, which are very similar in many aspects¹¹⁷. The use of engineered Fc regions can result in improved pharmacokinetics and effector functions (for reviews, see refs. 118,119), including bispecific IgG, in which engineering of two different Fc regions allows only heterologous pairing^{120,121}.

Other approaches to generate bispecific antibodies build upon the observation that some scFv fragments form bivalent dimers

(diabodies)¹²², trimers^{123,124} and even tetramers¹²⁵ when the V_H/V_L linker is shortened. Additional bispecific antibody designs are reviewed elsewhere¹²⁶. Even more radically, completely novel biochemical entities have been added to antigen-binding fragments. Fusions of scFv and Fab fragments to heterologous proteins, such as interleukins and cytokines^{127,128}, apoptotic ligands, enzymes, toxins or RNases (see refs. 129,130 for reviews) have allowed novel therapeutic paradigms. Many of the above candidate therapeutic antibody constructs arose from antibody genes initially isolated from mouse hybridomas, but this is expected to change as more human antibodies are made available from engineered repertoires.

Microinjected antibodies have long been used to knock out intracellular functions¹³¹. Antibody fragments can be expressed within target cells and targeted to various subcellular compartments^{116,132} by adding suitable signal sequences, allowing visualization or functional modification of proteins in different compartments. Removing the standard leader sequence results in cytoplasmic expression, whereas the addition of a nuclear localization signal results in antibodies being translocated to the nucleus. The combination of a leader sequence and the endoplasmic reticulum (ER) retention sequence retains expressed antibodies in the ER and has been used to prevent the expression of membrane proteins by sequestration in the ER. These include human interleukin 2 receptor, the ErbB2 receptor, β -amyloid precursor protein, vascular adhesion molecule 1 and many others^{133–136}. The advantage of this strategy is that it requires antibodies that bind to any accessible epitope to provide the functional knockout, as opposed to the functional activity required of cytoplasmically expressed antibodies. Functional studies of membrane receptors or secreted proteins can thus be attempted by a single standardized subcloning step immediately after *in vitro* antibody selection, providing equivalence to RNA interference knockdowns at the protein level.

Although expression in the secretory pathway is straightforward, folding of antibody fragments in the cytoplasm is far more challenging, owing to the reducing environment and the absence of specific chaperones, which prevents disulfide bond formation¹³⁷. Despite these problems, there are examples where cytoplasmic proteins have been targeted with intracellular scFvs^{78,138}. The success of this approach has been improved by the creation of libraries of particularly stable scFvs^{139–141}, preselecting antibodies for functional cytoplasmic expression^{142,143}, or by using binder libraries based on

molecular scaffolds that do not rely on disulfide bond formation, such as engineered ankyrin repeat proteins^{144,145}. An important advantage of using such protein-based allosteric blockers is their ability to generate very specific binders capable of distinguishing between closely related family members. Although the need to genetically modify the target cell is a major disadvantage, this has been partly alleviated by fusion to internalizing sequences that allow antibodies to enter the cell from the outside¹⁴⁶.

High-throughput selection by *in vitro* display methods

The ease with which antibodies can be selected, screened and produced by *in vitro* display technologies enables simpler and faster generation and screening of antibodies than with hybridomas. Typically, a panel of ELISA-positive monoclonal antibody fragments can be generated within 2 weeks. Early experiments demonstrated the feasibility of semi-automated selection and/or screening of phage antibody libraries^{147–149} on small numbers of targets. More recently, selections on >400 different antigens were successful, with 54% of bacterially produced and 88% of mammalian-produced antigens³⁹ yielding antibodies. The differences between the two protein classes probably arise from differences in the levels of correct folding.

In a recent international comparative study, antibodies were raised to 20 different human Src-homology 2 (SH2) domains using hybridoma or phage display. Results from two of the participating phage display laboratories^{60,61} show that antibodies (some with sub-nanomolar affinities) were generated against all antigens, with 55% of positive antibodies specific for target SH2 domains when assessed against the entire SH2 panel. These antibodies were validated in a broad range of assays, including microarray analysis, immunoblotting, immunofluorescence and immunoprecipitation.

Prospects

If antibodies selected by *in vitro* methods are so powerful, why are they not more widely perceived as valuable research reagents? Part of the answer lies in the difficult patent situation, which resulted in restriction of this technology to the high-margin therapeutic markets for commercial use. It is worth noting in this regard that hybridoma technology was never patented, and achieved relatively wide acceptance within a short period. The situation for some of the core phage-display patents is now changing rapidly, as most platform patents have either expired or will do so over the next few years¹⁵⁰. The technology may become more widely disseminated as a result.

Although largely unrecognized by the research community, it is worth emphasizing that some commercial 'monoclonal antibodies' are actually recombinant antibodies selected by phage display and then reformatted to look like traditional murine antibodies by the fusion of Fc regions to human variable regions (e.g., the sulfotyrosine antibody described above⁴⁵). Indeed, unmodified recombinant Fab fragments selected by phage display are commercially available. It therefore seems that the most important impediments to widespread adoption are a lack of knowledge of the capabilities of this technology, coupled with limited expertise and library availability. Furthermore, the number of companies willing to carry out *in vitro* selection for a fee is vanishingly small compared with the 180 companies willing to generate antibodies by immunization¹⁶.

Another explanation of why the strength of display technologies appears to be underappreciated relates to the difficulties encountered in expressing many of these antibodies. Although some of the specificities described above are remarkable, the expression and stability of antibody fragments varies enormously—from exceptionally stable scFv fragments used in clinical trials¹⁵¹ to other fragments with extremely low expression

levels. A typical selection almost always generates several different binders to any well-folded antigen. Among these, usually at least one is sufficiently stable and well produced for research use. Furthermore, it is expected that stability and expression levels will improve as libraries are based on more stable scaffolds¹⁵². The studies described above indicate that this goal can now be met in highly parallelized screening setups with low effort per antigen^{60,61}, provided that libraries of sufficient diversity and optimized protocols are used. Furthermore, stability and expression screening can be easily included as part of the high-throughput screening process. An additional issue with antibodies derived *in vitro* is that they are either not glycosylated if expressed in bacteria, or incorrectly glycosylated if expressed in standard yeast strains. If correct glycosylation is necessary, this can be overcome by expression in human cells or yeast modified to give human glycosylation patterns¹⁵³.

Once an antibody is generated, it can be defined precisely by sequence and even 'distributed' in this way. Gene synthesis is progressing at a remarkable pace, with the cost-per-base of synthesized genes falling dramatically. In fact, genes corresponding to the sequences of specific antibody fragments can now be synthesized for less than the cost of purchase of some antibodies from traditional vendors.

The present state of this field can be compared to the situation for sequencing technologies at the start of the human genome project. Just as enormous technical advances occurred in the human genome project once it was started and rigorous industrial processes were applied, so we anticipate dramatic improvement in all aspects of selection, screening, downstream use and distribution of affinity reagents derived *in vitro* once a proteome-scale project is initiated and financed. In summary, *in vitro* display technologies permit the facile generation of antibodies by providing access to billions of potential binders in large 'universal' or immune display libraries. The technologies facilitate production, screening and maturation of selected binders, allowing selection on target conformations and formats not possible by more traditional routes based on immunization. Furthermore, the easy availability of the gene sequence not only provides a definitive description of the product but also allows electronic sharing and re-creation of the binding molecule through gene synthesis. The last 20 years have witnessed the successful application of display technologies to the development of therapeutic antibody candidates. In the coming decade we expect to also see increased realization of the benefits of this technology within the research and diagnostic markets.

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