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

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Abstract

In vitro display technologies, best exemplified by phage and yeast display, were first described for the selection of antibodies some twenty years ago. Since that time a large number of antibodies, some with remarkable properties, have been selected and improved upon using these methods. The first antibodies derived using *in vitro* display methods are now in the clinic, with many more waiting in the wings. Here we discuss the scope of the technology, some of the powerful antibodies selected, and the future potential in a post-genomic world. Unique advantages offered by *in vitro* display technologies include the ability to carefully define selection conditions, allowing the derivation of antibodies recognizing predefined epitopes or conformations, the further improvement of selected antibodies, the potential for high throughput applications and the immediate availability of genes encoding the selected antibody. We anticipate that the high throughput potential of these technologies will soon lead to their use to select antibodies against all human proteins.

Introduction

For the past 35 years, hybridoma technology has enhanced our capacity for research and diagnostics by providing monoclonal antibody reagents allowing tracking, detection and quantitation of target molecules in cells and serum. Recently, a number of more advanced methods to harness the immune response have also been developed[1,2,3] that significantly increase the number of antibody producing cells that can be screened. Alongside these "traditional" method of making monoclonal antibodies, a quiet revolution has been brewing in the generation of antibodies using *in vitro* display technologies, which offer a number of advantages, including a greater degree of control over the nature of the derived antibodies. The success of these technologies has relied upon many previous advances, including the conception and implementation of phage display[4,5], the expression of antibody fragments in bacteria[6] and PCR-mediated amplification of antibody genes and libraries[7,8,9,10,11]. The most popular technologies, antibody phage[8,12,13] and yeast display[14,15], which are complementary in their properties, can be used with naïve, immunized or synthetic repertoires.

As a direct consequence of genome sequencing, and the advent of high throughput biology, the demand for large numbers of renewable high quality affinity reagents, recognizing ever-greater numbers of proteins, for affinity reagent based proteomic scale experiments, is expected to increase dramatically. *In vitro* methods have the potential to deliver enormous improvements from parallelization, automation and miniaturization. In contrast, further advances in animal immunization technologies are expected to be slim. Furthermore, it is generally accepted that, irrespective of the source, there is an urgent need to improve antibody quality, as reflected by a raft of recent papers[16,17,18,19,20,21,22] showing 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, there is an urgent need to resolve this problem. The high throughput potential of *in vitro* technologies make them ideal platforms for large scale projects to derive antibodies for all human proteins, which once completed are likely to have impacts perhaps as great as the completion of the human genome.

By carefully controlling selection and screening conditions, display technologies allow the generation of antibodies to defined antigen conformations or epitopes, for example, by the presentation of specific antigen conformations, or the inclusion of competitors to direct selection towards specific targets or epitopes (figure 1). Moreover, when variable regions from immunized sources are used with display technologies, specificities not detectable by traditional immunological techniques can often be selected[23]. During the process of *in vitro* antibody selection, the gene encoding the antibody is cloned at the same time as the antibody is selected, providing many advantages to the recombinant approach (Fig. 1). The availability of the antibody gene allows the creation of alternative constructs with added functionality by simple subcloning (see below). Libraries of mutagenized variants can be created and the same selection process repeated to yield variants that are improved, both in terms of specificity and affinity. The improvement of antibody affinity to picomolar levels[24,25,26,27,28] has become relatively routine, with one study describing an antibody in the femtomolar range[29]. 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[30,31]. In addition, antibody specificities can be broadened or narrowed by appropriate selection and screening.

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 binder generation[32,33]. *In vitro* methods also overcome immunological tolerance, allowing the selection of affinity reagents that recognize highly conserved targets such as ubiquitin[34], histones[35], hemoglobins[36] and post-translational modifications[37,38,39]. While tricks can be used to overcome tolerance during immunization[40,41], none are required to select antibodies against conserved proteins using *in vitro* display methods. Remarkably, the selection of hundreds of different antibodies from naïve human antibody repertoires against many different individual human targets has not been problematic[32,42,43].

Most of the examples described below relate to antibody fragments. However, display technologies have allowed the development of alternative, non-antibody scaffolds and they

too will provide affinity reagents with similar, or in some applications, superior properties to those described here. Selection platforms[44,45,46] and different scaffold proteins[47,48,49], including antibody fragments[50], have been widely dealt with in previous reviews. Our goal here is not to reiterate the ways in which such libraries are made or used, but to illustrate how *in vitro* display methods have yielded antibodies with remarkable properties some of which have rarely, if ever, been obtained by immunization. This will be carried out by describing a number of different classes of unique and interesting antibodies, as well as outlining the enormous advantages provided by immediate access to cloned antibody genes.

Recognition of chemical modifications and small molecules

Monoclonal and polyclonal antibodies with specificities for small molecules have been obtained by traditional immunization[51,52,53,54,55]. However, the ability of display methods to tailor both affinity and specificity has led to significantly better antibodies than can be obtained by immunization (Table 1 and Fig. 2).

Sulfotyrosine is a post-translational modification (PTM) predicted to occur in 30% of all secretory and membrane proteins[65]. Despite decades of immunization, it has proved impossible to generate antibodies recognizing this PTM by traditional means. This is probably due to the innate tolerance immune systems have 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[38,39]. These antibodies recognized sulfotyrosinated proteins in western blotting, immunofluorescence, 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 hydrolysates[66] or mass spectrometry (MS)[67], with the presence of sulfate groups often inferred, rather than proven.

Specificity for protein sequences and conformations

Phage display allows the generation of antibodies against nearly any target, including toxins, pathogens, non-immunogenic, or highly conserved antigens. With respect to protein targets, antibodies have been selected with exquisite specificity, differentiating, for example, between chicken and quail lysozyme[68] that differ by a single surface amino acid, and the SH2 domains of ABL1 and ABL2[69,70]. Phage antibody libraries have been widely used to select antibodies against infectious agents. These include antibodies that discriminate between different strains of Hantavirus[71], Dengue virus[72], influenza[73,74], Ebola[75] and Venezuelan equine encephalitis virus[76]. Given that many of these viruses are classified serologically, the ability to select phage antibodies with similar specificities is not surprising, but unlike antibodies generated by immunization, these have the potential to be used therapeutically. Human antibodies, some of which are protective in animal models[77,78,79], have also been selected against a number of bacterial biothreat targets, including *Brucella melitensis*[80], *Burkholderia mallei* and *Burkholderia pseudomallei*[81],

anthrax toxins[77,82,83] and spores[84], and Botulinum toxin[24,85]. One library[79] was generated from servicemen 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 study[36], 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 cells[95].

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 proteins conformers are powerful tools for probing the details of cell signaling. However, the generation of such antibodies by immunization is complicated by the difficulty of maintaining a particular protein conformation in an immunized animal. In contrast, *in vitro* selection technologies are ideally suited for these applications. Negative selections can be used to deplete non-specific binders, and affinity maturation strategies can be employed to fine-tune specificity. In one study, scFvs specific to the GTP-bound form of the small guanosine triphosphatase (GTPase) Rab6 were generated by performing selections against a GTP-locked mutant[96]. In another study[97], small molecules were used 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 the cell surfaces for conformational changes in response to various stimuli[101].

Finally, phage display has enabled the generation of antibodies recognizing *structured* RNA molecules[100], which are essentially non-immunogenic, and not as amenable to simple nucleotide probes. 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 hold great promise, as they establish general methods applicable to the generation of antibodies against other structured RNAs, and will be useful to decipher the biological roles of the vast numbers of noncoding RNAs found in metazoan transcriptomes.

Antibodies to cell surface receptors

Communication between cells is driven and controlled by interactions between cell surface receptors and the ligands they recognize. Antibodies can modify such interactions and many therapeutic antibodies exert their effect by interfering in communications at the cell surface using different mechanisms (Fig. 3). *In vitro* display technologies provide a powerful route

to generating functional antibodies that interfere in 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, over 1200 different antibodies directed towards B Lymphocyte Stimulator (BlyS) were generated by phage display[102]. This large panel was subsequently screened in 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 sub-nanomolar affinities were isolated directly from the naïve antibody-phage display library[102]. One of these antibodies, specific only for the secreted form of BlyS (BENLYSTA), was affinity matured[103] 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 (i.e. antagonistic or agonistic) antibodies selected *per target*[43].

An alternative strategy to block receptor signaling is to target the ligand binding sites on the receptors, thereby preventing the natural ligand from acting (Fig. 3b). This was used in a recent study, where antibodies were selected that prevented the interaction of insulin-like growth factor type 1 (IGF-1) with the IGF-1 receptor[106]. 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 EGF receptor (Erb-B1) have also shown competition with ligand binding. However, antibodies can also block receptor signaling by alternative mechanisms[120]. Erb-B1 has four extracellular domains, which 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 antibodies such as cetuximab stabilize the receptor in the closed conformation (Fig. 3d) while zalutumumab keeps intracellular domains apart preventing phosphorylation (Fig. 3c). Among the anti-Erb-B2 antibodies, Pertuzumab appears to work by preventing dimerization while trastuzumab (Herceptin) prevents receptor shedding and forms inactive tetramers[120]. While 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 juxta-membrane negative regulatory region (NRR) exposing 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 NRR domains stabilized the “closed” confirmation of the Notch receptor (Fig. 3d) preventing the proteolytic cleavage and translocation of the intracellular domain[107,108].

Dimeric antibodies targeting ligand-binding domains sometimes mimic the natural ligand, causing receptor activation rather than inhibition. This is the case for antibodies recognizing Met[109], with monomeric antibodies being antagonistic. However, in the case of TRAIL receptor 1 (TRAIL-R1) and TRAIL receptor 2 (TRAIL-R2)[119], an analysis of over 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 the TRAIL receptor leading to apoptosis, particularly in tumor cells over-expressing the receptor.

Antibodies also have great potential in blocking protein interactions associated with viral entry into target cells, illustrated by antibodies selected from naïve antibody libraries against recombinant H5 hemagglutinin influenza ectodomain[112,113]. Structural analysis of one of the antibodies showed 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 VH gene usage and neutralizing activity have been selected from phage antibody libraries created from recently infected individuals[23], 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 internalization[117]. 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 identification of the recognized antigen is usually carried out after selection. However, the use of mammalian cells transfected with the target of interest [118], or yeast displaying targets of interest on their surface[114], 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[121,122].

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, which facilitate rapid screening for potentially rare functional binders.

Improving antibody affinity and specificity

While 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. The most prevalent application of secondary libraries is the improvement of affinity, and all three major display formats (phage, yeast and ribosome) have been applied to develop extremely tight affinities that exceed those possible with natural antibodies (Table 4). Both stepwise[123] and computational[124] methods have also been developed that are able to generate similarly high affinity antibodies, but they have not been as widely used as the *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

cycles 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 sub-nanomolar range[26]. While yeast-displayed libraries are smaller than phage and ribosome libraries, they allow quantitative and exhaustive screening by fluorescence activated cell sorting (FACS). Coupled with sequential rounds of error-prone PCR, modest libraries of 10^5–10^7 unique clones were sufficient to affinity mature an anti-fluoroscein scFv into the low femtomolar range[29].

Specificity for a single antigen is generally the goal of antibody engineering. 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, due to tolerance. In contrast, *in vitro* libraries are unaffected by immune tolerance and antibodies targeting conserved sites across species have proven to be the rule rather than the exception. In the case of highly conserved proteins, such as vascular endothelial growth factor (VEGF), human/mouse cross-reactive antibodies have been obtained directly from naïve libraries[128],[129]. 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 naïve libraries and evolved to be highly cross-reactive[130]. Similar approaches have been used to generate antibodies recognizing two closely related chemokines (CXLC10 and CXCL9)[125] thereby permitting neutralization of 2 human chemokines with a single antibody.

Most specificity engineering examples involve the improvement of pre-existing weak recognition, due to homology between the recognized targets. In perhaps the most extreme case of engineered cross-reactivity, Herceptin has been evolved to recognize a very different protein, VEGF, as well as its original target, Erb-B2[126]. After significant evolution the affinities for both targets were comparable to those of therapeutic antibodies (K_d = 3/0.2 nM for VEGF/Erb-B2). The antibody inhibited both VEGF and Erb-B2-mediated cell proliferation *in vitro* and tumor progression in mouse models. The structures of the bispecific Fab in complex with Erb-B2 or VEGF revealed a common paratope, with the Erb-B2 functional paratope located predominantly on VH, and that for VEGF on VL (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[131],[132]. *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 (BoNT). Phage antibody libraries from immunized mice and humans resulted in the isolation of three antibodies recognizing non-overlapping epitopes on BoNT[133]. 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, *all* the serotypes afflicting man[127].

Exploiting the recombinant nature of in vitro selected antibodies

All *in vitro* selection systems immediately provide the genes, and corresponding sequences, of antibodies selected against a particular target, providing ready access to additional antibody formats by simple sub-cloning. Functions adopted using this "gene-based" approach include dimerization[134], multimerization[135],[136], and fusions to enzymes[137], tags[138] or fluorescent proteins[139]. 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[32],[137]. Short peptides acting as *in vivo* biotinylation tags[138], placed at the carboxy terminus of antibody fragments, allow stoichiometrically defined site specific antibody biotinylation, as well as a straightforward multimerization method[140]. Antibody fragments can also be transformed into full-length antibodies[141], or scFv-Fc fusions, which are very similar in many aspects[142]. The use of engineered Fc regions can result in improved pharmacokinetics and effector functions (for reviews see [143],[144]), including bispecific IgG, in which engineering of two different Fc regions allow only heterologous pairing[145],[146].

Other approaches to generate bispecific antibodies build upon the observation that some scFv fragments form bivalent dimers (diabodies)[147], trimers[148],[149] and even tetramers[150] when the VH/VL linker is shortened. Various other bispecific antibody designs have also been created (see [151] for a review). 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[152],[153], apoptotic ligands, enzymes, toxins or RNases (see [154],[155] 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 been long used to knock out intracellular functions[156]. Antibody fragments can be expressed within target cells and targeted to various subcellular compartments[141],[157] by adding suitable signal sequences, allowing visualization or functional modification of proteins in different compartments. Removing the standard leader sequence results in cytoplasmic expression while the addition of a nuclear localization signal targets 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 ErbB-2 receptor, s-amyloid precursor protein, vascular adhesion molecule 1 and many others[158],[159],[160],[161]. 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 RNAi knockdowns at the protein level.

While expression in the secretory pathway is straightforward, folding of antibody fragments in the cytoplasm is far more challenging, due to the absence of specific chaperones, and the reducing environment, which prevents disulfide bond formation[162]. Despite these problems, there are examples where cytoplasmic proteins have been targeted with intracellular scFvs[96,163]. The success of this approach has been improved by the creation of libraries of particularly stable scFvs[164,165,166], preselecting antibodies for functional cytoplasmic expression[167,168], or by using binder libraries based on molecular scaffolds that do not rely on disulphide bond formation, such as engineered ankyrin repeat proteins[169,170]. One major advantage of such protein based allosteric blockers is the ability to generate very specific binders, able to distinguish between closely related family members. While 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 outside[171].

High throughput selection by in vitro display methods

The ease with which antibodies can be selected, screened and produced by *in vitro* display technologies, makes generation and screening of antibodies rapid and simple compared to hybridomas. Typically a panel of ELISA positive monoclonal antibody fragments can be generated within two weeks. Early experiments demonstrated the feasibility of semi-automated selection/screening of phage antibody libraries[172,173,174] on small numbers of targets. More recently, selections on over 400 different antigens were successful with 54% of bacterial, and 88% of mammalian-produced antigens[32] yielding antibodies, with the differences between the two protein classes probably due to the levels of correct folding. In a recent international comparative study antibodies were raised to 20 different human SH2 domains using hybridoma or phage display. Results from two of the participating phage display labs[69,70], show that antibodies (some with sub-nanomolar affinities) were generated to 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 microarrays, immunoblots, immunofluorescence and immunoprecipitation.

The future vision of affinity reagents generated by display technologies

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 perhaps significant 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 in the process of rapid change as most platform patents have either expired, or will do so over the next few years[175], and it is possible that the technology will become more widely disseminated as a result.

Largely unnoticed by the research community, some commercial “monoclonal antibodies” are actually recombinant antibodies selected by phage display, reformatted to look like traditional murine antibodies by the fusion of Fc regions to human variable regions (e.g. the sulfotyrosine antibody described above[38]). ABDSerotec also sells a number of unmodified recombinant Fab fragments selected by phage display. 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 as a fee for service is vanishingly small compared to the 180 companies willing to generate antibodies by immunization[16].

However, one commonly cited issue relates to the ability to express these antibodies. While the specificities obtained, and described herein, are remarkable, the expression and stability of antibody fragments varies enormously, from exceptionally stable scFv fragments used in clinical trials[176] to other fragments that are poorly expressed. A typical selection almost always generates a number of different binders to any well-folded antigen. Among these there is usually at least one that 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 scaffolds[177]. The studies described above indicate that this goal can now be met in highly parallelized screening setups with low effort per antigen[69],[70], provided that libraries of sufficient diversity and optimized protocols are used. Furthermore, stability and expression screening can be easily included as part of the HT screening process. An additional issue with *in vitro* derived antibodies 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 patterns[178].

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 with 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 *in vitro* derived affinity reagents once a proteomic 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 recreation of the binding molecule through gene synthesis. Over the last 20

years display technologies have been applied successfully to the development of therapeutic antibody candidates. In the coming decade we expect to see increased realization of the benefits of this technology within the research and diagnostic markets as well.

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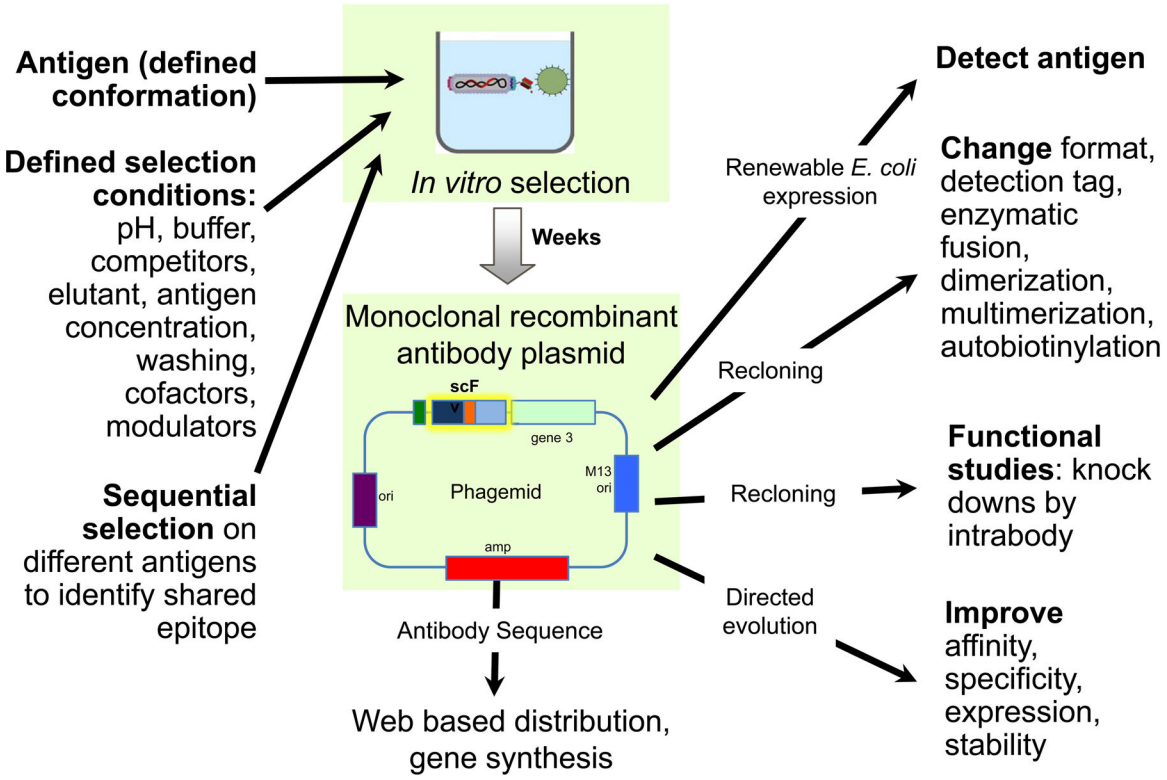

Figure 1.

The additional capabilities of *in vitro* selection offer a new approach to antibody generation, allowing the selection of antibodies with particular properties by predefining panning conditions. Variations in salt and pH conditions, the conformational form of the target and the presence of closely related proteins help determine the biochemical properties, fine specificity, cross-reactivities and affinity of resulting binders. Further, the immediate availability of the antibody gene provides significant additional value. Complemented by a more rapid antibody generation cycle, this will broadly change the manner in which antibodies will be made and used for research in the near future.

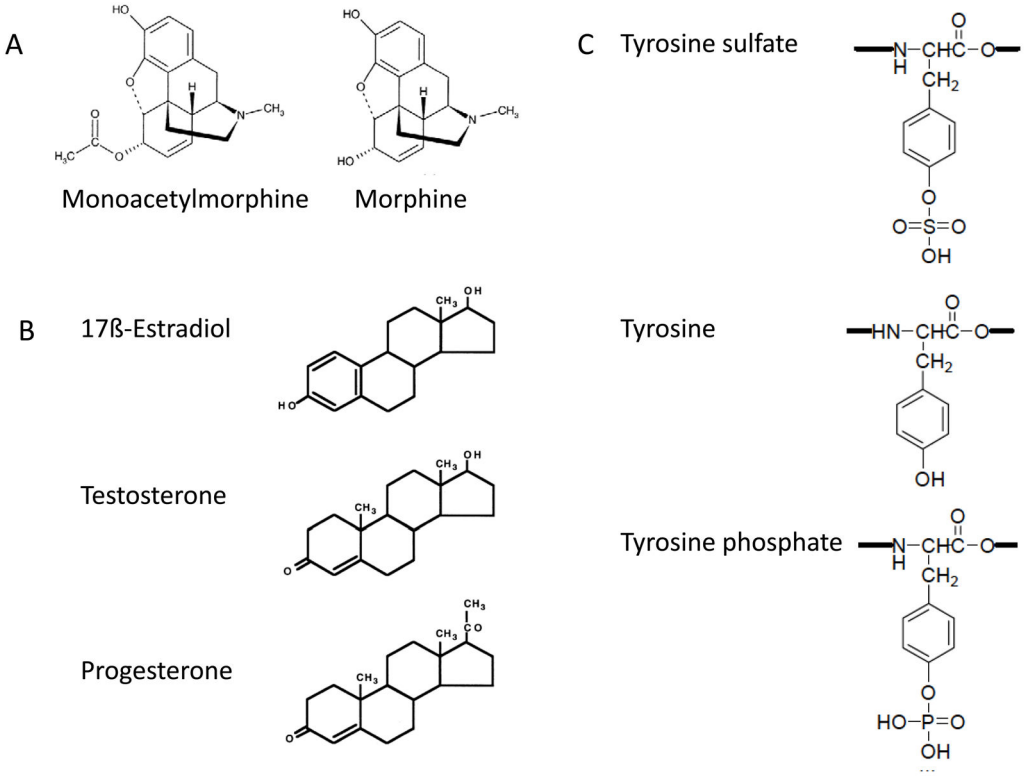

Figure 2.

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 morphine[56]. B) Many different antibodies have been selected and subsequently had both affinity and specificity matured to recognize each of the represented steroids (for references see Table 1). C) Antibodies against tyrosine sulfated modified proteins do not recognize proteins containing either tyrosine or tyrosine phosphate[38,39].

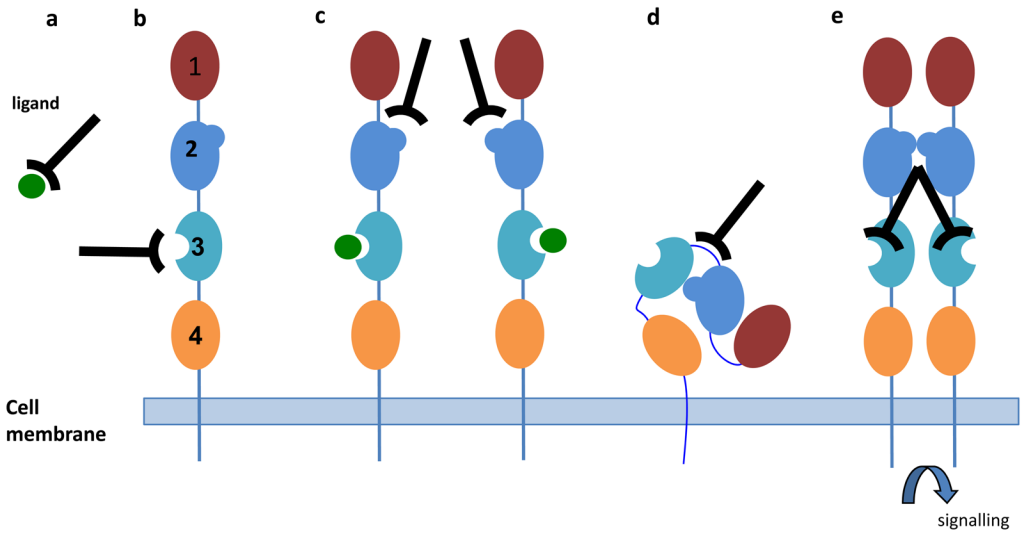

Figure 3.

Mechanisms for blocking or activating receptor signaling using antibodies. The EGF receptor is used to exemplify mechanisms by which antibodies can block signaling in different classes of receptor. The EGF receptor is a single trans-membrane domain with multiple extra-cellular domains (represented as different colored ovals) having different functional domains. 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. Antibodies can block signaling by **a**. binding to the ligand and preventing interaction with receptor, **b**. binding the ligand binding site of the receptor and preventing interaction with ligand, **c.** preventing dimerization by binding the dimerization domain or sterically blocking the interaction **d**. stabilising the closed conformation of the receptor. **e**. Activation can occur by binding the ligand-binding site typically with bivalent antibodies.

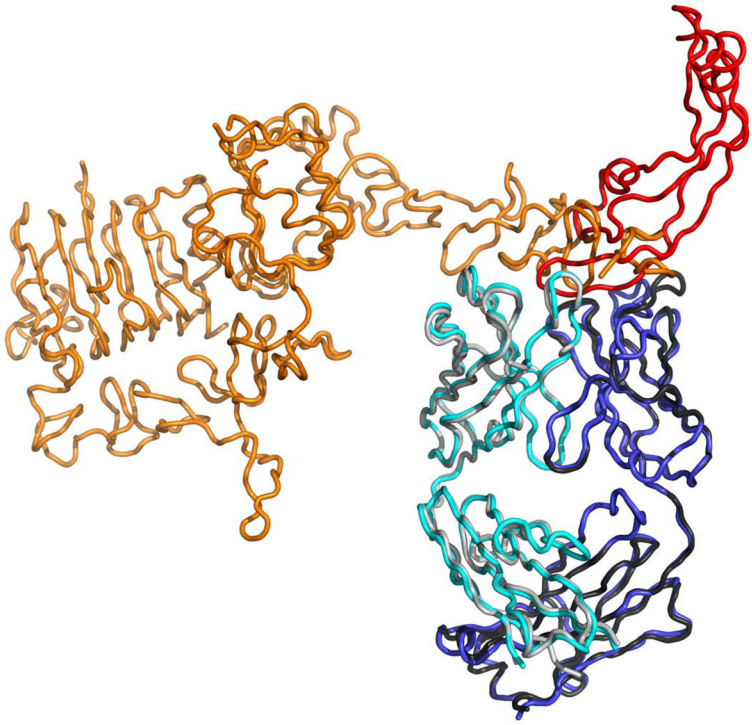

Figure 4.

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

Table 1

In vitro selected antibodies recognizing small molecules and modifications

Targets	Notes	Refs
6-monoacetylmorphine and morphine	Competition with morphine during panning to avoid crossreactivity	56
Fluorescein	Affinity matured to 48 fM by yeast display	29
testosterone, progesterone and 17β oestradiol		57,58,59,60,61
Sulfotyrosine as a post-translational modification	Antibodies recognize all sulfo-tyrosinated proteins and peptides	38,39
Sulfur mustard–modified keratin		62
Fluorogenic dyes	Antibody binding increases dye fluorescence up to 15,000 times by limiting conformational movement	63
Metallic gold		64

Table 2

In vitro selected antibodies recognizing protein sequences and conformations

Target	Notes	Ref
Peptide MHC complexes (similar to T cell receptor recognition)	Similar antibodies obtained by immunization[86,87] have lower affinities.	88,89,90,91
Fibronectin splice variants, EDA and EDB	Selection directed towards recognition of both human and mouse variants, allowing same antibody to be used in both models and clinical studies.	92,93,94
Fetal hemoglobin		36
Fetal nucleated red cells		95
GTP-bound Rab6	Antibodies were used to track activated Rab6 in the cell as GFP fusions	96
Caspase 1	Antibodies recognize either the “on” or “off” forms	97
Integral membrane proteins	CitS from *Klebsiella pneumoniaei* and KcsA from *Streptomyces lividans*. KcsA antibodies used as crystallization chaperones	98,99
RNA	Structured domain from *Tetrahymena* group I intron. Antibody used as crystallization chaperone	100
ABL1 versus ABL2	Differ by only 11%	69,70
Chicken versus quail lysozyme	Differ by only four amino acids, of which only one surface exposed	68

Table 3

In vitro selected antibodies recognizing cell surface receptors

Target	Notes/therapeutic indication	Ref
Blys	Systemic lupus erythematosis	102
Tumor necrosis factor a	Phage display was used to convert a murine mAb 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	104,105
IGF-1 receptor	Blocking of ligand-binding site of receptor and receptor down-regulation by endocytosis. Potential application in cancer	106
Notch	Prevent proteolysis of juxtamembrane NRR domain	107,108
Met	Dimeric antibodies are agonistic, monomeric ones are antagonistic, and prospected for non-small cell lung cancer	109
MuSK	Agonsitic antibodies demonstrate that MuSK activation is capable of triggering a key event in neuromuscular junction formation	110
CD40	Agonistic antibodies which activate normal human B suppress HIV-1 infection in vitro	111
Hemagglutinin	Antibodies recognize a previously unknown conserved conformational epitope. Isolated from both naïve and immunized libraries.	23,112,113
EphA2 and CD44	Selected from phage antibody library on yeast displayed antigen, followed by selection for internalization on cells	114
CD166	Internalizing antibodies selected directly for internalization on cancer cells (CD166 on prostate, ErbB2 and Transferrin receptor on breast, EGFR on A431). Antigen identified after selection. Potential utility for internalization of chemotherapeutics.	115,116
ErbB2		117
Transferrin receptor		117
EGFR		118
TRAIL-R	Over 500 different scFvs and Fabs isolated by phage display.	119

Table 4

Affinity and specificity maturation of antibodies by in vitro selection methods

Target	Notes	Ref
Affinity maturation		
HIV	CDRs targeted for mutation, 15pM affinity	28
c-erbB-2	CDRs targeted for mutation, 13pM affinity	27
Insulin	Ribosome display, random errors, 82pM affinity	26
Fluorescein	Affinity matured to 48 fM by yeast display	29
Specificity modification, recognition specificities		
CXCL10 & CXCL9	Antibody selected against CXCL10 and evolved to also recognize CXCL9	125
VEGF and Erb-B2	Antigens are completely unrelated, and antibody binds with 3/0.2nM affinity to VEGF/Erb-B2 respectively	126
Botulinum toxin A, B, E and F	One antibody able to recognize all Botulinum types afflicting man was selected by yeast display.	127