

In vitro display technologies reveal novel biopharmaceutics

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ABSTRACT Display technologies are fundamental to the isolation of specific high-affinity binding proteins for diagnostic and therapeutic applications in cancer, neurodegenerative, and infectious diseases as well as autoimmune and inflammatory disorders. Applications extend into the broad field of antibody (Ab) engineering, synthetic enzymes, proteomics, and cell-free protein synthesis. Recently, *in vitro* display technologies have come to prominence due to the isolation of high-affinity human antibodies by phage display, the development of novel scaffolds for ribosome display, and the discovery of novel protein-protein interactions. *In vitro* display represents an emerging and innovative technology for the rapid isolation and evolution of high-affinity peptides and proteins. So far, only one clinical drug candidate produced by *in vitro* display technology has been approved by the FDA for use in humans, but several are in clinical or preclinical testing. This review highlights recent advances in various engineered biopharmaceutical products isolated by *in vitro* display with a focus on the commercial developments.—Rothe, A., Hosse, R. J., Power, B. E. *In vitro* display technologies reveal novel biopharmaceutics. *FASEB J.* 20, 1599–1610 (2006)

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IN VITRO DISPLAY OF recombinant antibodies has led to the isolation of a vast range of engineered Ab-based reagents specifically targeted to clinically significant markers (1, 2). The field of application has been broadened and the potential of *in vitro* display technologies has been clarified through recent key events—for instance, the selection of high-affinity human Ab fragments by phage display (3), the display of alternative scaffolds by ribosome display (4), the discovery of novel protein-protein interactions by mRNA display (5), the establishment of *in vitro* compartments mimicking cellular compartmentalization (6), and the generation of stable DNA-protein complexes by covalent DNA display to tolerate harsh selection conditions (7). The requirement of a bacterial transformation step in phage display library construction imposes a technical limit on the actual size of library generated. No such limit is placed on the other *in vitro* display approaches. In theory, the ability to search through a larger amount of sequence

space should require fewer rounds of selection to access very high-affinity ligands without resorting to molecular evolution and affinity maturation.

The linkage of phenotype to genotype is the fundamental concept on which all *in vitro* display technologies are based. Phage, ribosome, mRNA, and DNA display all comprise a direct linkage, whereas *in vitro* compartmentalization achieves a steric proximity by establishing compartments in a water-oil emulsion (Fig. 1) (8). This principle is also naturally established on a cellular level by arrangement of plasma membrane, cytoplasm, and nucleus, enabling selection for favorable properties. *In vitro* display technology effectively supersedes natural in-cell selection by somatic hypermutation and B cell selection, which rely on the association of the fundamental diversification to the mutated gene, which is passed on to the following cell generations. *In vitro* display technologies reveal their obvious impact on the discovery of new biological entities.

The most widely used display technology is phage display (9, 10), which in essence links the displayed protein (binding activity) and its encoded sequence (genotype) within the same bacteriophage particle (Fig. 2a). Each particle displays one or more copies of a unique protein fused to either its pIII or pVIII coat proteins on its surface, allowing the enrichment of ligands with high specificity and affinity *in vitro*. Phage display is widely used for affinity maturation as discussed by Fernandez-Gacio *et al.* (11). Phage display has been used to isolate very high-affinity antibodies from human Fab libraries with synthetic diversification in the key antigen binding regions even without the need for affinity maturation (3). Synthetic diversity in heavy chain complementary determining regions 1 and 2 (CDR1 and 2) was combined with naturally occurring diversity in the heavy chain CDR3 and light chains. Affinities could be obtained in the low nanomolar range, which improved after conversion from Fab to whole IgG, possibly due to avidity. For detailed protocols and further information on phage display, see also ref 12.

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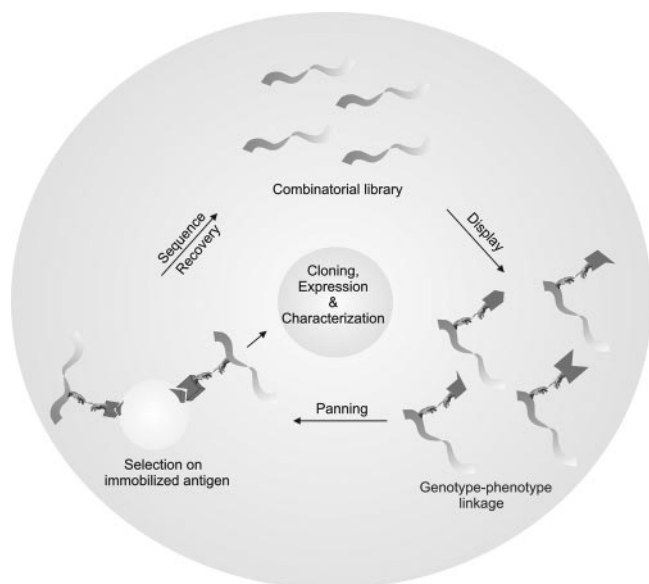


Figure 1. Schematic cycle of *in vitro* display and selection on immobilized antigen. *In vitro* transcription and translation for ribosome, mRNA, DNA display, and IVC or expression of fusion proteins on the surface of bacteriophage for phage display facilitates the linkage of displayed protein to its encoded sequence, symbolized by Michelangelo's "The hands of god and Adam." These complexes are panned on immobilized antigen to enrich for specific binders. Sequence recovery for the next selection round is achieved by an amplification step (PCR-based or reinfection of bacteria with eluted phage, depending on the methodology). Enriched clones are subjected to expression and further characterization.

In ribosome display, originally described by Mattheakis et al. (13), a cell-free transcription and ribosome-based translation system generates stable ternary complexes of mRNA, ribosome and protein totally *in vitro* (14, 15) (Fig. 2*b*). No transformation of living cells is required, allowing generation of very large libraries of up to 10^{14} members. A C-terminal spacer sequence reduces steric hindrance between the displayed protein and the ribosome and allows correct protein folding. Transcription and translation strategies using rabbit reticulocyte lysate or *Escherichia coli* extract are compared by Hanes et al. (16). One selection cycle can be performed in 1 day. The introduction of mutations inherent in this method is ideal for affinity maturation and molecular evolution (17, 18). Recently, ribosome display also advanced the display of alternative nonimmunoglobulin scaffolds with different surface topologies to generate diversity (19). For detailed protocols on ribosome display, see He and Taussig (20), Hanes et al. (16).

Yet another mRNA display involves the covalent puromycin linkage of RNA and protein, generating stable mRNA-peptide fusions, which are purified from the ribosomes before selection (21) (Fig. 2*c*). Puromycin serves as a stable mimic of amino-acyl tRNA. As it enters the ribosomal A site, it inhibits translation and binds covalently to the nascent peptide as a result of the peptidyl transferase activity of the ribosome. 10–40%

of the mRNA template can be converted into fusion product (22). The mRNA-puromycin-peptide complexes can be reverse-transcribed to cDNA to improve the stability before the consecutive selection process. mRNA display has been applied to *in vitro* selection of large peptide and protein libraries of $>10^{13}$ members. It has been used to identify novel peptide ligands for RNA (23), small molecules (24, 25), peptides (26), and proteins (5). Short random peptide aptamers without disulfide bonds and with superior solubility and stability were isolated (27). The identification of new protein-protein interactions in cellular pathways was also achieved by mRNA display (28). Unnatural amino acids have been incorporated, offering a greater sequence space for combinatorial libraries (29). Detailed protocols on mRNA display are published elsewhere (27).

In vitro compartmentalization (IVC) provides an alternative way of linking phenotype and genotype, coming close to the natural principle of evolution, since the organization of all life is dependent on a form of compartmentalization. The genetic information has to be linked to the phenotypic benefit, otherwise a selection advantage would be lost. As discussed by Tawfik and Griffiths (6), IVC enables selection of molecules requiring a direct linkage to their genes. The substrate for the selected enzyme was its own gene, protecting the encoded sequence from cleavage and thus providing a selection advantage. IVC is based on water-in-oil emulsions (30) (Fig. 2*d*). 1 ml of water-in-oil emulsion contains $\sim 10^{10}$ droplets, each with a single gene to be transcribed and translated. It is well suited for high throughput applications, e.g., selection by fluorescence-activated cell sorting (8). Recently, DNA-modifying and other highly active enzymes have been selected for substrate recognition and turnover by IVC (31, 32).

Selection systems consisting of DNA-peptide fusions have been developed, relying on *in vitro* transcription and selection and following different strategies to link the encoding DNA covalently or noncovalently to the nascent peptide (33, 34). The bacteriophage endonuclease P2A was exploited to generate a new system for covalent linkage of single chain Fv fragments (scFvs) to their encoding DNA, using the capability of this enzyme to bind the 5'-phosphate of its own DNA (cis-activity) after introducing a single strand discontinuity (nick) (35). Covalent display technology, also referred to as CIS display, uses the ability of the bacterial plasmid DNA-replication initiation protein RepA to bind to its template DNA after *in vitro* translation (7). During translation, the cis-element causes the ribosome to pause. The translated RepA then interacts noncovalently with its Ori and thus forms DNA-nascent protein complexes (Fig. 2*e*).

Selection and screening

The selection of antibodies with high affinity and specificity from diverse combinatorial libraries mimics the *in vivo* development of a humoral immune reaction after antigen exposure (1). Common to all *in vitro*

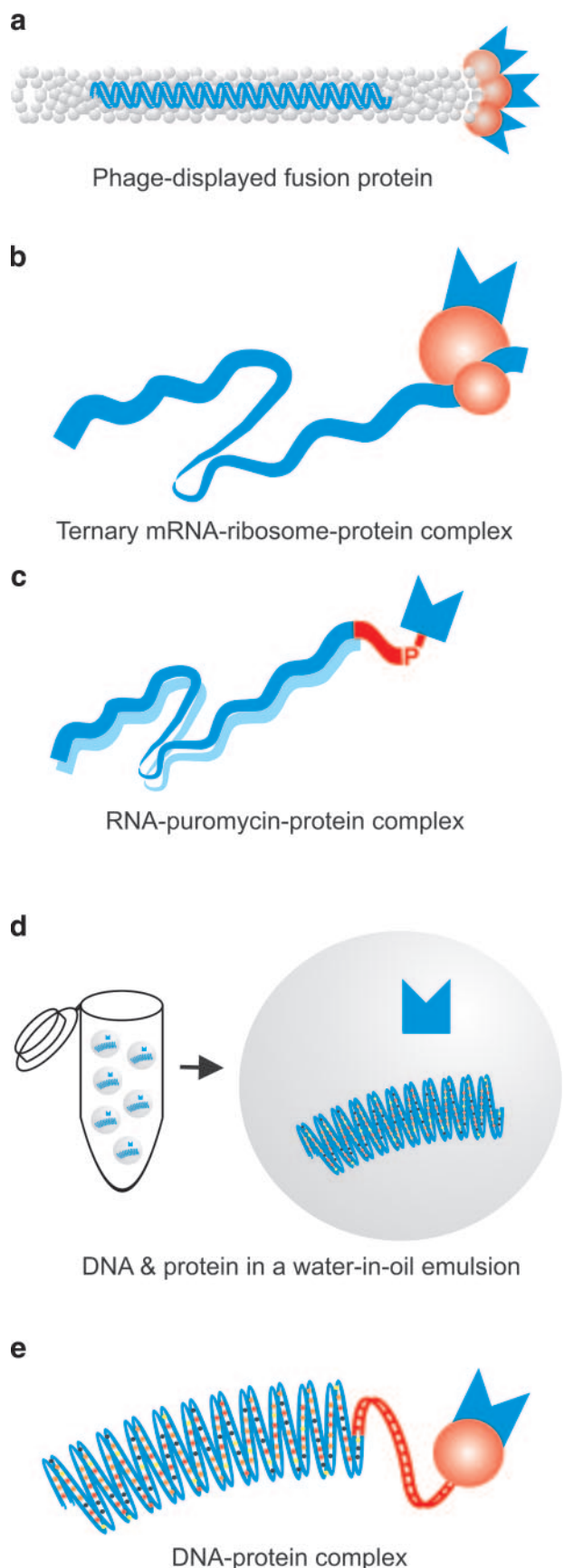


Figure 2. Cartoon representation of the phenotype-genotype linkage of different *in vitro* display technologies. *a*) Phage display: the protein (blue) is displayed as a fusion with the phage coat protein III (red). The phage coat, mainly consisting

display technologies, selection pressure applied by binding a displayed protein to its target and eliminating nonspecific reagents by stringent washing results in the enrichment of molecules with target specificity over several cycles. However, selection strategies have been taken one step further. For favorable biophysical properties such as stability, solubility, and expression efficiency, selection conditions can be optimized in sophisticated ways to apply well-defined selective pressure (36, 37). Moreover, binding affinity and on and off rates of selected reagents can be optimized independently according to the selection strategy and their desired biomedical application. Target antigens have been presented in various ways, e.g., immobilized on plates or beads, as membrane fractions, as whole cells, and even in living animals depending on availability and *in vitro* display method (1). Binding and functional assays of the enriched constructs often require high-throughput screening methods (38). Panning of naive Ab repertoires commonly yields low-affinity binders, which may have to be affinity matured, whereas a library generated from an immunized donor can lead to the isolation of higher affinity molecules.

Affinity reagents for target recognition

Antibodies and their fragments

The last decade has witnessed an enormous diversification of Ab formats (2). In the search to obtain the smallest functional binding unit, the whole IgG molecule was reduced to Ab fragments of decreasing size, increasing the ease of protein display and opening up new opportunities for target binding, pharmacokinetics, production efficiency, and biophysical properties. As the Fc region of immunoglobulins was not necessary for target specificity and, in many applications, even confers undesirable effects, Fab and scFvs have been designed for library construction. Ab engineering also led to the generation of multispecific and multivalent formats (39). The continuous domain clipping has led to single variable domains (V-domains). Naturally occurring variable heavy chain V_H-domains contain long

of the major coat protein VIII (gray), contains the sequence of the displayed protein integrated into the phage genome (blue spiral). *b*) Ribosome display: the nascent protein (blue) is linked to its mRNA (blue ribbon) by the ribosome subunits (red), which stalls in the absence of a stop-codon, forming a ternary complex. *c*) mRNA display: the nascent protein (blue) is covalently linked to its mRNA (blue ribbon), which has been reverse-transcribed to first-strand cDNA (light blue ribbon) by a puromycin linker (red). *d*) *In vitro* compartmentalization: a water droplet (gray) emulsified in oil contains DNA (blue double strand) for transcription and translation, as well as the nascent protein (blue). *e*) Covalent DNA display as an example for stable DNA-protein linkage: after translation, the bacterial protein Rep A (red), which is fused to the displayed protein (blue), interacts noncovalently with its own origin-of-replication (red double strand) integrated into the template DNA (blue double strand).

CDR3 loops enabling the access of immunosilent and cryptic epitopes on viral surfaces, in enzyme active sites, and G-protein-coupled receptors (40). Furthermore, single V-like domains have been advanced by their presence in Ab lineages of camelids (40) and cartilaginous fish (41). *In vivo* studies have demonstrated that small-sized Ab fragments show improved tissue penetration with a more homogeneous distribution, faster blood clearance, but less tumor uptake compared to whole IgG antibodies (42). Single domain antibodies (~15 kDa), scFv (~27 kDa), and diabodies (~55 kDa) are below the kidney threshold and subject to quick renal clearance. Therefore, they exhibit short circulating half-lives of a few hours and low injected dose per gram values. The aim to achieve specific targeting, good tissue penetration properties, and rapid systemic clearance can be achieved by tailoring the Ab fragments for the desired pharmacokinetic and biodistribution properties required for therapeutic or imaging use.

Phage display has demonstrated suitability for most Ab formats as recently reviewed by Bradbury and Marks (43). Evaluation of the more recent ribosome, mRNA, and DNA display, restricted to single-copy display and fewer suitable Ab formats (in particular, scFv and single-domain antibodies), has not progressed as far (1). *In vitro* compartmentalization still awaits its validation with Ab fragments.

Nonimmunoglobulin scaffolds

Although the different immunoglobulin (Ig) formats are the classic example of natural and engineered binding proteins, they are facing increasing competition by alternative nonimmunoglobulin frameworks for protein display as reviewed by Binz et al. (4). This new class of scaffolds includes small, stable, and efficiently expressed proteins of highly diverse origin, most of them involved in binding events under natural conditions. Libraries based on these scaffolds, which are often superior to antibodies in terms of stability and expression characteristics, have been screened by most *in vitro* display technologies and have yielded novel molecular tools for applications ranging from affinity purification (44), protein microarray technology (45), bioimaging (46), enzyme inhibition (47) to potential drug delivery vehicles (48, 49), and drug candidates in clinical trials (50).

Avimers (**Fig. 3a**) represent a recently developed therapeutic protein platform based on a nonimmunoglobulin library scaffold. They consist of A-domains, which naturally occur as strings of multiple domains in cell surface receptors binding to >100 different targets. 217 human A-domains are known so far. They average ~35 amino acids (~4 kDa) and are stabilized by three disulfide bonds and calcium binding. Silverman and colleagues (51) constructed a phage display library of ~10¹⁰ clones and chose an interesting selection strategy resulting in avidity and multimers (=avimers). The single domain (monomer) library was panned against a

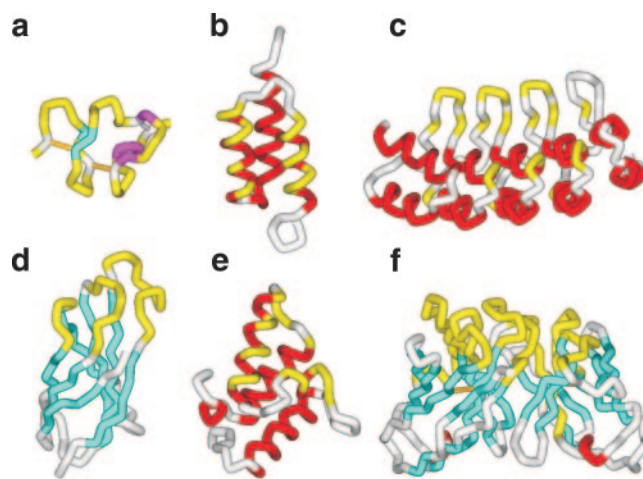


Figure 3. *In vitro* display scaffolds. Representative scaffolds for each *in vitro* display technology are shown. *a–e*) Alternative nonimmunoglobulin scaffolds; *f*) an example for a scFv Ab. α -Helices are depicted in red, β -sheets in blue, residues used for randomization and/or involved in binding events under natural conditions in yellow and disulfide bonds in orange. PDB IDs used to generate this figure are shown in brackets. *a*) Avimer: A-domain (1AJJ). Based on a consensus sequence derived from a family of human receptor domains, avimer libraries represent a modular approach whereby additional domains are sequentially added after each set of phage display and biopanning to achieve a multiple domain format for avidity and increased specificity. *b*) Affibody: Z-domain of protein A (1Q2N). Affibody molecules are derived from bacterial surface receptors and have been selected by phage display against different targets. *c*) Repeat-motif protein: ankyrin repeat protein (1SVX). Designed ankyrin repeat proteins (DARPin), based on one of the most common protein-protein interaction motifs, represent a modular approach to library construction and have been selected by ribosome display. *d*) Tenth fibronectin type III domain (1FNA). mRNA display has been used for presentation and screening of a library based on the human tenth fibronectin type III domain (¹⁰FN3), and high-affinity binders against TNF- α have been obtained and affinity matured. *e*) Immunity protein: Im9 (1EMV). IVC has been used for selection of immunity protein 9 (Im9) mutants with a novel specificity for the DNase domain of ColE7. *f*) scFv Ab (1A14). scFv antibodies have been used in a novel covalent Ab display technology linking the scFv to its DNA by the endonuclease P2A. See text for details and references.

given target and selected binders were displayed as dimers by fusion to an additional random A-domain library for a second set of pannings against the same target. Specific dimers were subsequently engineered into trimers; selection and screening cycles were repeated and finally led to multiple domain proteins binding to different epitopes on the target. Avimers with variable domain numbers and subnanomolar affinities were obtained against five different targets, among which interleukin 6 (IL-6) appears most relevant for therapeutic applications. An avimer that inhibits IL-6 with 0.8 pM IC₅₀ in cell-based assays proved to be active in two animal models (51).

Affibody molecules (**Fig. 3b**) derived from bacterial surface receptors have been selected by phage display against different targets (52), and their affinity and

avidity have been further increased by helix shuffling and multimerization, respectively (53). They now represent an established source of small binding molecules (6 kDa) for biotechnological and biotherapeutic applications ranging from protein microarrays to targeting the extracellular domain of the HER2 receptor in breast cancer (46, 54). Another group of alternative scaffolds that seems to be well-suited for display on phage are kunitz domain inhibitors, small, irregular protease inhibitors. Steps toward the development of a high-affinity, high specificity protein drug have already been undertaken for DX-88 (50), selected from a phage display library based on variants of the first Kunitz domain of human lipoprotein-associated coagulation inhibitor (LACI). DX-88 binds with high affinity to human plasma kallikrein, a serine protease, an important mediator in the pathophysiology of hereditary angioedema. The start of a clinical phase III trial had been planned for the first half of 2005 (www.dyax.com).

Members of the lipocalin protein family have been successfully displayed and selected by phage as well as ribosome display. Phage display libraries based on the bilin binding protein (BBP) from *Pieris brassicae* or the human apolipoprotein D (ApoD) have yielded “anticalins” against fluorescein, digoxigenin (55, 56), and hemoglobin (57), respectively. Ribosome display, on the other hand, enabled Lamla and Erdmann to isolate streptavidin-binding peptides of low nanomolar affinity using the bovine fatty acid binding protein as a carrier for this N-terminal peptide library (44, 58).

Designed ankyrin repeat proteins (DARPin) (Fig. 3c), based on one of the most common protein-protein interaction motifs, have been selected by ribosome display. The most recent screening of DARPins libraries yielded high-affinity inhibitors of aminoglycoside phosphotransferase (3′)-IIIa (APH) (47).

mRNA display has been used for presenting and screening of a library based on the trypsin inhibitor from the squirting cucumber *Ecballium elaterium* (EETI-II), a member of the so called “knottin family” (26), or the human tenth fibronectin type III domain (¹⁰FN3) (Fig. 3d) (59), whereas recently IVC has been used for selection of immunity protein nine mutants (Im9) (Fig. 3e), with a novel specificity for the DNase domain of ColE7 (32).

The suitable *in vitro* display technology has to be determined for every new scaffold. This necessity has been further emphasized by a recent study comparing phage display and ribosome display for the presentation of a mammalian receptor domain. In this study, ribosome display facilitated and stabilized correct protein folding whereas aggregation of the receptor domain prevented display on filamentous phage (60). Providing a different “binding surface footprint,” libraries of nonimmunoglobulin scaffolds will allow the isolation of novel binding surfaces for ligand interaction. Alternative molecules can expand the therapeutic options for patients not eligible for conventional Ab therapy, for instance, due to side effects. The features

of alternative scaffolds and how they compare to antibodies, have recently been reviewed by ref 61.

Molecular evolution

Affinity maturation implies the improvement of the binding characteristics of a low-affinity binding molecule (Fig. 4). Entirely *in vitro* performed display systems provide ideal tools for inherent affinity maturation, as each amplification step involves numerous (error-prone) polymerase chain reaction (PCR) cycles, large library sizes, and rapid panning cycles. Random mutagenesis, as well as designed interface mutations in the complementary determining regions (CDRs) and the exchange of whole heavy or light chain domains by chain shuffling, have proved effective for affinity maturation by *in vitro* display (62–64). Affinity-matured antibodies can exhibit increased biological efficacy (1) and reduction of immunogenicity (65). Thus, toxic side effects due to allergic reactions may be reduced. However, high-affinity antibodies can also reduce efficacy due to impaired tissue penetration into solid tumors (66). Affinity increases of 500- to 1000-fold were achieved for ribosome-displayed antibodies with designed and random mutagenesis (67,68). The fully human Ab Belimumab (LymphoStat-B™, chloroamphenicol acetyltransferase (CAT) & Human Genome Sciences) directed against B-lymphocyte stimulator (BLyS, implicated in the pathogenesis of autoimmune diseases) was isolated from a naive human phage display library (69). Affinity optimization was performed by randomization of the last six amino acids of the heavy-chain CDR-3 region. Belimumab is currently tested in clinical phase 2 trials for treatment of systemic lupus erythematosus. Germline hot-spot residues are

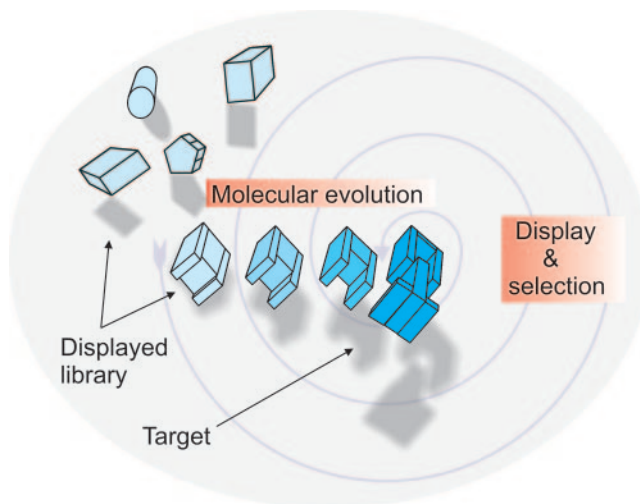


Figure 4. Principle of molecular evolution. A gene repertoire in the form of a library is displayed and selected against a given target. Consecutive rounds of display, selection, and recovery are performed. The binding characteristics of enriched proteins are improved stepwise during the selection process with regard to the interaction with the target molecule.

most likely to have a major impact on affinity based on statistical analysis of *in vivo* mutation rates. Consequently, designed hot-spot mutation in the light chain CDR1 of the anti-CD22 immunotoxin BL22 led to an up to 10-fold increase in activity (70).

***In vitro* display technologies in the development of diagnostics and therapeutics**

There is a strong trend in emerging medical science to evolve strategies to visualize and control disease-related processes via targeted agents, and *in vitro* display technologies are utilized to develop targeted therapeutics and diagnostics for various fields such as cancer, autoimmune and inflammatory diseases, and metabolic and allergic disorders (1, 2, 67, 71, 72). Using the massive data pool created by genomics and proteomics (73), they also facilitate the discovery of new drug targets and disease-related genes among ~20–25,000 human genes (74). Among 18 monoclonal antibodies currently on the market, only one was developed by *in vitro* display technologies. The anti-TNF- α Ab Adalimumab (Humira®) was humanized by guided selection with phage display (75) and is used to treat rheumatoid and psoriatic arthritis. Not all of these antibodies have been successful. In 2005, the fully human phage display-derived anti-TGF β Ab Lerdelimumab (Trabio®, CAT) failed in its second pivotal clinical trial (Phase III) to meet the primary end point of improving the outcome of surgery for glaucoma. Another issue to consider is immunogenicity. Since not all Ab fragments and non-immunoglobulin scaffolds are of human origin, they may be immunogenic during the course of multiple administrations. However, immunogenicity, even of human protein scaffolds, always needs to be addressed (1).

The potential of engineered antibodies becomes evident by the prediction that they will account for >30% of the profits in the biotechnological market by 2008 (2). The first wave of alternative scaffold-based products can be expected in the next 5–7 years (76), and targeted peptides could evolve into a new class of biologicals (72). Partnerships are forming between companies and technology providers to combine resources in protein engineering and access to feasible drug targets (77). Many companies based in the field of engineered protein-based biopharmaceuticals use *in vitro* display as platform technologies (Table 1) advanced by several drug candidates in preclinical or clinical evaluation. Among >100 antibodies in clinical trials (2), several were subjected to *in vitro* display technologies (Table 2). A variety of proof-of-principle approaches widens the spectrum of possible applications. The following section highlights several representative examples and advances and may not be comprehensive.

Target discovery

The identification of tumor-specific cell surface and intracellular antigens, as well as the characterization of

novel protein-protein interactions, is crucial for the development of targeted therapeutics and a challenging application for *in vitro* display technology. In a study aimed at reconfirming the overexpression of genes in colorectal cancer, which was detected by gene expression profiling on mRNA level, Fab fragments against five peptides were isolated from a phage display library (78). Targets were detected in cell lysate as well as on tissue cryosections by immunohistochemistry. With the introduction of cDNA expression libraries to phage display, more complex biological targets than single molecules have become available as biopanning targets (79), and high-throughput analysis becomes possible. In mRNA or ribosome display, the accessibility of the encoding single-stranded nucleic acid allows the hybridization with complementary sequences. Recently, mRNA-peptide fusions have been hybridized to an oligonucleotide array, creating a protein chip displaying a large library (80). However, based on previous experience the synthesis of correctly folded disulfide-bonded proteins in cell-free translation systems remains a challenge (81). A study of a proteome-wide scale led to the isolation of both novel and known calmodulin binding proteins from a human proteome library with mRNA display (82). Proteome libraries were also generated from human liver, kidney, and bone marrow transcripts (25). Affinity selection against the immunosuppressant FK506, immobilized as biotin-conjugate on magnetic beads, was performed with an mRNA-displayed library mix. The natural cytosolic receptor of FK506 (a 12 kDa protein known as FKBP12) and mutated binders were identified. FKBP12 was shown to be present in kidney, liver, and bone marrow libraries by recovery with tissue-specific primer sets. Recently, a mammalian receptor scaffold, which could not be displayed on phage was successfully presented using ribosome display. Randomized libraries of the Nogo receptor (NgR), which is involved in the inhibition of axon regeneration in the mammalian central nervous system (CNS), were selected against its binding partner Nogo-66 (83). Detailed mapping of the receptor's binding epitope (referred to as affinity fingerprinting) has been performed, which could lead to the development of receptor blocking agents to promote functional recovery after CNS injury. These strategies outline the potential of *in vitro* display technology for the characterization of novel protein-protein interactions. The information gained can now be used to develop new diagnostic or therapeutic approaches through a deeper insight into cellular signaling pathways.

Biologicals in diagnosis

The biotechnological progress in the development of human antibodies has helped overcome adverse immune reactions associated with the application of rodent antibodies for radio-immunoimaging. Targeted agents developed by *in vitro* display technologies are currently exploited in optimizing imaging strategies, and Ab probes are commonly clipped to fragments or

TABLE 1. Companies using constructs developed by *in vitro* display technology*

Pharmaceutical company	<i>In vitro</i> display technology	Scaffold	Web address
Abbott Laboratories	Phage display	Antibodies	http://www.abbottimmunology.com
Ablynx	Phage and ribosome display	Domain antibodies	http://www.ablynx.com
Affimed	Phage display	Bispecific antibodies	http://www.affimed.com
Affitech	Phage display	Antibodies	http://www.affitech.com
Avidia	Phage display	Undisclosed human protein scaffold (Avimer®)	http://www.avidia.com/default.aspx
Bioinvent	Phage display	Antibodies	http://www.bioinvent.com
Biosite	Phage display	Antibodies	http://www.biosite.com
Cambridge Antibody Technology	Phage and ribosome display	Antibodies	http://www.cambridgeantibody.com
Compound Therapeutics Inc.	mRNA display	Fibronectin type III domain	http://www.compoundtherapeutics.com
Crucell	Phage display	Antibodies	http://www.crucell.com
Discerna	Ribosome display	Antibodies	http://www.discerna.co.uk
Domantis	Phage display	Domain antibodies	http://www.domantis.com
Dyax	Phage display	Antibodies, peptides, Kunitz domain	http://www.dyax.com
Enzon	Phage display	scFv	http://www.enzon.com
Evogenix	Ribosome display	Antibodies	http://www.evogenix.com
Genentech	Phage display	Antibodies	http://www.gene.com
Genzyme Corp.	Phage display	Antibodies incl. Fab, scFv	http://www.genzyme.com
GPC-Biotech	Phage display	Antibodies	http://www.gpc-biotech.com
Hermes Biosciences	Phage display	Antibodies, scFv	http://www.hermesbio.com
Human Genome Sciences	Phage display	Antibodies	http://www.hgsi.com
ImClone Systems	Phage display	Antibodies	http://www.imclone.com
Isogenica Ltd.	Covalent display	Peptides, Antibodies	http://www.isogenica.com
Medimmune	Phage display	Antibodies	http://www.medimmune.com
Micromet	Phage display	Bispecific scFv	http://www.micromet.de
Molecular Partners	Ribosome display	Ankyrin repeats	http://www.molecularpartners.com
Morphosys	Phage and ribosome Display	Antibodies	http://www.morphosys.com
Peptech	Phage display	Antibodies	http://www.peptech.com
Phylogica	Phage display	Peptides of bacterial and plant origin	http://www.phylogica.com
Pieris Proteolab AG	Phage display	Lipocalins	http://www.pieris-ag.de
Tanox	Phage display	Antibodies	http://www.tanox.com
Wyeth	Phage and ribosome display	Antibodies	http://www.wyeth.com
Xoma	Phage display	Antibodies	http://www.xoma.com

*This table is based on information in the public domain. The authors want to acknowledge that it is not comprehensive, due to the fact that some of the company information is confidential.

are of human origin (84). These agents can be generated in a shorter time frame than hybridoma technology and animal immunizations. To detect severe acute respiratory syndrome coronavirus (SARS-CoV), which causes SARS, an epidemic viral pneumonia with high mortality rate, sensitive diagnostic are needed. Human scFv antibodies to the N-protein of SARS-CoV were selected from a phage library on immobilized antigen (85). They could even detect SARS-CoV particles in infected cells and therefore may contribute to the development of reagents for rapid detection of the SARS-CoV N-protein and virus particles.

Antibodies linked to fluorescent semiconductor nanocrystals (quantum dots) represent new mediators for high-resolution cellular imaging (86) and further development to targeted agents for the clinic is applicable. Thus, engineered proteins are valuable vehicles

in bioimaging, e.g., for *in vitro* or *in vivo* tumor targeting.

Biologicals in therapy

The research focus on *in vitro* display technologies was driven by the enormous potential of Ab based therapies and, at the same time, the disadvantages of mouse-derived molecules due to their immunogenicity. Human antibodies are the only increasing portion of antibodies entering clinical studies that include mouse, chimeric, and humanized antibodies (87). Antibodies have been fused to radionuclides, toxins, and enzymes to deliver effector functions to their specific targets (42). Recently, reagents isolated by phage display have been used to generate immunoconjugates, as demon-

TABLE 2. Antibodies and alternative constructs derived from *in vitro* display technology^a

<i>In vitro</i> display technology	Name (generic)	Format	Target
Phage display	Humira (adalimumab)	IgG1	TNF α
Phage display	GC-1008	IgG	TGF β
Phage display	CAT-213 (bertilimumab)	IgG4	eotaxin1
Phage & ribosome display	CAT-354	IgG4	IL-13
Phage display	CAT-192 (metelimumab)	IgG4	TGF β 1
Phage display	ABT-874	IgG1	IL-12
Phage display	LymphoStat-B (belimumab)	IgG1	Blys
Phage display	HGS-ETR1 (mapatumumab)	IgG1	TRAIL-R1
Phage display	HGS-ETR2	IgG1	TRAIL-R2
Phage display	ABthrax TM	IgG1	
Phage display	cAb-CEA5:: β L (Nanobody)	VhH-B-lactamase fusion (camelid)	CEA
Phage display	Nanobody	VhH (camelid)	Maf1
Phage display	DX-890/EPI-hNE4	Kunitz domain	Neutrophil Elastase
Phage display	DX-88	Kunitz domain	Kallikrein
Phage display	DX2240	Undisclosed	Tie-1
Phage display	IMC-1121B	IgG1	VEGFR2
Phage display	IMC-11F8	IgG1	EGFR
Phage display	IMC-A12	IgG1	Insulin-like growth factor receptor
Phage display	BL-22	Fv-Pseudomonas exotoxin (Immunotoxin)	CD22
Phage display	dAb	human Variable domain	TNF α
Phage display	BiTE	Bispecific scFv	EpCAM, CD3
Phage display	Nanobody	VhH (camelid)	vWF
Phage display	L19- γ IFN	Diabody (VH-VL)2	EDB domain of fibronectin
Phage display	C6.5K-A	Diabody (VH-VL)2	Her2/neu
Phage display	F5scFv-PEG immunoliposome	ScFv-PEG fusion	Her2/neu
Phage display	B4/12	Fab	Gp120
Phage display	G8	Fab	MHC peptide
Phage display	Fab12	Fab	VEGF
Phage display	A4.6.1	IgG1	VEGF
Phage display	1D09C3	CD38	MHC-II
Phage display	MOR101	Fab	ICAM-1
Phage display	MOR102	IgG4	ICAM-1

^aThis table is based on information in the public domain. The authors want to acknowledge that it is not comprehensive, as some of the company information is confidential.

strated by radiolabeling of the human anti-Her2 diabody C6.5 with the beta-emitter ⁹⁰Y (88). This radio-immunoconjugate showed tumor growth inhibition in mice. Tumor-specific drug delivery was also improved by immunoliposomes, e.g., generated from phage display-derived anti-Her2 scFv F5 (89). F5-PEG-lipid conjugates facilitate binding and internalization of drug-loaded liposomes to Her2 overexpressing carcinomas (90). An interesting strategy used mRNA display to enhance penicillin efficacy by linking a penicillin moiety to the library molecules. This led to the isolation of peptide-drug hybrids with 100-fold higher activity than the parent antibiotic (91). Recently, the efficacy of two fully human phage display-derived antibodies (HGS-ETR1 and HGS-ETR2, CAT & Human Genome Sciences) binding to the death receptors TRAIL-R1 and

TRAIL-R2, was shown for the antibodies alone and in combination with chemotherapy (92). The natural ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor superfamily, and induces apoptosis on receptor binding via activation of a caspase-dependent pathway. Both agonistic antibodies led to preferential killing of lymphoma cells. Cell death of >10% was achieved in ~70%, and of >20% in ~30% of the samples with comparable results for cell lines and primary tumor cells. Additive effects were observed in combination with the anthracycline doxorubicin and the vascular endothelial growth factor receptor Ab Bortezomib for both constructs. HGS-ETR1 and HGS-ETR2 are being evaluated in clinical lymphoma studies. The efficacy of a human anti-human interleukin-13 Ab (CAT-354, Cambridge Ab Technology, Cambridge,

TABLE 2. (continued)

Indication	Stage	Company/developer
Autoimmune diseases	Approved	CAT/Abbott
Idiopathic pulmonary fibrosis	Phase I	CAT/Genzyme
Allergic disorders		CAT
Asthma	Phase I	CAT
Scleroderma	Phase I	CAT/Genzyme
Autoimmune diseases	Phase II	CAT/Abbott
Rheumatoid Arthritis, Lupus	Phase II	CAT/HGSI
Solid & hematological malignancies	Phase II	CAT/HGSI
Advanced cancers	Phase I	CAT/HGSI
Anthrax	Preclinical	CAT/HGSI
Cancer imaging / therapy	Preclinical	Ablynx
Dandruff (Malassezia furfur)	Preclinical	Ablynx
Cystic fibrosis	Phase II	Dyax/Debiopharm
Hereditary angioedema	Phase II	Dyax
Vascular targeting	Preclinical	Dyax
Solid tumors	Phase I	ImClone/Dyax
Solid tumors	Phase I	ImClone/Dyax
Solid tumors	Phase I	ImClone
Chronic lymphatic leukemia, Hairy cell leukemia	Phase I	Enzon
Autoimmune diseases	Preclinical	Domantis/Peptech
Colorectal Cancer	Preclinical	Micromet
Thrombophilia	Preclinical	Ablynx
Antiangiogenesis	Preclinical	University of Siena. Italy
Breast and ovarian cancer	Preclinical	University of California, San Francisco, USA
Drug delivery in breast cancer	Preclinical	University of California, San Francisco, USA
HIV	Undisclosed	Scripps Research Inst., La Jolla, CA, USA
Undisclosed	Undisclosed	University of Maastricht, Netherlands
Revascularization	Undisclosed	Genentech
Revascularization	Undisclosed	Genentech
Multiple myeloma	Phase I	Morphosys
Skin burn	Preclinical	Morphosys
Psoriasis	Preclinical	Morphosys

UK) was assessed in a murine model for allergic asthma (93). CAT-354 was developed with phage and ribosome display. The intratracheal injection of IL-13 increased metacholine sensitivity for obstruction of the airways, goblet cell metaplasia, mucus production, and esophageal and lung eosinophilia. Pretreatment with the human Ab significantly reduced airway hypersensitivity and eosinophilia. In a different study, human T cell receptors (TCRs), stabilized by a non-native interchain disulfide bond, have been displayed on the surface of bacteriophage. The directed evolution of high-affinity TCRs, specific for markers complexed with human leukocyte antigens (human T cell lymphotropic virus type 1 (HTLV-1) and tumor-associated peptide NY-ESO-1) led to the isolation of TCRs with picomolar affinities and high specificity (94). The authors con-

clude that these engineered TCRs could be valuable tools for blocking T cell activation, e.g., in cancer or autoimmune diseases.

Single domain antibodies are stable, soluble, and easy-to-express entities consisting of one Ig variable domain. Examples are camelid-derived Nanobodies™ (Ablynx, Ghent, Belgium) and human domain antibodies (dAbs, Domantis, Cambridge, MA, USA). They combine the benefits of small molecules in terms of versatility with those of antibodies in terms of diversity and specificity. Phage-displayed Nanobodies™ and dAbs are currently undergoing preclinical evaluation against clinically significant markers, such as CEA (95) and TNF- α (www.domantis.com), respectively. The nanobody AU/VWFa-11, selected from an immunized llama-derived phage library, recognizes specifically ac-

tivated von-Willebrand factor (vWF), providing a tool to investigate the role of vWF in the development of thrombocytopenia (96). Monomeric dAbs are also re-engineered into larger molecules to create drugs with prolonged serum half-lives or other pharmacological characteristics (97).

Expanding the range of *in vitro* displayed reagents against clinically significant markers further, peptides specific for the murine *v-abl* tyrosine kinase were isolated from a random mRNA-displayed library as well as from a proteomic library derived from human bone marrow cells (98). The murine *v-abl* gene is related to the human proto-oncogene *c-abl*, which is involved in the pathogenesis of chronic myelogenous leukemia after translocation with the *bcr* gene. The authors propose that new inhibitors of the ABL tyrosine kinase family could be evolved from the isolated substrates by molecular evolution. Thrombin binding peptides were isolated from a random library of $\sim 10^{11}$ individual members after 10 rounds of affinity enrichment using mRNA display (99). This extensive selection procedure demonstrates the applicability of a high number of selection rounds, and at the same time leaves room to reflect parameters that may interfere with a stringent selection and quick enrichment. Isolated clones of high affinity in the nanomolar range may be candidates for the development of antithrombotic agents.

An alternative scaffold, a small, irregular protease inhibitor, has already been used to develop therapeutic protein drugs. For instance, phage-selected DX-88, based on the first Kunitz domain of human lipoprotein-associated coagulation inhibitor (LACI), binds with high affinity to human plasma kallikrein, a serine protease that is an important mediator in the pathophysiology of hereditary angioedema (50). A Phase III trial is planned to begin soon.

CONCLUSIONS

During the past years, there has been growing scientific and commercial interest in *in vitro* display systems. They represent fast developing emerging technologies for the discovery and molecular evolution of novel high-affinity biologicals to various targets. A broad spectrum of biotechnological, medical, and proteomic applications is conceivable and elucidates the impact of *in vitro* display technologies on the development of new biopharmaceutical entities for *in vitro* and *in vivo* diagnostics and therapeutics in various disease fields. An increasing number of novel drugs developed by *in vitro* display technologies and based on engineered antibodies, Ab fragments, alternative scaffolds, or peptides are currently tested in clinical or preclinical studies. However, their efficacy has to be validated. There appears to be a clear trend from whole antibodies toward smaller formats, providing access to new molecular characteristics and cost-efficient production. In contrast to phage display, younger technologies such as *in vitro* compartmentalization, ribosome, mRNA, and DNA display still

have to consolidate their role while closely following their pathfinder. Though all of these technologies are successful, the ultimate choice of which to use will always depend on the parameters imposed by the final application. FJ

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