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Ribosome-display technology: applications for directed evolution of functional proteins

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In vitro **display technologies, especially ribosome display, are valuable tools for many applications. In this paper, ribosome display technology and its applications for directed evolution of functional proteins will be reviewed. Ribosome display has 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. Ribosome display is carried out fully *in vitro*, which overcomes some of the limitations of cell-based display systems. We anticipate that ribosome display will have a great impact on applications in biotechnology, medicine and proteomics.**

In the past decade, recombinant antibody engineering has emerged as one of the most promising approaches for the design, selection and production of reagents for basic research, medicine and the pharmaceutical industry [1–3]. Now, the first antibody derived from phage display technology has been approved in the USA by the FDA (Table 1). Antibody engineering is continuously developing new and robust discovery platforms and novel antibody formats, which points to the versatility of antibodies as therapeutic and diagnostic agents.

The *in vitro* selection and evolution of antibody library technology now provides us with powerful tools for producing antibodies without using animals and the human immune system. Several display methods have been used for antibody selection. They include cell-based systems, such as phage display [4] and cell-surface display [5,6], and cell-free methods, such as ribosome display [7–9] and mRNA display [10].

In the last few years, cell-free display technologies, in particular ribosome display technology, have been gaining attention from researchers from different areas of science [7]. Many of the limitations of phage display (such as the inability to select under conditions different from the cellular environment, problems with the selection of proteins that are toxic, cells circumventing selection pressure and low transformation efficiency) can be circumvented by using *in vitro* ribosome display [11], a strategy initially described for peptides by Mattheakis *et al.* [7]. This was followed by Gersuk *et al.* [12] who selected high-affinity peptide ligands to prostate-specific antigen from a random peptide library. Ribosome display has the prospect of becoming an increasingly important tool in the advancement of antibody therapeutics and can become a very powerful method for the directed evolution of proteins.

The principle of ribosome display

Ribosome display is a technique used to perform *in vitro* protein evolution to create proteins that can bind to a desired ligand (Figure 1). The process results in translated proteins that are associated with their mRNA progenitor that is used, as a complex, to bind to an immobilized ligand in a selection step. The mRNA–protein hybrids that bind well are then reverse-transcribed to cDNA and their sequence amplified using PCR. The end result is a nucleotide sequence that can be used to create tightly binding proteins. The key issue of the strategy is dependent on coupling individual nascent proteins to their corresponding mRNA for selection. This selection enables the isolation of proteins against virtually any antigen, including self-molecules, certain drugs, potent toxins and haptens, which would normally be impossible to raise with *in vivo*-based systems because of toxicity and/or lack of immunogenicity [13–15]. Molecular evolution can be mimicked

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TABLE 1

US and EU therapeutic mAb approvals to date[a]

Generic	Company/location	Trade	Description	Therapeutic category	Approval date
Muromonab-CD3	Johnson and Johnson	Orthoclone OKT3	Murine, IgG2a, anti-CD3	Immunological	19/06/1986 (US)
Abciximab	Centocor	ReoPro	Chimeric, IgG1, anti-GPIIb/IIIa; Fab	Hemostasis	22/12/1994 (US)
Rituximab	Genentech	Rituxan	Chimeric, IgG1κ, anti-CD20	Oncological	26/11/1997 (US) 02/06/1998 (EU)
Daclizumab	Hoffmann-La Roche	Zenapax	Humanized, IgG1κ, anti-CD25	Immunological	10/12/1997 (US) 26/02/1999 (EU)
Basiliximab	Novartis	Simulect	Chimeric, IgG1κ, anti-CD25	Immunological	12/05/1998 (US) 09/10/1998 (EU)
Palivizumab	MedImmune	Synagis	Humanized, IgG1κ, anti-respiratory syncytial virus	Anti-infective	19/06/1998 (US) 13/08/1999 (EU)
Infliximab	Centocor	Remicade	Chimeric, IgG1κ, anti-tumor necrosis factor (TNFα)	Immunological	24/08/1998 (US) 13/08/1999 (EU)
Trastuzumab	Genentech	Herceptin	Humanized, IgG1κ, anti-HER2	Oncological	25/09/1998 (US) 28/08/2000 (EU)
Gemtuzumab ozogamicin	Wyeth	Mylotarg	Humanized, IgG4κ, anti-CD33; immunotoxin	Oncological	17/05/2000 (US)
Alemtuzumab	Genzyme	Campath-1H	Humanized, IgG1κ, anti-CD52	Oncological	07/05/2001 (US) 06/07/2001 (EU)
Ibritumomab tiuxetan	Biogen Idec	Zevalin	Murine, IgG1κ, anti-CD20; radiolabeled (Yttrium 90)	Oncological	19/02/2002 (US) 16/01/2004 (EU)
Adalimumab	Abbott	Humira	Human, IgG1κ, anti-TNFα	Immunological	31/12/2002 (US) 1/09/2003 (EU)
Omalizumab	Genentech	Xolair	Humanized, IgG1κ, anti-IgE	Immunological	20/06/2003 (US)
Tositumomab-I131	Corixa	Bexxar	Murine, IgG2aλ, anti-CD20; radiolabeled (Iodine 131)	Oncological	27/06/2003 (US)
Efalizumab	Genentech	Raptiva	Humanized, IgG1κ, anti-CD11a	Immunological	27/10/2003 (US) 20/09/2004 (EU)
Cetuximab	Imclone Systems	Erbitux	Chimeric, IgG1κ, anti-Epidermal growth factor receptor	Oncological	12/02/2004 (US) 29/06/2004 (EU)
Bevacizumab	Genentech	Avastin	Humanized, IgG1, anti-vascular endothelial growth factor	Oncological	26/02/2004 (US) 12/01/2005 (EU)
Natalizumab[a]	Biogen Idec	Tysabri	Humanized, IgG4κ, anti-α4-integrin	Immunological	23/11/2004 (US)

[a] Adapted from Ref. [47].

in vitro either by using an error-prone polymerase or by shuffling the library DNA between rounds of selection to make new combinations of antibody fragments. Generally, the number and affinity of the antibodies generated to a particular antigen is a function of library size and diversity, with larger libraries yielding a greater number of high-affinity antibodies [16,17].

Ribosome display offers two important advantages. First, large libraries can be made rapidly because there is no need to transform large numbers of mutant plasmids into a host and, second, additional mutations can be introduced at every round because a PCR step is included in each selection cycle rather than an *in vivo* amplification step [18].

Construction and screening of ribosome-displayed antibody libraries

Construction of scFv ribosome display libraries

One of the most powerful applications of ribosome display has been the isolation of recombinant antibodies with high affinity specificity. In this regard, ribosome display technology can be used: (i) to isolate human antibodies from patients exposed to certain viral pathogens to understand better the immune response during infection and how protective antibodies are generated; (ii) to generate human antibodies, significant for cancer immunotherapy; and (iii) elucidate the specificity of autoimmune antibodies. A protocol of production of human single-chain Fv antibodies (scFvs) by ribosome display is established by He *et al.* [9,19,20] (Figure 2). With a cell-free display system, all the steps are nucleic-acid-based, which this allows the assembly of a large library with up to 1×10^{15} members. In ribosome-display strategy, naïve repertoires [8], immune repertoires [21] and synthetic repertoires [22] are the three main types of libraries.

Choice of cell-free system

The choice of a cell-free system for protein synthesis is generally based on the origin of the proteins to be expressed and their downstream applications. The commonly used rabbit reticulocyte lysates, wheat germ and *Escherichia coli* S30 extracts are commercially available in both coupled and uncoupled formats (coupled cell-free expression systems use DNA as a template, uncoupled cell-free expression systems use RNA as a template). Coupled systems

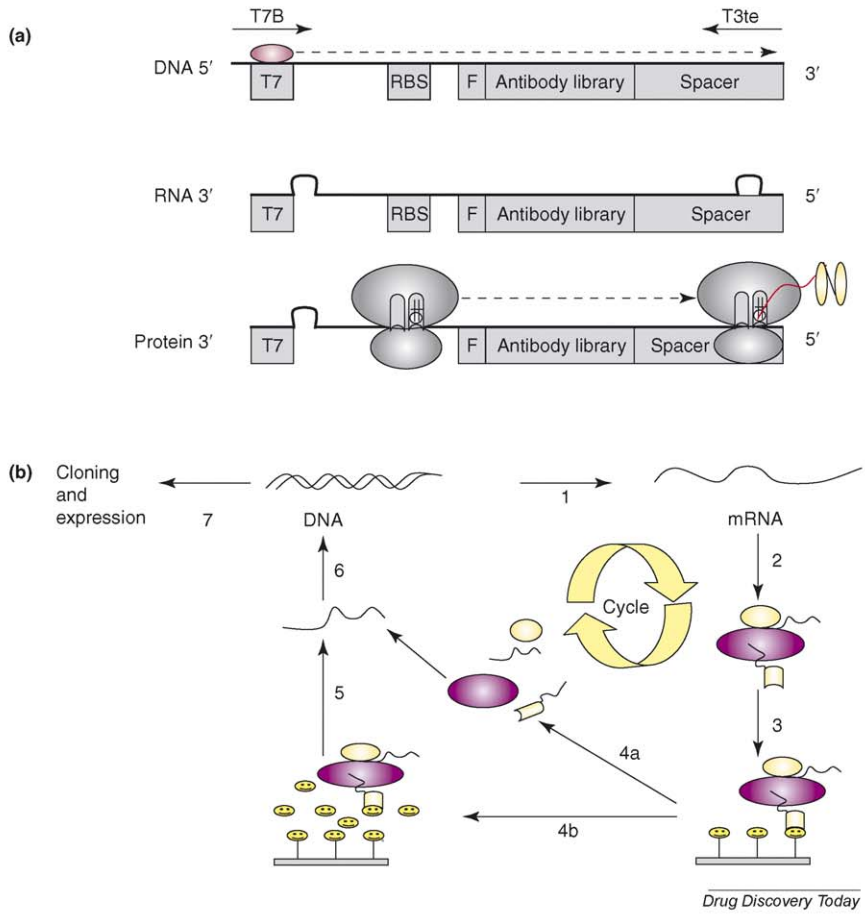

FIGURE 1

Principle of *in vitro* ribosome display. Library construction **(a)** and selection cycle **(b)**. DNA is amplified using terminal primers and is transcribed by T7 RNA polymerase. RNA forms stem loops which promote ribosome binding to the ribosome binding site (RBS). The ribosome translates RNA into a polypeptide consisting of a FLAG tag (F), the antibody protein and a spacer peptide and remains bound due to the absence of a stopsignal codon. (b) Step 1, a DNA scFv library is first amplified by PCR, whereby a T7 promoter, ribosome binding site, and stem-loops are introduced, and then transcribed to RNA. Step 2, after purification, mRNA is translated *in vitro* in an *Escherichia coli* S-30 system in the presence of different factors enhancing the stability of ribosomal complexes and improving the folding of the scFv antibodies on the ribosomes. Step 3, the desired ribosome complexes are affinity selected from the translation mixture by binding of the native scFv to the immobilized antigen. Step 4, the bound ribosome complexes can then be dissociated by EDTA (step 4b), or whole complexes can be specifically eluted with antigen (step 4a). Step 5, RNA is isolated from the complexes. Step 6, isolated mRNA is reverse transcribed to cDNA, and cDNA is then amplified by PCR. Step 7, cloning and expression.

are generally simpler and more efficient; they also avoid problems of mRNA degradation and mRNA secondary structure [23].

The choice of cell-free system can affect the production of a particular protein. Parallel expression of five different coding sequences of bacterial and eukaryotic origin in either *E. coli* S30 extract, wheat germ extract or rabbit reticulocyte lysate systems has revealed that, although predominantly full-length products of all five sequences tested were generated in the two eukaryotic systems, many incomplete nascent polypeptides accompanied the full-length product in *E. coli* S30 extracts [24,25]. It was suggested that the generation of these incomplete polypeptides was caused by pausing of the *E. coli* ribosome [25]. In addition, co- and post-translational modifications can only be carried out in eukaryotic systems [24]. A comparison between the efficiency of *E. coli* ribosome-display system and coupled rabbit reticulocyte ribosome-display system showed the efficiency of the *E. coli* ribosome display system was 100-fold [26].

Applications of ribosome display in drug discovery

Because ribosome display avoids the problems of cytotoxicity, soluble protein expression and secretion bias in cell-based systems, it could be an ideal means by which to display functional (single chain) proteins for drug discovery applications like target discovery and functional identification. With the completion of genome sequences, it is possible to design general cDNA libraries for ribosome display. In combination with high-throughput protein arrays [27,28], the screening power of ribosome display could be further increased, permitting library-versus-library screening and genome-wide analysis of protein–protein interactions. Because the antibody–ribosome–mRNA (ARM)display procedure is contained within a single tube [9,29], it could be developed into an automated process for proteomics applications where high-throughput manipulation is necessary. A reconstituted cell-free system has been described [30] that could be useful for further optimization and improvement of ribosome-display technology.

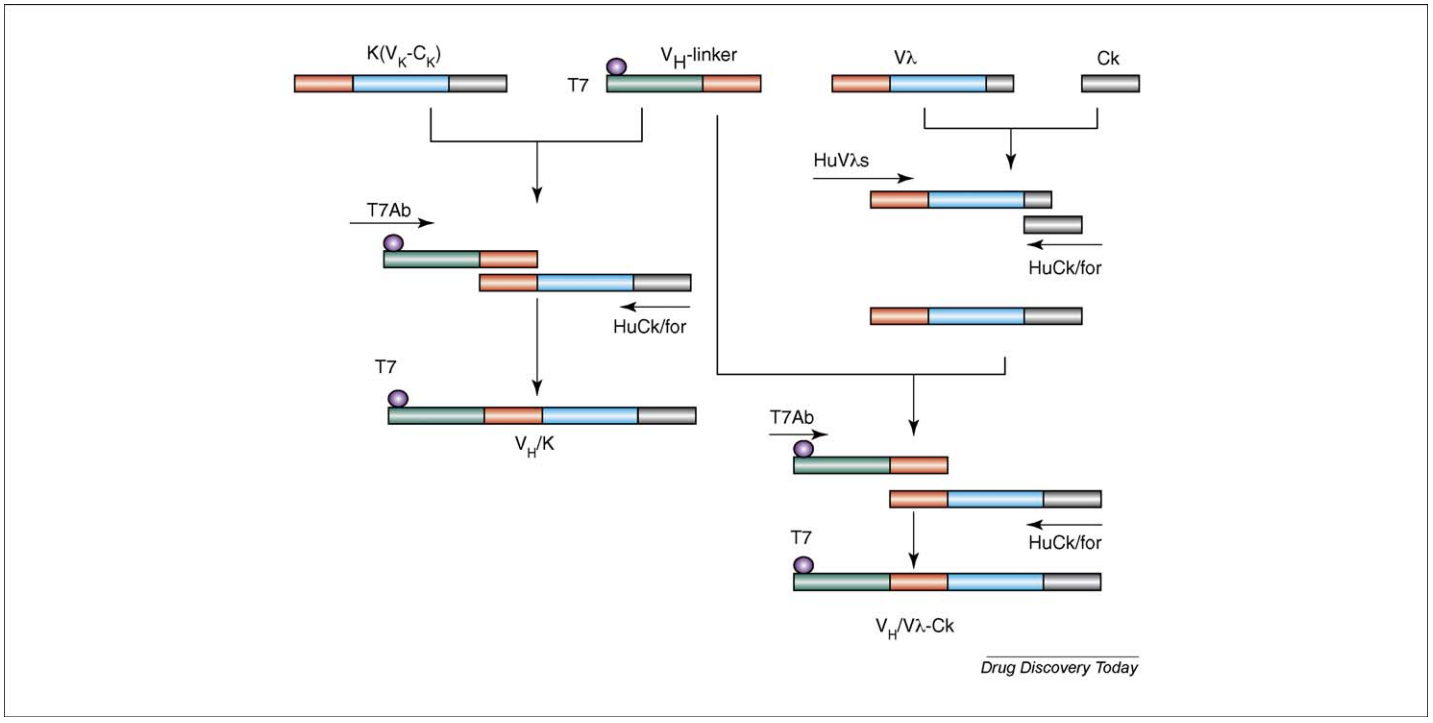

FIGURE 2

A strategy for the construction of V_H/K and V_H/Vλ-Cκ PCR libraries from mRNA. V_H/K is generated by linking the heavy-chain variable domain (VH) to the complete κ chain. V_H/Vλ-Cκ is made by linking VH to a fusion of variable region of λ chain (Vλ) and constant region of κ chain (Cκ). The heavy-chain 'elbow' region, which is a continuation of the VH domain, is used as the peptide linker to join heavy chains with light chains. The Cκ domain provides a spacer region to allow an antibody fragment to be displayed on the surface of ribosome and to offer priming sites for RT-PCR recovery after selection. Adapted, with permission from Ref. [20].

Ribosome display has been exemplified in both prokaryotic and eukaryotic systems and successfully used for selection and evolution of peptides [7,31–34], single-chain antibodies [8,9,11,19,20,35], enzymes [36,37], and ligand-binding proteins [38–42].

Peptides

Mattheakis *et al.* [7] first published the concept of a polysome display system for identifying ligands from very large peptide libraries. They successfully constructed a pool of DNA sequences encoding 10^{12} random decapeptides in an *E. coli* S30 coupled transcription–translation system, the binding affinities of enriched peptides for mAb D32.39 ranged from 7.2 nM to 140 nM. Lamla and Erdmann [31] constructed a synthetic library based on the scaffold of bovine heart fatty-acid-binding protein (FABP) with 1.1×10^{14} independent members. Ribosome display was applied to select streptavidin-binding peptides *in vitro* from 2×10^{13} molecules of the library each encoding FABP with 15 contiguous random amino acid residues at its N terminus. The selection yielded several different binding peptides. The best binder possessed a dissociation constant of 4 nM.

A wheat-germ ribosome-display system was used to display a 20-mer random library, which was selected for binding to prostate-specific antigen (PSA), a tumor marker [12]. After four rounds of selection, several peptides showing higher affinity to PSA than to bovine serum albumin or gelatin were isolated, but no quantitative data were reported. A randomized region in the cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) scaffold was also used as the basis for a library which was selected against lysozyme. Although binders were found, no quantitative data were reported [32].

Recently, ribosome display was used to identify the main antigenic polypeptides of *Staphylococcus aureus* [33]. Wu *et al.* [34] further investigated the roles of type IVB pili of *Salmonella enterica* serovar Typhi (*S.* Typhi), a 12-mer peptide (RQERSSLSKPVV), binding to the structural protein PilS of the type IVB pili of *S.* Typhi, was isolated with a ribosome-display system. The specific binding constant K_a between the 12-mer peptide R and PilS protein was between 0.4×10^5 and 2.2×10^5 $Lmol^{-1}$. Their findings suggest that the type IV pili-binding peptide R has potential as an antibacterial peptide effective against *S.* Typhi infections, in terms of prevention and therapeutic treatment.

Single-chain antibodies

Hanes and Plückthun [8] reported a system with which a correctly folded complete protein and its encoding mRNA remain attached to the ribosome and can be enriched for the ligand-binding properties of the native protein. They successfully selected a single-chain variable fragment (scFv) of an antibody that has a 10^8-fold specific enrichment of target mRNA using five cycles of transcription, translation, antigen-affinity selection, and PCR. He and Taussig [9] developed the simple and rapid eukaryotic ARM display method, which completes each cycle in 8 h, including analysis. We also successfully screened the human single-chain antibody library for lung cancer using ribosome-display technology, the target antigens are human vascular endothelial growth factor receptor 2 (VEGFR-2) and digoxin [35] (X.H. Yan, PhD thesis, Zhejiang University, 2006). After three selection cycles, anti-VEGFR-2 scFv DNA and anti-digoxin scFv DNA were obtained successfully [35]. The affinity constant of anti-VEGFR-2 scFv and anti-digoxin scFv were

7.7×10^{-10} M and 8.3×10^{-8} M, respectively, using a noncompetitive enzyme-linked immunosorbent assay.

Many methods, such as DNA shuffling, site-directed mutagenesis, error-prone PCR and off-rate selection, are used for affinity maturation and stability enhancement of scFv molecules [18,43–46].

Enzymes

Amstutz *et al.* [36,37] first reported the in *vitro* selection for catalytic activity based on catalytic turnover using ribosome display. They designed and synthesized a mechanism-based inhibitor of β-lactamase, biotinylated ampicillin sulfone, appropriate for selection of catalytic activity of the ribosome-displayed β-lactamase. This derivative of ampicillin inactivated β-lactamase in a specific and irreversible manner. Under appropriate selection conditions, active RTEM-β-lactamase (penicillin amido-β-lactam hydrolase, EC 3.5.2.6) was enriched relative to an inactive point mutant >100-fold per ribosome-display selection cycle. Selection for binding, carried out with β-lactamase inhibitory protein, gave results similar to selection with the suicide inhibitor, indicating that ribosome display was similarly efficient in catalytic activity and affinity selections. The capacity to select directly for enzymatic activity using an entirely *in vitro* process could allow for a significant increase in the explorable sequence space relative to existing strategies.

Ligand-binding proteins

Lamla and Erdmann [38] first described the use of a protein with a binding property, which is not an antibody, for ribosome display. This stands in contrast to previous work that used phenotypic selection for ligand-binding with antibodies. Another new feature that was introduced was the use of an affinity tag for isolating protein–ribosome–mRNA complexes, thus establishing the ribosome display technique. After nine cycles of transcription, translation, affinity selection and reverse-transcription PCR, the protein with the His tag could be enriched 10^8-fold. Binz *et al.* [39] designed ankyrin repeat (AR) protein libraries of varying repeat numbers using a consensus design strategy. They showed the successful selection of binding molecules from these libraries. The properties of the designed AR proteins perfectly match the criteria for alternative scaffolds, unlike previously presented scaffolds [40,41].

Concluding remarks

Ribosome display is a powerful method for screening very large antibody libraries. Each step of ribosome display is carried out *in vitro*, thus circumventing limitations associated with *in vivo* systems. Libraries can be further diversified during PCR steps in ribosome display using low-fidelity polymerases. The past three years have seen several advances in library design, antibody capture and arraying technologies, which leave recombinant antibodies ideally poised for widespread application in proteomics. We anticipate that ribosome display will be of particular importance in the future for directed evolution of proteins through many generations, yielding versatile molecules for a large variety of applications.

References

1 Ross, J.S. *et al.* (2003) Anticancer antibodies. *Am. J. Clin. Pathol.* 119, 472–485
2 Valle, R.P. and Jendoubi, M. (2003) Antibody-based technologies for target discovery. *Curr. Opin. Drug Discov. Devel.* 6, 197–203
3 Russeva, M.G. and Adams, G.P. (2004) Radioimmunotherapy with engineered antibodies. *Expert Opin. Biol. Ther.* 4, 217–231
4 Winter, G. *et al.* (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.* 12, 433–455
5 Georgiou, G. *et al.* (1997) Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. *Nat. Biotechnol.* 15, 29–34
6 Shusta, E.V. *et al.* (1999) Biosynthetic polypeptide libraries. *Curr. Opin. Biotechnol.* 10, 117–122
7 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
8 Hanes, J. and Plückthun, A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U. S. A.* 94, 4937–4942
9 He, M. and Taussig, M.J. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibodies combining sites. *Nucleic Acids Res.* 25, 5132–5134
10 Roberts, R.W. and Szostak, J.W. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc. Natl. Acad. Sci. U. S. A.* 94, 12297–12302
11 Schaffitzel, C. *et al.* (1999) Ribosome display: an *in vitro* method for selection and evolution of antibodies from libraries. *J. Immunol. Methods* 231, 119–135
12 Gersuk, G.M. *et al.* (1997) High-affinity peptide ligands to prostate-specific antigen identified by polysome selection. *Biochem. Biophys. Res. Commun.* 232, 578–582
13 Fjällman, T. and Hall, J.C. (2005) Antibody engineering-a valuable asset in preventing closed environment epidemics. *Acta Astronaut.* 57, 81–88
14 Moghaddam, A. *et al.* (2001) Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naïve recombinant antibody libraries. *J. Immunol. Methods* 254, 169–181
15 Zhou, B. *et al.* (2002) Human antibodies against spores of the genus Bacillus: a model study for detection of and protection against anthrax and the bioterrorist threat. *Proc. Natl. Acad. Sci. U. S. A.* 99, 5241–5246
16 Moghaddam, A. *et al.* (2003) Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. *J. Immunol. Methods* 280, 139–155
17 Vaughan, T.J. *et al.* (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.* 14, 309–314
18 Thom, G. *et al.* (2006) Probing a protein–protein interaction by *in vitro* evolution. *Proc. Natl. Acad. Sci. U. S. A.* 103, 7619–7624
19 He, M. *et al.* (1999) Selection of a human anti-progesterone antibody fragment from a transgenic mouse library by ARM ribosome display. *J. Immunol. Methods* 231, 105–117
20 He, M. *et al.* (2004) Production of human single-chain antibodies by ribosome display. *Methods Mol. Biol.* 248, 177–189
21 Hanes, J. *et al.* (1998) Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14130–14135
22 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
23 Alimov, A.P. *et al.* (2000) Cell-free synthesis and affinity isolation of proteins on a nanomole scale. *Biotechniques* 28, 338–344
24 Jackson, A.M. *et al.* (2004) Cell-free protein synthesis for proteomics. *Brief. Funct. Genomic Proteomic* 2, 308–319
25 Ramachandiran, V. *et al.* (2000) Expression of different coding sequences in cell-free bacterial and eukaryotic systems indicates translational pausing on E. coli ribosome. *FEBS Lett.* 482, 185–188
26 Hanes, J. *et al.* (1999) Comparison of *Escherichia coli* and rabbit reticulocyte ribosome display systems. *FEBS Lett.* 450, 105–110
27 Pandey, A. and Mann, M. (2000) Proteomics to study genes and genomes. *Nature* 405, 837–846
28 He, M. and Taussig, M. (2001) Single step generation of protein arrays from DNA by cell-free expression and *in situ* immobilisation (PISA method). *Nucleic Acids Res.* 29, e73
29 Taussig, M. *et al.* (2000) ARM complexes for *in vitro* display and evolution of antibody combining sites. In *Monoclonal Antibodies: A Practical Approach* (Shepherd, P. and Dean, C., eds), pp. 91–109, Oxford University Press, Oxford

30 Shimizu, Y. *et al.* (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* 19, 751–755
31 Lamla, T. and Erdmann, V.A. (2003) Searching sequence space for high affinity binding peptides using ribosome display. *J. Mol. Biol.* 329, 381–388
32 Coia, G. *et al.* (2001) Panning and selection of proteins using ribosome display. *J. Immunol. Methods* 254, 191–197
33 Weichhart, T. *et al.* (2003) Functional selection of vaccine candidate peptides from *Staphylococcus aureus* whole-genome expression libraries *in vitro*. *Infect. Immun.* 71, 4633–4641
34 Wu, H.Y. *et al.* (2005) Functional selection of a type IV pili-binding peptide that specifically inhibits *Salmonella* Typhi adhesion to/invasion of human monocytic cells. *Peptides* 26, 2057–2063
35 Yan, X.H. and Xu, Z.R. (2005) Production of human single-chain variable fragment (scFv) antibody specific for digoxin by ribosome display. *Indian J. Biochem. Biophys.* 42, 350–357
36 Amstutz, P. *et al.* (2001) *In vitro* display technologies: novel developments and applications. *Curr. Opin. Biotechnol.* 12, 400–405
37 Amstutz, P. *et al.* (2002) *In vitro* selection for catalytic activity with ribosome display. *J. Am. Chem. Soc.* 124, 9396–9403
38 Lamla, T. and Erdmann, V.A. (2001) *In vitro* selection of other proteins than antibodies by means of ribosome display. *FEBS Lett.* 502, 35–40
39 Binz, H.K. *et al.* (2004) High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat. Biotechnol.* 22, 575–582
40 Nygren, P.Å. and Uhlén, M. (1997) Scaffolds for engineering novel binding sites in proteins. *Curr. Opin. Struct. Biol.* 7, 463–469
41 Skerra, A. (2000) Engineered protein scaffolds for molecular recognition. *J. Mol. Recognit.* 13, 167–187
42 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
43 Zahnd, C. *et al.* (2004) Directed *in vitro* evolution and crystallographic analysis of a peptide binding single chain antibody fragment (scFv) antibody with low picomolar affinity. *J. Biol. Chem.* 279, 18870–18877
44 Jermutus, L. *et al.* (2001) Tailoring *in vitro* evolution for protein affinity or stability. *Proc. Natl. Acad. Sci. U. S. A.* 98, 75–80
45 Wang, X.B. *et al.* (2004) A new approach for rapidly reshaping single-chain antibody *in vitro* by combining DNA shuffling with ribosome display. *J. Biochem. (Tokyo)* 136, 19–28
46 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
47 Reichert, J.M. *et al.* (2005) Monoclonal antibody successes in the clinic. *Nat. Biotechnol.* 23, 1073–1078