

Harnessing phage and ribosome display for antibody optimisation

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Therapeutic antibodies have become a major driving force for the biopharmaceutical industry; therefore, the discovery and development of safe and efficacious antibody leads have become competitive processes. Phage and ribosome display are ideal tools for the generation of such molecules and have already delivered an approved drug as well as a multitude of clinical candidates. Because they are capable of searching billions of antibody variants in tailored combinatorial libraries, they are particularly applicable to potency optimisation. In conjunction with targeted, random or semi-rational mutagenesis strategies, they deliver large panels of potent antibody leads. This review introduces the two technologies, compares them with respect to their use in antibody optimisation and highlights how they can be exploited for the successful and efficient generation of putative drug candidates.

Generating potent therapeutic antibodies today

Thirty years after monoclonal antibodies (mAbs) were made available through the development of hybridoma cell lines [1] they have become a major driving force for the biopharmaceutical industry. Owing to their ability to bind to virtually every kind of target molecule with high affinity and specificity, they are exploited as potent diagnostic and therapeutic agents. The best selling antibody drugs, such as Remicade[®] (infliximab; <http://www.remicade.com/>), Rituxan[®] (rituximab; <http://www.rituxan.com/>) or Humira[®] (adalimumab; <http://www.humira.com/>), today account for sales of several billion US\$, a market growing faster than all others in the sector [2] – altogether there are currently 18 mAbs approved for the market and >150 in clinical development [3]. Lower attrition rates for antibody leads in clinical trials compared with small molecule compounds as well as recent successes in treating cancer or inflammatory diseases with mAbs have triggered the interest of all the major pharmaceutical companies; through acquisitions, in-licensing or collaborations, each one has gained access to technology and expertise for their discovery and development [4]. Although this has fuelled the antibody sector financially it has also made it competitive, and companies not only have to bolster failures with reasonably sized portfolios but they also need to be efficient and fast in bringing safe and efficacious antibody leads to the clinic.

To become a safe and efficacious drug, an antibody first needs to bind to a target, also termed antigen, central to

the cause or progression of the disease to be treated [5]. More precisely, it should recognize a surface area – the epitope – that is biologically relevant and thereby impact, agonistically or antagonistically, on a disease-related function. The mAb should recognize the antigen with great specificity to avoid triggering unwanted reactions by cross-reacting with other molecules possibly present at higher concentrations. Generally, it should also have the highest possible affinity for the antigen to be efficacious even at low concentrations. Failure to achieve this would require administration at higher and/or more frequent doses, which would be costly, inconvenient for the patient and possibly even dangerous because of the reduced therapeutic window and, thus, the prevalence of side effects. Important exceptions to this rule are mAbs against solid tumour targets, for which high affinity has potentially been shown to decrease tumour penetration and, therefore, the efficacy of the drug [6].

The classical hybridoma technology has been adapted to generate therapeutic mAbs through either humanising the molecules obtained from immunising rodents [7] or by using mice harbouring human immunoglobulin genes instead of their own [8,9]. Despite its successful history, this method has some inherent limitations: it is not applicable to antigens that are toxic to the animals or conserved across species (and thus non-immunogenic); the isolation of antigen-specific antibodies can be slow; the selection conditions *in vivo* are difficult to control; the number of isolated antibodies, their recognized epitopes and their specificity and affinity can be unpredictable; and it can suffer from an affinity ceiling. Although antibodies with dissociation constants <100 pM have been reported following immunisation [10], they are not actively selected because their slower dissociation rates cannot be discriminated from the intrinsic B-cell receptor internalisation rate [11].

A faster, more flexible and more reliable alternative for the generation of therapeutic mAbs is represented by the creation of large, diverse combinatorial libraries of antibody heavy and light chain variable domains (V_H and V_L, respectively), followed by their interrogation using display technologies *in vitro*. Antibody fragments, such as single-chain Fv (scFv), single domain or Fab fragments, can be presented on phage or yeast surfaces as well as on ribosomes, while the encoding nucleotide sequence is incorporated within or is physically attached. This link of phenotype to genotype enables selection and enrichment of molecules with high specific affinities for a given antigen followed by identification of the co-selected gene.

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Box 1. Concept and steps of phage and ribosome display

- **Phage display:** the DNA library is imported into *E. coli* cells for *in vivo* synthesis of the encoded scFv fused, for example, to the gene III coat protein of filamentous phage. In a phagemid system, the DNA lacks essential factors for phage production, demanding phage rescue with a helper phage, which supplies the missing factors *in trans*. The resulting phage particles display the proteins on the surface, and the encoding DNA is contained within. Following selection, the genotype of selected variants is recovered and amplified by infecting and subsequent culturing of fresh *E. coli* cells, respectively. Phage display and its applications for isolation and engineering of antibodies have been reviewed in detail [74–77] and are outlined in Figure 1.
- **Ribosome display:** the DNA library constructs contain all the signals required for cell-free *in vitro* transcription and translation. In the most widely used version, the absence of a stop codon at the end of the coding sequence prevents the release of the mRNA and the nascent polypeptide from the ribosomes. Low temperatures and an elevated level of magnesium ions further stabilize the ternary mRNA–ribosome–polypeptide complex. An unstructured tether is added to the C-terminus of the constructs to enable correct folding of the nascent polypeptides outside the ribosome tunnel. After selection, mRNA molecules are recovered by reverse transcription (RT) and amplified by polymerase chain reaction (PCR). Alternative coupling methods include addition of chloramphenicol to the translation reaction [28], fusion of the ricin A chain to the polypeptide [78] or, in a variation termed mRNA display, the direct linking of mRNA and polypeptide using an RNA-binding protein [79] or puromycin [80,81]. For general reviews of ribosome and mRNA display and their applications, see [60,82–85] and Figure 1.

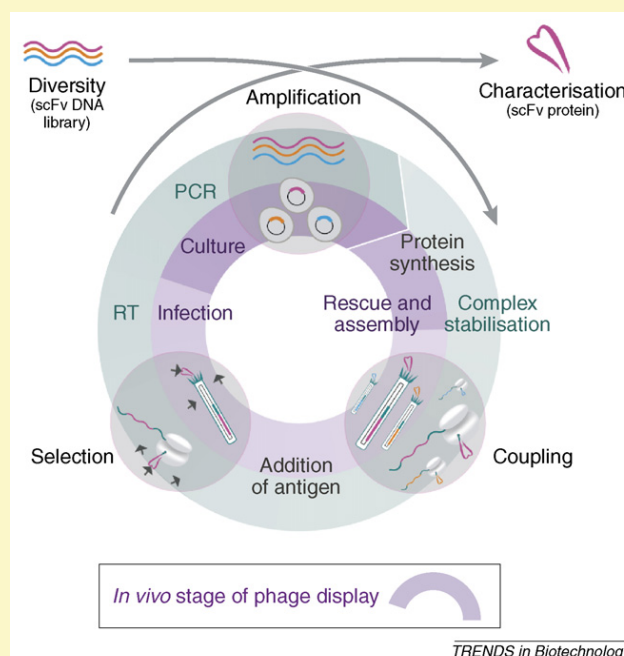


Figure 1. Illustration of the phage (purple) and ribosome display (green) cycles. All display technologies, including phage and ribosome display, follow the same iterative concept. A combinatorial DNA library is prepared that encodes diverse protein variants, for example, single-chain Fv antibody fragments (Diversity). The polypeptides with their individual phenotypes are synthesized and coupled to their encoding genotypes (Coupling). Controlled selection pressure is applied to capture those variants with a defined property, such as specific binding to a target antigen, whereas non-binders are eliminated (Selection). The genotype of the selected variants is recovered and amplified for another round of selection (Amplification). The process is repeated several times to enable enrichment of individual variants to be characterized in analytical and functional assays (Characterisation).

Consequently, the need for screening is reduced to a minimum, and large diverse panels of unique antibodies can be identified within weeks [12]. Usually, targeting a variety of epitopes with distinct binding specificities and affinities, they can often present a greater choice of putative drug candidates than is obtainable from *in vivo* immunisation.

In particular, display technologies are ideal for *in vitro* optimisation of antibodies that recognize the correct epitope on a specific antigen but are not potent enough for diagnostic or therapeutic applications. To this end, a library of mutants is created, and improved antibodies are isolated in a way analogous to the natural affinity maturation processes of somatic hypermutation and affinity selection [13]. Carried out in the test tube, display technologies enable the comparatively easy generation of new sequence repertoires, selection under tightly controlled conditions and, finally, isolation of antibodies with affinities beyond those routinely achievable *in vivo*.

In this review, we introduce and compare the two most widely used display technologies – phage and ribosome display – with respect to their use in antibody affinity maturation. We highlight their individual characteristics and explain how they might be exploited for the successful and efficient generation of potent antibody leads. The concept and steps of phage and ribosome display are

explained in Box 1. Of all the other display technologies, yeast display has been applied most successfully to antibody optimisation [14–16]; however, to the best of our knowledge, no side-by-side comparison with phage or ribosome display has so far been reported. We therefore incorporate only some selected yeast display antibody optimisation articles and refer to [17,18], and references therein, for more information on the technology itself.

Phage display: a well-established selection technology

In 1985, M13 phage displaying a specific peptide antigen on its surface was isolated from a population of wild-type phage, based on the affinity of a specific antibody for the peptide [19]. Antibody variable domains were successfully displayed by McCafferty *et al.* in 1990, enabling the selection of the antibodies themselves [20]. During the past 15 years, phage display has been exploited successfully to isolate and optimize antibody molecules such as the human anti-TNF α antibody marketed as Humira[®] and many more mAbs currently in the clinic.

The major advantages of phage display compared with other display technologies are its robustness, simplicity, and the stability of the phage particles, which enables selection on cell surfaces [21], tissue sections [22] and even *in vivo* [23]. However, because the coupling of genotype and phenotype (i.e. protein synthesis and assembly of phage

particles) takes place in bacteria, the encoding DNA needs to be imported artificially. Library size is therefore restricted by transformation efficiency. Despite great improvements in this area, the largest reported libraries still comprise no more than $\sim 1 \times 10^{10}$ to $\sim 1 \times 10^{11}$ different members. This is believed to be a disadvantage for isolating high affinity antibodies because the size of the library is directly linked to the probability of selecting them [24]. With respect to use in antibody optimisation, it further means that the number of mutants, and thus the sequence diversity that can be interrogated in selections, is limited. Finally, the coupling of phenotype and genotype and the amplification of selected variants *in vivo* can lead to considerable negative selection pressures. Antibody fragments that are toxic for the host, poorly expressed or folded, inefficiently incorporated into the phage particle or susceptible to proteolysis or aggregation slow down the bacterial growth and display less efficiently. This reduces the library diversity and enables a low potency but fast-growing clone to dominate a whole population after just a few rounds of selection.

Ribosome display: a powerful alternative

As an alternative, researchers have developed *in vitro* display technologies [25], the most widely used of which is ribosome display [26,27]. Here, a DNA library that encodes peptides or proteins is transcribed and translated *in vitro* using prokaryotic or eukaryotic cell-free expression systems such as *E. coli*, rabbit reticulocyte or wheat germ extracts. The combination of the absence of a stop codon, an elevated level of magnesium ions and low temperature stalls the ribosome at the end of the mRNA while the nascent polypeptide folds and is presented outside the ribosome tunnel.

In 1994, Mattheakis *et al.* presented a large library of synthetic peptides on ribosomes and selected them for binding to a specific antibody [28]. Efficient display and selection of antibody fragments was shown by Plückthun's group in 1997 [29]. Since then, cell-free expression systems have been made commercially available and protocols improved to facilitate the wider use of the technology [30]. Most recently, highly efficient ribosome display selections have been reported using an *in vitro* expression system based on purified proteins, thus lacking RNases, proteases and other unknown inhibitory factors [31]. In spite of this, the use of ribosome display still remains challenging under certain conditions as well as on cell surfaces or when using non-purified antigens.

Ribosome display has a particular advantage compared with phage and all other cell-surface display technologies for *in vitro* optimisation of antibodies and proteins in general: the DNA does not have to be imported into a host because phenotype–genotype coupling and amplification both take place *in vitro*. Hence, libraries of more than 1×10^{13} members are possible, the limit being the maximal number of ribosomes that can be provided routinely in selections. The libraries are also less prone to the negative selection pressures associated with *in vivo* systems. Finally, and most notably, additional sequence diversification during the selection process – and, thus, a directed evolution following the Darwinian principle of variation

and selection – is greatly facilitated by the inherent PCR step at each round.

Successful antibody potency optimisation using phage and ribosome display

For *in vitro* affinity maturation, both phage and ribosome display selections are preferentially performed on an antigen in solution to control its concentration and reduce avidity effects. When labelled with biotin, for example, the antigen and the bound scFv–phage or scFv–ribosome–mRNA complexes can be pulled down with streptavidin-coated magnetic beads. The antigen concentration chosen should be below the dissociation constant (K_d) of the parent antibody at the first round of selection and then reduced incrementally during subsequent cycles to enrich for variants with lower K_d [32,33]. Selections have been performed in the presence of an excess of competitor antigen or antibody, resulting specifically in variants with lower off-rates [32,34,35]. Correspondingly, on-rate selections are conceivable using phage and ribosome display; however, this has so far only been realized with yeast display [14], which profits from using flow cytometric cell sorting to finely discriminate variants with specified binding kinetics [36,37]. The selected antibodies can be tested for increased affinity but should preferentially be screened for improved potency in a relevant cell-based assay because the sequence diversification and selection process might also have enriched variants with increased folding efficiency and thermodynamic stability, both contributing to potency and, ultimately, efficacy.

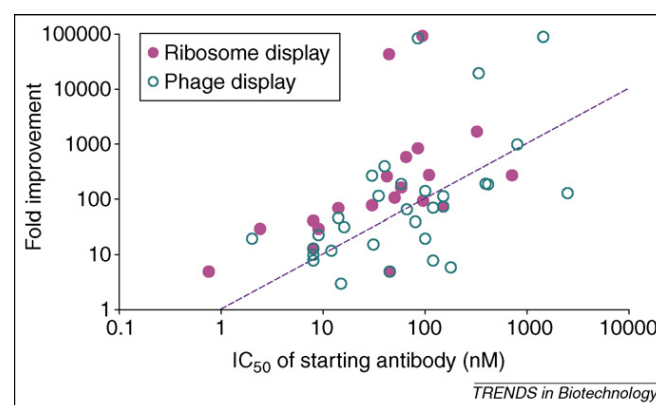


Figure 1. Potency improvements of antibodies optimized using phage and ribosome display. The graph correlates the maximum potency improvements achieved by antibody optimisation with the starting potencies of the respective parent antibodies. Each parent was obtained from phage display selections on naive libraries before being optimized using phage or ribosome display as indicated. The starting potencies of the parent antibodies are shown as the nanomolar (nM) antibody concentrations resulting in 50% antigen inhibition in a functional assay (IC_{50}). The potency gains are represented as fold improvements in IC_{50} in the same assays. The starting potencies as well as the potency improvements varied considerably because of the diversity of the antibody–antigen interactions and the assay formats. Generally, however, greater potency improvements could be observed when starting with lower potency antibodies. Most antibody optimisations using either phage or ribosome display resulted in final IC_{50} values in the picomolar range, as represented by the circles above the broken line. Depending on the assay format, the mode of action of the antibody and the biology of the target, this is usually potent enough for therapeutic application. The affinities of such antibodies are generally in the low picomolar range. It is notable that some optimized antibodies below the line might have been the result of abandoned optimisation efforts in favour of another lineage against the same target.

We have used both phage and ribosome display for *in vitro* optimisation of therapeutic antibodies. As shown in Figure 1, the achieved potency improvements in relevant functional assays are comparable for the two technologies, despite the considerable variations from antibody to antibody. In agreement with published data, many antibodies could be optimized to the picomolar potencies usually required for therapeutic application.

Targeted approaches to antibody affinity maturation

With tools such as phage and ribosome display available to isolate rapidly specific high-potency antibodies from large variant populations, a major key to efficient and successful *in vitro* antibody optimisation is the introduction of the appropriate sequence diversity into the starting antibody. Generally, two approaches can be taken: either amino acid residues in the antibody sequence are substituted in a targeted way or mutations are generated randomly.

Antibodies are ideal candidates for targeted sequence diversification because they share a high degree of sequence similarity and their conserved immunoglobulin protein fold is well studied. Many *in vitro* affinity maturation efforts using combinatorial libraries in conjunction with display technologies have targeted the complementarity-determining regions (CDRs) harbouring the antigen-binding site. Normally, amino acid residues are fully randomized with degenerate oligonucleotides. If applied to all positions in a given CDR, however, this approach would create far more variants than can be displayed on phage or even ribosomes – saturation mutagenesis of a CDR of 12 residues, for example, would result in 1×12^{20} different variants. In addition, the indiscriminate mutation of these residues creates many variants that no longer bind the antigen, reducing the functional library size. Scientists have therefore restricted the number of mutations by targeting only blocks of around six consecutive residues per library [38] or by biasing the mutagenesis randomly towards the parent sequence and/or chemically or sterically conservative changes, a strategy sometimes referred to as parsimonious mutagenesis [39–42]. Mutagenesis has also been focussed on natural hotspots of somatic hypermutation [43], or the residues to be targeted were chosen based on mutational or structural analyses as well as molecular modelling [39,40,44].

Further affinity improvements have been achieved by recombining mutations within the same or different CDRs of improved variants [15,39,44,45]. In spite of having yielded some substantial gains, such an approach is unpredictable. An alternative is the so-called CDR walking. Here, the highest affinity antibody from the maturation of one CDR is used as the template for mutagenesis of another CDR [46–48]. Although this might result in greater improvements, it is time consuming and permits only one set of amino acid changes to recombine with new mutations. Alternatively, whole populations of, for example, pre-selected V_H and V_L CDR3-targeted variants can be recombined, and the resulting combinatorial library subjected to further rounds of selection. This enables the pairing of hundreds of thousands of improved V_H variants with a similar number of V_L variants and thus provides a much greater chance of creating outstanding synergistic

potency improvements of up to 40 000-fold higher than for the best variant from V_H or V_L mutagenesis alone (unpublished data).

Introducing sequence diversity randomly

In addition to the targeted strategies, several random mutagenesis methods can be used to improve antibody potency. One is the shuffling of gene segments, where V_H and V_L populations, for example, can be randomly recombined with each other (as described above) or, as previously reported, by keeping one of the domains constant [33,40,42,49]. Alternatively, the shuffle can be performed with CDRs [50,51] or random parts of the scFv, based on a concept initially described by Stemmer *et al.* [34,52,53]. Another approach is the indiscriminate mutation of nucleotides using the low-fidelity *Taq* DNA polymerase [54], error-prone PCR [32,34,55,56], the error-prone Q β RNA replicase [57] or *E. coli* mutator strains [58,59] before and in-between rounds of selection. Shuffling and random point mutagenesis are particularly useful when used in conjunction with targeted approaches because they enable the simultaneous evolution of non-targeted regions [38,60]; in addition, they are powerful when performed together because individual point mutations can recombine and cooperate, again leading to synergistic potency improvements. This has created some of the highest affinity antibodies produced so far, with dissociation constants in the low picomolar [35] and, in a study using yeast display, even the femtomolar range [16].

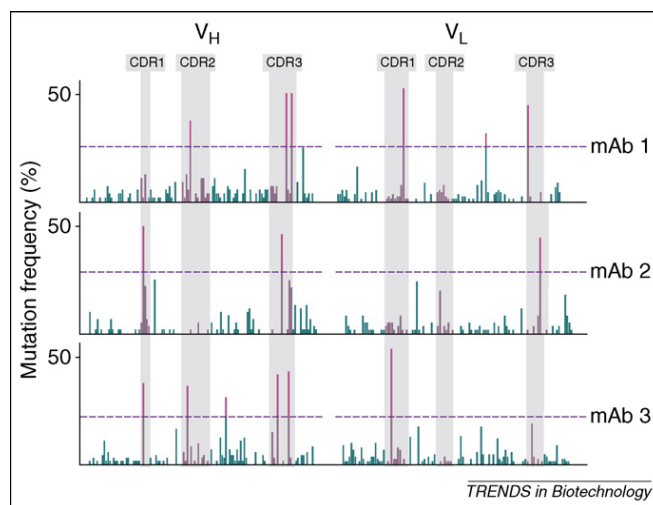


Figure 2. Identification of mutation hotspots by random mutagenesis and affinity selections. The figure illustrates possible mutation frequencies for each amino acid residue in the variable domains (V_H and V_L) of antibody variants of improved potency. Three exemplary antibody optimisations are depicted (mAb 1, mAb 2 and mAb 3). The location of the complementarity-determining regions 1 to 3 (CDR1 to CDR3) in the primary structure of each domain (N- to C-termini, from left to right) is indicated by grey shades, CDR residues are depicted in red, and framework residues in green. Random mutagenesis of the scFv using error-prone polymerase chain reaction (PCR) followed by ribosome-display affinity selections and screening for improved potency leads to an accumulation of mutations in a few distinct hotspots, located mainly in the CDRs. Their number and exact position vary considerably from case to case and can therefore not be predicted. With the power of display technologies to interrogate large mutant libraries, however, they can be identified without the need for any structural information. A frequency threshold (broken line) can be set to select hotspots for targeted saturation mutagenesis in a second-generation library followed by further rounds of selection.

When performed separately, random mutagenesis can help identify mutation hotspots, defined as amino acid residues mutated frequently in a population. To this end, a variant library generated by error-prone PCR, for example, might be subjected to affinity selections followed by the sequencing of improved scFvs. In a manner similar to somatic hypermutation, this method leads to the accumulation of mutations responsible for potency gains mainly in CDRs, despite having been introduced randomly throughout the whole scFv-coding sequence (Figure 2) [38]. The likelihood of non-human, potentially immunogenic residues in the conserved framework regions is therefore minimal; in any case, their contribution to the immunogenicity of therapeutic mAbs could not yet be fully elucidated [61]. It is notable that selection for increased folding efficiency or thermodynamic stability, for example, might result in a different distribution pattern. In any case, the number and location of these hotspots differ from antibody to antibody. Without the need for any structural information, the residues to be targeted preferentially in a semi-rational manner can thus be rapidly identified on a case-by-case basis. Although error-prone PCR only mutates a few of the hotspots in any one antibody, and the number of accessible amino acid variants is limited owing to the redundancy in the genetic code, they can now be fully randomized in parallel using oligonucleotide-based saturation mutagenesis. This enables the sampling of maximum sequence space and the creation and selection of mutations that might synergistically improve antibody potency further.

The continual need for display technologies and combinatorial libraries

Both targeted and random mutagenesis strategies have produced high-affinity antibodies *in vitro* [62]; however, because every antibody–antigen pair is different, it is not obvious which approach is superior with respect to the affinity achieved and the fold-improvement compared with the starting antibody. Although individually tailored strategies are, therefore, required each time, some general trends exist. After a set of key contact residues at the centre of the antigen-binding site has been selected during the antibody isolation phase, affinity maturation results in a tightening of the antibody–antigen interaction through more peripheral, or ‘second-sphere’ changes [38,63–65]. This involves the removal of sterically hindering side-chains, the addition of contact points and the reorientation of the binding site geometry [66]. Reducing the flexibility and enhancing the packing of the CDR loops might also decrease the entropy loss upon antigen binding [67,68]. Even distal mutations might provide the subtle structural alterations in the variable domains and their interface that are necessary to refine antibody–antigen interactions to low picomolar or femtomolar affinities [16,35,69]. Somatic hypermutation in addition to the approach presented in Figure 2 are, therefore, focussed on, but not limited to, the CDRs.

Despite the growing knowledge around antibody structures and protein–protein interactions, and the rapid development of *in silico* evolution, molecular modelling and protein–protein docking tools [70–73], it is still nearly

impossible to predict the multitude of mutations resulting in improved antibody potency. Moreover, specific structural information – on the antibody to be optimized, its antigen and their interaction – is rarely available or lacks the high resolution required to determine accurately important details such as side-chain conformations, hydrogen-bonding patterns and the position of water molecules. Therefore, the most effective way to improve antibody potencies remains the use of display technologies to interrogate large variant populations, using either targeted or random mutagenesis strategies. In either case, we recommend recombining beneficial mutations, followed by further rounds of selection to increase the chance of identifying synergistic potency gains. To reach high potencies, the use of ribosome display combined with random mutagenesis strategies should be envisaged, either alone or as part of a targeted approach, to enable additional sequence diversification during the selection process. We also recommend the semi-rational targeting of experimentally determined mutation hotspots for efficient potency optimisation without the need for any specific structural information. The combination of targeted, semi-rational and random approaches can finally contribute to the mapping of the precise antigen-binding site, also termed paratope, and help understand the specific molecular mode of action of the antibody [38].

A matured ribosome display technology, which has recently delivered the first optimized antibody to the clinic (CAT-354; <http://www.cambridgeantibody.com>), along with the well-established phage display have become the tools of choice for *in vitro* optimisation of antibodies. Elaborate mutagenesis strategies and strictly controlled selection conditions facilitate fast generation of a multitude of high potency antibody molecules. This enables the efficient development of safe and efficacious antibody drug candidates in an ever more competitive environment.

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References

- 1 Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497
- 2 Pavlou, A.K. and Belsey, M.J. (2005) The therapeutic antibodies market to 2008. *Eur. J. Pharm. Biopharm.* 59, 389–396
- 3 Reichert, J.M. *et al.* (2005) Monoclonal antibody successes in the clinic. *Nat. Biotechnol.* 23, 1073–1078
- 4 Baker, M. (2005) Upping the ante on antibodies. *Nat. Biotechnol.* 23, 1065–1072
- 5 Presta, L.G. (2005) Selection, design, and engineering of therapeutic antibodies. *J. Allergy Clin. Immunol.* 116, 731–736
- 6 Weiner, L.M. and Carter, P. (2005) Tunable antibodies. *Nat. Biotechnol.* 23, 556–557
- 7 Clark, M. (2000) Antibody humanization: a case of the ‘Emperor’s new clothes’? *Immunol. Today* 21, 397–402
- 8 Kellermann, S.A. and Green, L.L. (2002) Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Curr. Opin. Biotechnol.* 13, 593–597
- 9 Lonberg, N. (2005) Human antibodies from transgenic animals. *Nat. Biotechnol.* 23, 1117–1125
- 10 Rathanaswami, P. *et al.* (2005) Demonstration of an *in vivo* generated sub-picomolar affinity fully human monoclonal antibody to interleukin-8. *Biochem. Biophys. Res. Commun.* 334, 1004–1013

- 11 Foote, J. and Eisen, H.N. (1995) Kinetic and affinity limits on antibodies produced during immune responses. *Proc. Natl. Acad. Sci. U. S. A.* 92, 1254–1256
- 12 Edwards, B.M. *et al.* (2003) The remarkable flexibility of the human antibody repertoire; isolation of over one thousand different antibodies to a single protein, BlyS. *J. Mol. Biol.* 334, 103–118
- 13 Neuberger, M.S. (2002) Novartis Medal Lecture. Antibodies: a paradigm for the evolution of molecular recognition. *Biochem. Soc. Trans.* 30, 341–350
- 14 Razai, A. *et al.* (2005) Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. *J. Mol. Biol.* 351, 158–169
- 15 Rajpal, A. *et al.* (2005) A general method for greatly improving the affinity of antibodies by using combinatorial libraries. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8466–8471
- 16 Boder, E.T. *et al.* (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10701–10705
- 17 Colby, D.W. *et al.* (2004) Engineering antibody affinity by yeast surface display. *Methods Enzymol.* 388, 348–358
- 18 Feldhaus, M.J. and Siegel, R.W. (2004) Yeast display of antibody fragments: a discovery and characterization platform. *J. Immunol. Methods* 290, 69–80
- 19 Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228, 1315–1317
- 20 McCafferty, J. *et al.* (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552–554
- 21 Liu, B. *et al.* (2004) Mapping tumor epitope space by direct selection of single-chain Fv antibody libraries on prostate cancer cells. *Cancer Res.* 64, 704–710
- 22 Tordsson, J. *et al.* (1997) Efficient selection of scFv antibody phage by adsorption to *in situ* expressed antigens in tissue sections. *J. Immunol. Methods* 210, 11–23
- 23 Pasqualini, R. and Ruoslahti, E. (1996) Organ targeting *in vivo* using phage display peptide libraries. *Nature* 380, 364–366
- 24 Ling, M.M. (2003) Large antibody display libraries for isolation of high-affinity antibodies. *Comb. Chem. High Throughput Screen* 6, 421–432
- 25 Leemhuis, H. *et al.* (2005) New genotype–phenotype linkages for directed evolution of functional proteins. *Curr. Opin. Struct. Biol.* 15, 472–478
- 26 Kawasaki, G.H. (1990) Cell-free synthesis and isolation of novel genes and polypeptides, *European patent* 494955
- 27 Kawasaki, G.H. (1991) Cell-free synthesis and isolation of novel genes and polypeptides, *US patent* 5658754
- 28 Mattheakis, L.C. *et al.* (1994) An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. U. S. A.* 91, 9022–9026
- 29 Hanes, J. *et al.* (2000) Selecting and evolving functional proteins *in vitro* by ribosome display. *Methods Enzymol.* 328, 404–430
- 30 Douthwaite, J.A. *et al.* (2006) An improved method for an efficient and easily accessible eukaryotic ribosome display technology. *Protein Eng. Des. Sel.* 19, 85–90
- 31 Villemagne, D. *et al.* (2006) Highly efficient ribosome display selection by use of purified components for *in vitro* translation. *J. Immunol. Methods* 313, 140–148
- 32 Hawkins, R.E. *et al.* (1992) Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J. Mol. Biol.* 226, 889–896
- 33 Schier, R. *et al.* (1996) Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J. Mol. Biol.* 255, 28–43
- 34 Jermutus, L. *et al.* (2001) Tailoring *in vitro* evolution for protein affinity or stability. *Proc. Natl. Acad. Sci. U. S. A.* 98, 75–80
- 35 Zahnd, C. *et al.* (2004) Directed *in vitro* evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J. Biol. Chem.* 279, 18870–18877
- 36 Boder, E.T. and Wittrup, K.D. (1998) Optimal screening of surface-displayed polypeptide libraries. *Biotechnol. Prog.* 14, 55–62
- 37 Feldhaus, M. and Siegel, R. (2004) Flow cytometric screening of yeast surface display libraries. *Methods Mol. Biol.* 263, 311–332
- 38 Thom, G. *et al.* (2006) Probing a protein–protein interaction by *in vitro* evolution. *Proc. Natl. Acad. Sci. U. S. A.* 103, 7619–7624
- 39 Yelton, D.E. *et al.* (1995) Affinity maturation of the BR96 anti-carcinoma antibody by codon-based mutagenesis. *J. Immunol.* 155, 1994–2004
- 40 Osbourn, J.K. *et al.* (1996) Generation of a panel of related human scFv antibodies with high affinities for human CEA. *Immunotechnology* 2, 181–196
- 41 Schier, R. *et al.* (1996) Identification of functional and structural amino-acid residues by parsimonious mutagenesis. *Gene* 169, 147–155
- 42 Thompson, J. *et al.* (1996) Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. *J. Mol. Biol.* 256, 77–88
- 43 Ho, M. *et al.* (2005) *In vitro* antibody evolution targeting germline hot spots to increase activity of an anti-CD22 immunotoxin. *J. Biol. Chem.* 280, 607–617
- 44 Chen, Y. *et al.* (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J. Mol. Biol.* 293, 865–881
- 45 Jackson, J.R. *et al.* (1995) *In vitro* antibody maturation. Improvement of a high affinity, neutralizing antibody against IL-1 beta. *J. Immunol.* 154, 3310–3319
- 46 Yang, W.P. *et al.* (1995) CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* 254, 392–403
- 47 Schier, R. *et al.* (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol.* 263, 551–567
- 48 Wu, H. *et al.* (1998) Stepwise *in vitro* affinity maturation of Vitaxin, an alpha₃beta₃-specific humanized mAb. *Proc. Natl. Acad. Sci. U. S. A.* 95, 6037–6042
- 49 Marks, J.D. *et al.* (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N. Y.)* 10, 779–783
- 50 Knappik, A. *et al.* (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296, 57–86
- 51 Jirholt, P. *et al.* (2001) A central core structure in an antibody variable domain determines antigen specificity. *Protein Eng.* 14, 67–74
- 52 Minshull, J. and Stemmer, W.P. (1999) Protein evolution by molecular breeding. *Curr. Opin. Chem. Biol.* 3, 284–290
- 53 Fermer, C. *et al.* (2004) Specificity rescue and affinity maturation of a low-affinity IgM antibody against pro-gastrin-releasing peptide using phage display and DNA shuffling. *Tumour Biol.* 25, 7–13
- 54 Hanes, J. *et al.* (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat. Biotechnol.* 18, 1287–1292
- 55 Gram, H. *et al.* (1992) *In vitro* selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. U. S. A.* 89, 3576–3580
- 56 Daugherty, P.S. *et al.* (2000) Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2029–2034
- 57 Irving, R.A. *et al.* (2001) Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J. Immunol. Methods* 248, 31–45
- 58 Irving, R.A. *et al.* (1996) Affinity maturation of recombinant antibodies using *E. coli* mutator cells. *Immunotechnology* 2, 127–143
- 59 Low, N.M. *et al.* (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J. Mol. Biol.* 260, 359–368
- 60 Groves, M.A. and Osbourn, J.K. (2005) Applications of ribosome display to antibody drug discovery. *Expert Opin. Biol. Ther.* 5, 125–135
- 61 Pendley, C. *et al.* (2003) Immunogenicity of therapeutic monoclonal antibodies. *Curr. Opin. Mol. Ther.* 5, 172–179
- 62 Hoogenboom, H.R. (2005) Selecting and screening recombinant antibody libraries. *Nat. Biotechnol.* 23, 1105–1116

- 63 Foote, J. and Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487–499
- 64 Tomlinson, I.M. *et al.* (1996) The imprint of somatic hypermutation on the repertoire of human germline V genes. *J. Mol. Biol.* 256, 813–817
- 65 Ramirez-Benitez, M.C. and Almagro, J.C. (2001) Analysis of antibodies of known structure suggests a lack of correspondence between the residues in contact with the antigen and those modified by somatic hypermutation. *Proteins* 45, 199–206
- 66 Chong, L.T. *et al.* (1999) Molecular dynamics and free-energy calculations applied to affinity maturation in antibody 48G7. *Proc. Natl. Acad. Sci. U. S. A.* 96, 14330–14335
- 67 Manivel, V. *et al.* (2000) Maturation of an antibody response is governed by modulations in flexibility of the antigen-combining site. *Immunity* 13, 611–620
- 68 Sagawa, T. *et al.* (2003) Thermodynamic and kinetic aspects of antibody evolution during the immune response to hapten. *Mol. Immunol.* 39, 801–808
- 69 Midelfort, K.S. *et al.* (2004) Substantial energetic improvement with minimal structural perturbation in a high affinity mutant antibody. *J. Mol. Biol.* 343, 685–701
- 70 Park, S. *et al.* (2004) Advances in computational protein design. *Curr. Opin. Struct. Biol.* 14, 487–494
- 71 Vajda, S. and Camacho, C.J. (2004) Protein–protein docking: is the glass half-full or half-empty? *Trends Biotechnol.* 22, 110–116
- 72 Gray, J.J. (2006) High-resolution protein–protein docking. *Curr. Opin. Struct. Biol.* 16, 183–193
- 73 Lazar, G.A. *et al.* (2006) Engineered antibody Fc variants with enhanced effector function. *Proc. Natl. Acad. Sci. U. S. A.* 103, 4005–4010
- 74 Carmen, S. and Jermutus, L. (2002) Concepts in antibody phage display. *Brief Funct. Genomic Proteomic* 1, 189–203
- 75 Hoogenboom, H.R. (2002) Overview of antibody phage-display technology and its applications. *Methods Mol. Biol.* 178, 1–37
- 76 Bradbury, A.R. and Marks, J.D. (2004) Antibodies from phage antibody libraries. *J. Immunol. Methods* 290, 29–49
- 77 Conrad, U. and Scheller, J. (2005) Considerations on antibody-phage display methodology. *Comb. Chem. High Throughput Screen* 8, 117–126
- 78 Zhou, J.M. *et al.* (2002) A novel strategy by the action of ricin that connects phenotype and genotype without loss of the diversity of libraries. *J. Am. Chem. Soc.* 124, 538–543
- 79 Sawata, S.Y. *et al.* (2004) A system based on specific protein–RNA interactions for analysis of target protein–protein interactions *in vitro*: successful selection of membrane-bound Bak–Bcl-xL proteins *in vitro*. *Protein Eng. Des. Sel.* 17, 501–508
- 80 Roberts, R.W. (1999) Totally *in vitro* protein selection using mRNA–protein fusions and ribosome display. *Curr. Opin. Chem. Biol.* 3, 268–273
- 81 Kurz, M. *et al.* (2000) Psoralen photo-crosslinked mRNA–puromycin conjugates: a novel template for the rapid and facile preparation of mRNA–protein fusions. *Nucleic Acids Res.* 28, E83
- 82 Lipovsek, D. and Pluckthun, A. (2004) *In-vitro* protein evolution by ribosome display and mRNA display. *J. Immunol. Methods* 290, 51–67
- 83 He, M. and Khan, F. (2005) Ribosome display: next-generation display technologies for production of antibodies *in vitro*. *Expert Rev Proteomics* 2, 421–430
- 84 Douthwaite, J. and Jermutus, L. (2006) Exploiting directed evolution for the discovery of biologicals. *Curr. Opin. Drug Discov. Devel.* 9, 269–275
- 85 Rothe, A. *et al.* (2006) Ribosome display for improved biotherapeutic molecules. *Expert Opin. Biol. Ther.* 6, 177–187

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