In vitro display technologies: novel developments and applications

Patrick Amstutz, Patrik Forrer, Christian Zahnd and Andreas Plückthun*

In vitro display techniques are powerful tools to select polypeptide binders against various target molecules. Novel applications include maturation of protein affinity and stability, selection for enzymatic activity, and the display of cDNA and random polypeptide libraries. Taken together, these display techniques have great potential for biotechnological, medical and proteomic applications.

Addresses
Biochemisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
*e-mail: plueckthun@biocfebs.unizh.ch

Current Opinion in Biotechnology 2001, 12:400–405

0958-1669/01/$ – see front matter

Abbreviations
PCR polymerase chain reaction
scFv single-chain Fv

Introduction

The growing interest of the research community and pharmaceutical companies in protein–protein interactions has led to an increasing demand for sophisticated methods for the rapid identification, characterization, and potential improvement of interaction partners. The most popular of these methods, namely the yeast two-hybrid system [1] and phage display [2] (see the article by Sidhu and Pelletier in this issue pp 340–347), are limited by the involvement of living cells in the process of library generation or screening. This is not the case for *in vitro* selection technologies. In these techniques the number of molecules that can be handled is not limited by cellular transformation efficiencies; thus, very large libraries of up to 10^{14} members can be built. This feature also facilitates directed evolution experiments, in which rounds of randomization and selection alternate, as transformation can be avoided between rounds. Furthermore, special reagents such as the reducing agent dithiothreitol or detergents can be added to select binders under conditions chosen by the experimentor.

Two main groups of *in vitro* selection technologies can be distinguished. The first group imitates the compartmentalization of living cells by performing translation and selection within a water-in-oil emulsion [3–5]; this method was recently summarized in an excellent review [6]. This compartmentalization ensures the coupling of genotype and phenotype — a prerequisite for any selection method. The second group, the *in vitro* display technologies, makes use of a physical link between messenger RNA (mRNA) and nascent polypeptide during translation to couple genotype and phenotype. The most popular *in vitro* display technologies are ribosome display and mRNA display (reviewed in [7–12]). This review focuses on the recent advances in the field of *in vitro* display methods and discusses the potential of these technologies for future applications in basic and applied research.

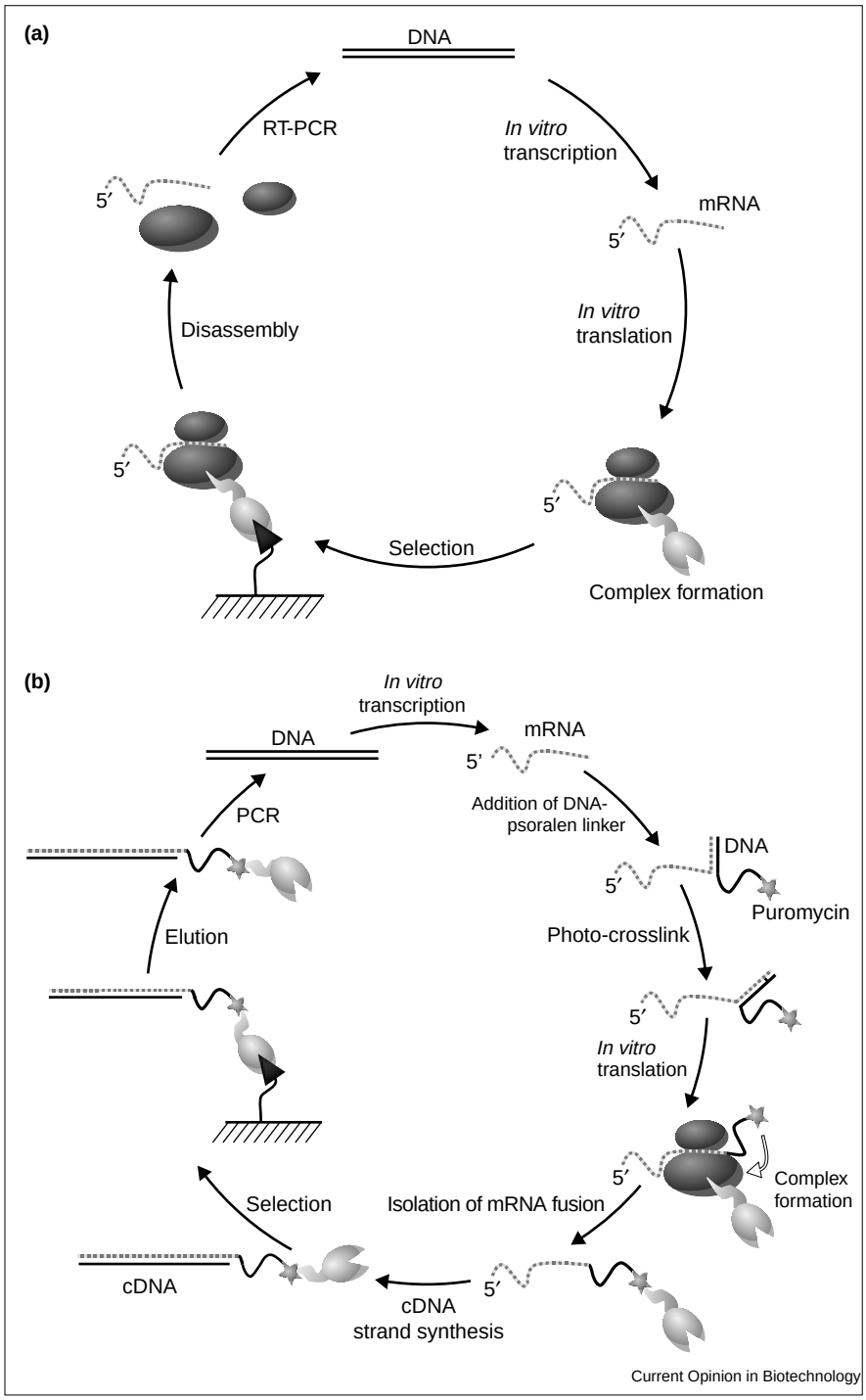
In vitro display technologies

Ribosome display (Figure 1a) was first developed by Mattheakis *et al.* [13] for the selection of peptides and further improved for the selection of folded proteins by Hanes and Plückthun [14] and He and Taussig [15]. This method relies on non-covalent ternary complexes of mRNA, ribosome and nascent polypeptide, ensuring the coupling of genotype and phenotype. A fusion protein is constructed in which the the domain of interest is fused to a C-terminal tether, such that this domain can fold while the tether is still in the ribosomal tunnel. This fusion construct lacks a stop codon at the mRNA level, thus preventing release of the mRNA and the polypeptide from the ribosome. High concentrations of magnesium and low temperature further stabilize the ternary complex. These complexes, which are formed during *in vitro* translation, can directly be used to select for the properties of the displayed protein.

The related technology of mRNA display (Figure 1b), which has also been termed 'mRNA–protein fusions' [16] or '*in vitro* virus' [17], was predominantly developed by Roberts and Szostak. This method relies on the covalent coupling of mRNA to the nascent polypeptide. The mRNA is first covalently linked to a short DNA linker carrying a puromycin moiety. The library is then translated *in vitro*, as in ribosome display. When the ribosome reaches the RNA–DNA junction the ribosome stalls and the puromycin moiety enters the peptidyltransferase site of the ribosome and forms a covalent linkage to the nascent polypeptide. The protein and the mRNA are thus coupled and are subsequently isolated from the ribosome and purified. In the current protocol, a cDNA strand is then synthesized to form a less sticky RNA–DNA hybrid and these complexes are finally used for selection.

The protocol of mRNA display has been significantly improved since it was first reported by extending the method from the display of short peptides to proteins [9]. The authors were also able to increase the yield of functional mRNA–protein fusions about 40-fold compared with the original protocol. Furthermore, the laborious linkage of mRNA to the puromycin-containing DNA linker, thus far an enzymatic ligation reaction, was also significantly improved [18,19]. In the new method, a DNA linker carrying a psoralen moiety is hybridized to the end of the mRNA and directly photo-crosslinked to the mRNA. These improvements have opened the door for mRNA display to handle complex protein libraries, as has been possible with ribosome display [11,15,20,21•,22••,23,24].

Figure 1



In vitro display technologies. (a) Schematic representation of a ribosome display selection round. A DNA library encoding the proteins of interest is genetically fused to a tether, which allows the protein to fold while the tether is still in the ribosomal tunnel. The resulting construct, which lacks a stop codon, is transcribed *in vitro* into mRNA and further translated *in vitro*. The translation is stopped such that stable ternary complexes of mRNA, ribosomes and nascent polypeptides are formed. These complexes are directly used for binding selection on the immobilized target. The mRNA of the bound complexes is rescued by dissociating the ribosome with EDTA. A reverse transcription reaction followed by PCR yields the genetic information of the selected clones. These clones can then be analyzed or used as input for the next selection round. (b) Schematic representation of a mRNA display selection round. A DNA library encoding the proteins of interest is transcribed *in vitro*. The resulting mRNA is covalently fused to a short DNA linker which carries a puromycin moiety at its 3′-end. This linking can be achieved by hybridization and subsequent photo-crosslinking of a psoralen-labeled DNA linker to the mRNA (as shown here) or by an enzymatic ligation reaction (not shown). The resulting construct is translated *in vitro*. During translation the ribosome reaches the RNA–DNA junction and stalls. This allows the puromycin moiety to bind to the ribosomal A site. Thereby, the nascent polypeptide is transferred to the puromycin moiety leading to a covalent mRNA–polypeptide fusion. The mRNA–fusion complex is subsequently purified and the first cDNA strand is synthesized. A selection for binding on immobilized ligand is then carried out and the bound complexes are eluted. The following PCR yields the genetic information of the selected clones. These clones can then be analyzed or used as input for the next selection round. In both (a) and (b), black lines correspond to DNA and dotted lines correspond to mRNA. In (b) the star represents the puromycin moiety. For simplicity, the crosslinked mRNA–DNA hybrid is not shown after the *in vitro* translation step.

The stability of mRNA was repeatedly discussed as a weak point of both ribosome and mRNA display, as RNA is susceptible to hydrolysis and nuclease degradation. Nevertheless, for ribosome display it has been shown that the ternary complexes are stable for up to at least 15 days (C Zahnd *et al.*, unpublished data). To improve the stability of mRNA display, Kurz *et al.* [25•] reported a method to replace the mRNA molecule within the mRNA–protein complex with its double-stranded cDNA. This methodology may be especially attractive for selections under harsh conditions (e.g. high temperatures).

Improved library quality by preselection

The success of selection experiments depends to a large extent on the quality of the library. Although the theoretical size of a library is virtually unlimited, the transformation

efficiency for yeast (10^7–10^8 cells/μg DNA) and for *Escherichia coli* (10^9–10^{10} cells/μg DNA) limits the achievable library size. By contrast, *in vitro* display technologies can handle libraries with up to 10^{14} members, depending only on the scale of the *in vitro* translation used. A common way to generate libraries involves the use of degenerate oligonucleotides; however, such oligonucleotides often contain deletions that result in frame-shifts and the appearance of stop codons, thus decreasing the effective library size. One way to avoid this is to use trinucleotides [26] as building blocks, because even if deletions do occur the correct reading frame is still maintained.

A generally useful approach to enlarge the percentage of correct (i.e. in-frame and full-length) clones in a library is to eliminate problematic sequences through preselection. Because of the large library size accessible in the *in vitro* display technologies, a useful library diversity is maintained even after preselection. The library is cloned between an N- and C-terminal tag and displayed such that selection for the occurrence of these tags will yield in-frame and full-length polypeptides. Premature stop codons cause the ribosome to dissociate, and frame-shifts will alter the C-terminal tag. Nonetheless, suppressor tRNA present in the extract or mistakes in the synthesis machinery appear to still let a fraction of undesired molecules slip through this 'filter'. Cho *et al.* [27•] performed such a preselection using mRNA display. In this way they were able to remove a large fraction of the non-functional proteins from a large-scale *in vitro* translation (10 ml), thus improving the proportion of correct proteins in three different libraries by up to two orders of magnitude. They still maintained a final complexity of about 10^{13} full-length molecules.

Selection experiments with two of these libraries have been published. From one such library, Keefe and Szostak [28••] were able to select polypeptides, presumably with a folded structure, that were able to bind ATP. This library consisted of a completely random stretch of 80 amino acids, which had been preselected as described above. All selected sequences were full-length, indicating that preselection was successful. From their results, the authors estimated the number of ATP binders in a random sequence to be 1 in 10^{11}, which underlines the necessity of having a high-quality full-length library, and a selection method capable of handling libraries of this size.

Using the second of these preselected libraries [27•], consisting of amphipathic α-helical or β-strand segments, Wilson *et al.* [29] selected for streptavidin binders. The highest affinity of the selected peptides for streptavidin was about 5 nM, in contrast to micromolar affinities of peptides selected in previous phage-display experiments [30]. However, all of the selected peptides were derived from frame-shifted sequences, even though preselection had been performed. Because the library was designed with restricted codon frequencies, such frame-shifted sequences had a 700-fold increased probability of containing the known streptavidin-binding consensus sequence His-Pro-Gln. These results also demonstrate how important the design of the initial library is, as the selected molecules had no similarity to the designed α-helical or β-stranded elements. Even though preselection was performed, the rare frame-shifted sequences prevailed over the much more abundant in-frame sequences.

Nevertheless, the preselection approach using *in vitro* display technologies may be a powerful tool for improving library quality in terms of enlarging the proportion of correct library members.

Directed evolution of proteins

Natural evolution has efficiently adapted proteins to their tasks under given environmental conditions. Nevertheless, the technological or medical application of proteins often places different demands on them; thus, their performance needs to be optimized. Using the Darwinian principle, evolution of polypeptides can now be conducted in the test tube: a pool of molecules (library) is subjected to alternating rounds of selection and randomization. If the randomization is carried out on the whole library, not only the original library is screened for the best molecules but the library composition is also adapted from round to round so that sequences not present in the original library become accessible to selection. With this approach, Hanes *et al.* [21•] selected, by ribosome display, a set of different antibody single-chain Fv (scFv) fragments from a synthetic naïve library, with up to 40-fold improved affinities when compared with the progenitor sequences present in the original library. All of the selected antibodies had accumulated mutations as a result of amplification with a low-fidelity DNA polymerase. This work demonstrated that protein evolution can be an intrinsic part of each ribosome display cycle. By including an additional diversification step in each round of ribosome display to increase the error rate even further, for example, by error-prone polymerase chain reaction (PCR) [31,32] (described in the article by Kurtzman *et al.* in this issue pp 361–370) or DNA shuffling [33], Jermutus *et al.* [22••] further confirmed the potential of ribosome display for directed *in vitro* protein evolution. They demonstrated that distinct and predictable biophysical characteristics of scFvs, affinity and stability, can be rapidly and efficiently evolved by combining these techniques (see below). Similarly, mRNA display in combination with error-prone PCR proved to be a powerful approach for *in vitro* evolution of proteins as shown by Keefe and Szostak [28••] (see above). Thus, the work of these groups demonstrated that the PCR amplification step inherent to ribosome and mRNA display can be directly exploited for *in vitro* protein evolution by relaxing the accuracy of pool replication during selection cycles. The large library size accessible by these *in vitro* display techniques further supports successful evolution experiments. Taken together, ribosome and mRNA display have great potential for directed protein evolution.

Maturation of protein affinity

Protein affinity maturation with molecular evolution technologies is an important step in producing selective and high-affinity binding proteins for applications in biotechnology and medicine [23]. Both ribosome display and mRNA display have allowed selection for binding proteins to a wide variety of targets, such as small compounds [22••,28••], peptides [20], whole proteins [21•,23,24,34••] or even a specific DNA structure [35••].

Recent work now also demonstrates that *in vitro* display technologies have great potential for the maturation of high-affinity protein binders. Hanes *et al.* [21•] isolated picomolar affinity scFvs from a synthetic naïve library by combining the intrinsic selection and evolution power of ribosome display. An 'off-rate' selection procedure may further favor protein affinity maturation [36]. In off-rate selection, a pool of polypeptides is bound to an immobilized ligand. By adding an excess of free ligand, every dissociating library member will be immediately trapped. After incubation, only those binders with the lowest off-rate will remain bound to the immobilized antigen. Thus, increasing the incubation time with the competitive ligand increases the selection pressure applied. As the on-rate normally only changes within a relatively small window, lowering the off-rate will result in increased binding affinity. Using such off-rate selections over a period of up to ten days, Jermutus *et al.* [22••] were able to improve an antifluorescein scFv that already had a high initial affinity of 1.1 nM a further 30-fold. Using the same strategy, peptide-binding scFvs were evolved to affinities in the low picomolar range (C Zahnd *et al.*, unpublished data). These results demonstrate that off-rate selection is a valuable tool to select high-affinity binders from libraries. Interestingly, the mRNA — normally thought to be a very labile entity — was stable under these experimental conditions for more than 15 days.

Taken together, these results demonstrate that *in vitro* display technologies are not only valuable tools for the selection of binding molecules, but also for protein affinity maturation — either of a given molecule or in conjunction with the selection process from the initial library. This may have important implications in biotechnological and medical applications.

Maturation of protein stability

A common requirement for most biotechnological and medical applications of proteins is that they possess an intrinsic high stability against denaturation. Stability engineering is still a difficult task [37–40]. Evolutionary methods to perform stability engineering have shown promise, especially methods that employ phage display (reviewed in [41]). In a model system using antibody scFv fragments, Jermutus *et al.* [22••] have shown that ribosome display may be a valuable tool for *in vitro* evolution of protein stability. Antibody scFv fragments were evolved that are stable in the absence of disulfide bonds, which are normally required for their stability [37]. When the disulfide bond was allowed to reform, these scFv mutants were more stable than the corresponding wild-type protein, as indicated by urea denaturation experiments. They gave higher yields of functional protein upon periplasmic expression in *E. coli*, where disulfide bonds do form. Most importantly, the selected scFv mutants could also be functionally expressed in the reducing environment of the cytoplasm; an uncommon feature of an antibody scFv fragment. This study illustrates the versatility of the ribosome display approach — expression and selection can take place in a cytoplasmic-like environment, when dithiothreitol is added. Such stable and well-behaved antibodies might find application in tumor targeting [42] and as effective intrabodies [43,44] for the intracellular inactivation of proteins. Stability engineering of proteins by using heat or proteases as selection pressure (reviewed in [41]) may also be achievable with *in vitro* display technologies, especially for mRNA display.

Selection for enzymatic activity

It has been stated several times [6,41,45,46] that a combination of directed evolution and the use of display technologies provides a powerful strategy to evolve improved biocatalysts. Although it is known that enzymes can be functionally displayed on the ribosome [47], ribosome display had so far not been used to select for enzymatic activity. In this technique the genetic information (i.e. the mRNA) is not covalently attached to the protein. Thus, the mRNA can be simply eluted, even in applications based on suicide inhibitors, where the selected protein is covalently bound to the target. P Amstutz *et al.* (unpublished data) have performed a selection for enzymatic activity using ribosome display. Using a β-lactamase suicide inhibitor, an active RTEM-β-lactamase was successfully enriched over an inactive mutant. In these experiments the efficiency of activity selection was comparable to selection for affinity using a β-lactamase ligand. Overall, *in vitro* display methods may open new roads for the selection of catalytically active proteins.

Display of cDNA products

Phage display and two-hybrid systems are well-established methods to screen or select cDNA libraries for binders [1,2,48,49]. Recently, two groups investigated the potential of *in vitro* display techniques for the display of cDNA products. Bieberich *et al.* [50] reported the specific isolation of the cDNA of sialyltransferase II by functional binding of the encoded enzyme to its substrate, ganglioside GD3, in a single-tube coupled ribosome display system. It remains unclear, however, if their ribosome display construct is free of a stop codon and if it contains an appropriate C-terminal tether. The demonstration that ribosome display can be performed in a single well of a microtiter plate may have implications for proteomic applications where automation and high-throughput screening are essential. By using cDNA product libraries displayed on mRNA, Hammond *et al.* [34••] isolated both previously known and several novel binders of the antiapoptotic protein Bcl-X_L. The binding affinities of these isolated proteins ranged from approximately 2 nM to 10 μM.

In contrast to phage display or two-hybrid systems, *in vitro* display techniques are not biased by cytotoxic or secretion-incompatible cDNA products. In addition, *in vitro* display libraries can be preselected (see above) to improve their quality. Taken together, *in vitro* display of cDNA product libraries may be an interesting approach for proteomic applications, where the ultimate objectives are to functionally display all proteins and to minimize any selection or expression bias.

Conclusions

In vitro display technologies, namely ribosome and mRNA display, prove to be valuable tools for many applications other than merely selecting polypeptide binders. They have great potential for directed evolution of protein stability and affinity, the generation of high-quality libraries by *in vitro* preselection, the selection of enzymatic activities, and the display of cDNA and random-peptide libraries. In addition, these technologies have several features that should make them amenable to standardization and automation: they comprise fast selection cycles, allow the processing of huge libraries, are not limited by cellular transformations, and are not biased by *in vivo* environments. We envision that *in vitro* display technologies will have a great impact on applications in biotechnology, medicine and proteomics.

Acknowledgements

We thank Markus Kurz and Philip W Hammond for sharing unpublished results and Stephen F Marino and Christiane Schaffitzel for critically reading the manuscript and helpful suggestions.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Mendelsohn AR, Brent R: Protein interaction methods – toward an endgame. *Science* 1999, 284:1948-1950.

2. Dunn IS: Phage display of proteins. *Curr Opin Biotechnol* 1996, 7:547-553.

3. Ghadessy FJ, Ong JL, Holliger P: Directed evolution of polymerase function by compartmentalized self-replication. *Proc Natl Acad Sci USA* 2001, 98:4552-4557.

4. Doi N, Yanagawa H: STABLE: protein–DNA fusion system for screening of combinatorial protein libraries ***in vitro***. *FEBS Lett* 1999, 457:227-230.

5. Tawfik DS, Griffiths AD: Man-made cell-like compartments for molecular evolution. *Nat Biotechnol* 1998, 16:652-656.

6. Griffiths AD, Tawfik DS: Man-made enzymes – from design to ***in vitro*** compartmentalisation. *Curr Opin Biotechnol* 2000, 11:338-353.

7. Plückthun A, Schaffitzel C, Hanes J, Jermutus L: ***In vitro*** selection and evolution of proteins. *Adv Protein Chem* 2000, 55:367-403.

8. Hanes J, Jermutus L, Plückthun A: Selecting and evolving functional proteins ***in vitro*** by ribosome display. *Methods Enzymol* 2000, 328:404-430.

9. Liu R, Barrick JE, Szostak JW, Roberts RW: Optimized synthesis of RNA–protein fusions for ***in vitro*** protein selection. *Methods Enzymol* 2000, 318:268-293.

10. Roberts RW: Totally ***in vitro*** protein selection using mRNA–protein fusions and ribosome display. *Curr Opin Chem Biol* 1999, 3:268-273.

11. Schaffitzel C, Hanes J, Jermutus L, Plückthun A: Ribosome display: an ***in vitro*** method for selection and evolution of antibodies from libraries. *J Immunol Methods* 1999, 231:119-135.

12. Jermutus L, Ryabova LA, Plückthun A: Recent advances in producing and selecting functional proteins by using cell-free translation. *Curr Opin Biotechnol* 1998, 9:534-548.

13. Mattheakis LC, Bhatt RR, Dower WJ: An ***in vitro*** polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci USA* 1994, 91:9022-9026.

14. Hanes J, Plückthun A: ***In vitro*** selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* 1997, 94:4937-4942.

15. He M, Taussig MJ: Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for ***in vitro*** display and evolution of antibody combining sites. *Nucleic Acids Res* 1997, 25:5132-5134.

16. Roberts RW, Szostak JW: RNA–peptide fusions for the ***in vitro*** selection of peptides and proteins. *Proc Natl Acad Sci USA* 1997, 94:12297-12302.

17. Nemoto N, Miyamoto-Sato E, Husimi Y, Yanagawa H: ***In vitro*** virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome ***in vitro***. *FEBS Lett* 1997, 414:405-408.

18. Kurz M, Kuang G, Lohse PA: An efficient synthetic strategy for the preparation of nucleic acid-encoded peptide and protein libraries for ***in vitro*** evolution protocols. *Molecules* 2000, 5:1259-1264.

19. Kurz M, Kuang G, Lohse PA: Psoralen photo-crosslinked mRNA–puromycin conjugates: a novel template for the rapid and facile preparation of mRNA–protein fusions. *Nucleic Acids Res* 2000, 28:E83.

20. Hanes J, Jermutus L, Weber-Bornhauser S, Bosshard HR, Plückthun A: Ribosome display efficiently selects and evolves high-affinity antibodies ***in vitro*** from immune libraries. *Proc Natl Acad Sci USA* 1998, 95:14130-14135.

21. • Hanes J, Schaffitzel C, Knappik A, Plückthun A: Picomolar affinity antibodies from a fully synthetic naïve library selected and evolved by ribosome display. *Nat Biotechnol* 2000, 18:1287-1292.
The authors selected a range of different scFvs with affinities up to 82 pM from a fully synthetic naïve antibody scFv library using ribosome display. All of the selected antibodies accumulated beneficial mutations throughout the selection cycles. This work demonstrated that ribosome display not only allows the selection of library members but also further evolves them, thereby mimicking the strategy of the immune system.

22. •• Jermutus L, Honegger A, Schwesinger F, Hanes J, Plückthun A: Tailoring ***in vitro*** evolution for protein affinity or stability. *Proc Natl Acad Sci USA* 2001, 98:75-80.
The authors demonstrate the potential of ribosome display for directed *in vitro* protein evolution. By combining ribosome display with DNA shuffling the authors improved an scFv 30-fold to a final affinity of 40 pM, using off-rate selections over a period of several days. In a second set of similar experiments they evolved an scFv to be functionally expressed under reducing conditions. Under these conditions the scFv evolved novel stabilizing structures to compensate for the loss of the disulfide bonds. The selected mutants, when allowed to reform disulfide bonds, showed improved stability (from an initial 24 kJ/mol to 54 kJ/mol).

23. Irving RA, Coia G, Roberts A, Nuttall SD, Hudson PJ: Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J Immunol Methods* 2001, 248:31-45.

24. He M, Menges M, Groves MA, Corps E, Liu H, Brüggemann M, Taussig MJ: Selection of a human anti-progesterone antibody fragment from a transgenic mouse library by ARM ribosome display. *J Immunol Methods* 1999, 231:105-117.

25. • Kurz M, Gu K, Al-Gawari A, Lohse PA: cDNA–protein fusions: covalent protein–gene conjugates for the ***in vitro*** selection of peptides and proteins. *Chem Biochem* 2001, in press.
The authors describe a method to replace the mRNA in the mRNA–protein fusion with its cDNA. The cDNA–protein complex is significantly more stable than the corresponding mRNA complex.

26. Virnekäs B, Ge L, Plückthun A, Schneider KC, Wellnhofer G, Moroney SE: Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucleic Acids Res* 1994, 22:5600-5607.

27. • Cho G, Keefe AD, Liu R, Wilson DS, Szostak JW: Constructing high complexity synthetic libraries of long ORFs using ***in vitro*** selection. *J Mol Biol* 2000, 297:309-319.
This paper describes a strategy for improving protein libraries, by selecting against frame-shifts and internal stop codons. mRNA display was used to perform such preselection of three libraries: a random sequence, a patterned sequence and an $(\alpha/\beta)_8$ (TIM) barrel library. Modules of these libraries were inserted between a C-terminal and N-terminal polypeptide tag. Selection for these tags yielded full-length in-frame protein modules, which were subsequently combined to form the libraries for selection. Using this strategy the proportion of correct full-length library members was increased by up to two orders of magnitude. The final library, originating from a 10 ml *in vitro* translation, had a complexity of around 10^{13} members.

28. •• Keefe AD, Szostak JW: Functional proteins from a random-sequence library. *Nature* 2001, 410:715-718.
From a library of 6 x 10^{12} polypeptides, consisting of a stretch of 80 completely random amino acids, four new ATP-binding folds were selected by mRNA display. By further mutagenesis and selection the behavior of these proteins was improved, yielding specific binders with affinities up to 100 nM. One selected fold seems to have a fold-stabilizing Zn^{2+}-binding site.

29. Wilson DS, Keefe AD, Szostak JW: The use of mRNA display to select high-affinity protein-binding peptides. *Proc Natl Acad Sci USA* 2001, 98:3750-3755.

30. Schmidt TG, Koepke J, Frank R, Skerra A: Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. *J Mol Biol* 1996, 255:753-766.

31. Zaccolo M, Gherardi E: The effect of high-frequency random mutagenesis on ***in vitro*** protein evolution: a study on TEM-1 β-lactamase. *J Mol Biol* 1999, 285:775-783.

32. Cadwell RC, Joyce GF: Mutagenic PCR. *PCR Methods Appl* 1994, 3:S136-S140.

33. Minshull J, Stemmer WP: Protein evolution by molecular breeding. *Curr Opin Chem Biol* 1999, 3:284-290.

34. •• Hammond PW, Alpin J, Rise CE, Wright M, Kreider BL: ***In vitro*** selection and characterization of Bcl-X_L-binding proteins from a mix of tissue-specific mRNA display libraries. *J Biol Chem* 2001, 276:20898-20906.
After four rounds of mRNA display using uniquely tagged cDNA libraries from different tissues, 71 protein binders were selected against the anti-apoptotic protein Bcl-X_L. Of these, only eight were identified as false positives, as they were derived from introns or wrong reading frames. In addition to known binders of Bcl-X_L, several proteins not previously demonstrated to interact with Bcl-X_L were identified and their biological relevance can now be tested. This is the first successful report where novel binders of a target protein were selected from a cDNA product library by mRNA display, illustrating the potential of *in vitro* display technologies for proteomic applications.

35. •• Schaffitzel C, Berger I, Postberg J, Hanes J, Lipps HJ, Plückthun A: ***In vitro*** generated antibodies specific for telomeric guanine-quadruplex DNA react with ***Stylonychia lemnae macronuclei***. *Proc Natl Acad Sci USA* 2001, in press.
This paper describes the ribosome display selection of high-affinity antibodies specific for guanine quadruplex DNA from a naïve library. Antibody scFv fragments recognizing different conformations of this DNA structure were selected and applied *in vivo* in ciliates. This work provides the first evidence for the occurrence of guanine quadruplex DNA in macronuclei of the ciliate *Stylonychia lemnae*.

36. Hawkins RE, Russell SJ, Winter G: Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol* 1992, 226:889-896.

37. Wörn A, Plückthun A: Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 2001, 305:989-1010.

38. Vieille C, Zeikus GJ: Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol Mol Biol Rev* 2001, 65:1-43.

39. Lehmann M, Pasamontes L, Lassen SF, Wyss M: The consensus concept for thermostability engineering of proteins. *Biochim Biophys Acta* 2000, 1543:408-415.

40. Colacino F, Crichton RR: Enzyme thermostabilization: the state of the art. *Biotechnol Genet Eng Rev* 1997, 14:211-277.

41. Forrer P, Jung S, Plückthun A: Beyond binding: using phage display to select for structure, folding and enzymatic activity in proteins. *Curr Opin Struct Biol* 1999, 9:514-520.

42. Houghton AN, Scheinberg DA: Monoclonal antibody therapies — a 'constant' threat to cancer. *Nat Med* 2000, 6:373-374.

43. Cattaneo A, Biocca S: The selection of intracellular antibodies. *Trends Biotechnol* 1999, 17:115-121.

44. Chames P, Baty D: Antibody engineering and its applications in tumor targeting and intracellular immunization. *FEMS Microbiol Lett* 2000, 189:1-8.

45. Petrounia IP, Arnold FH: Designed evolution of enzymatic properties. *Curr Opin Biotechnol* 2000, 11:325-330.

46. Olsen M, Iverson B, Georgiou G: High-throughput screening of enzyme libraries. *Curr Opin Biotechnol* 2000, 11:331-337.

47. Kolb VA, Makeyev EV, Spirin AS: Co-translational folding of a eukaryotic multidomain protein in a prokaryotic translation system. *J Biol Chem* 2000, 275:16597-16601.

48. Santi E, Capone S, Mennuni C, Lahm A, Tramontano A, Luzzago A, Nicosia A: Bacteriophage λ display of complex cDNA libraries: a new approach to functional genomics. *J Mol Biol* 2000, 296:497-508.

49. Fields S: Proteomics. Proteomics in genomeland. *Science* 2001, 291:1221-1224.

50. Bieberich E, Kapitonov D, Tencomnao T, Yu RK: Protein-ribosome-mRNA display: affinity isolation of enzyme-ribosome-mRNA complexes and cDNA cloning in a single-tube reaction. *Anal Biochem* 2000, 287:294-298.