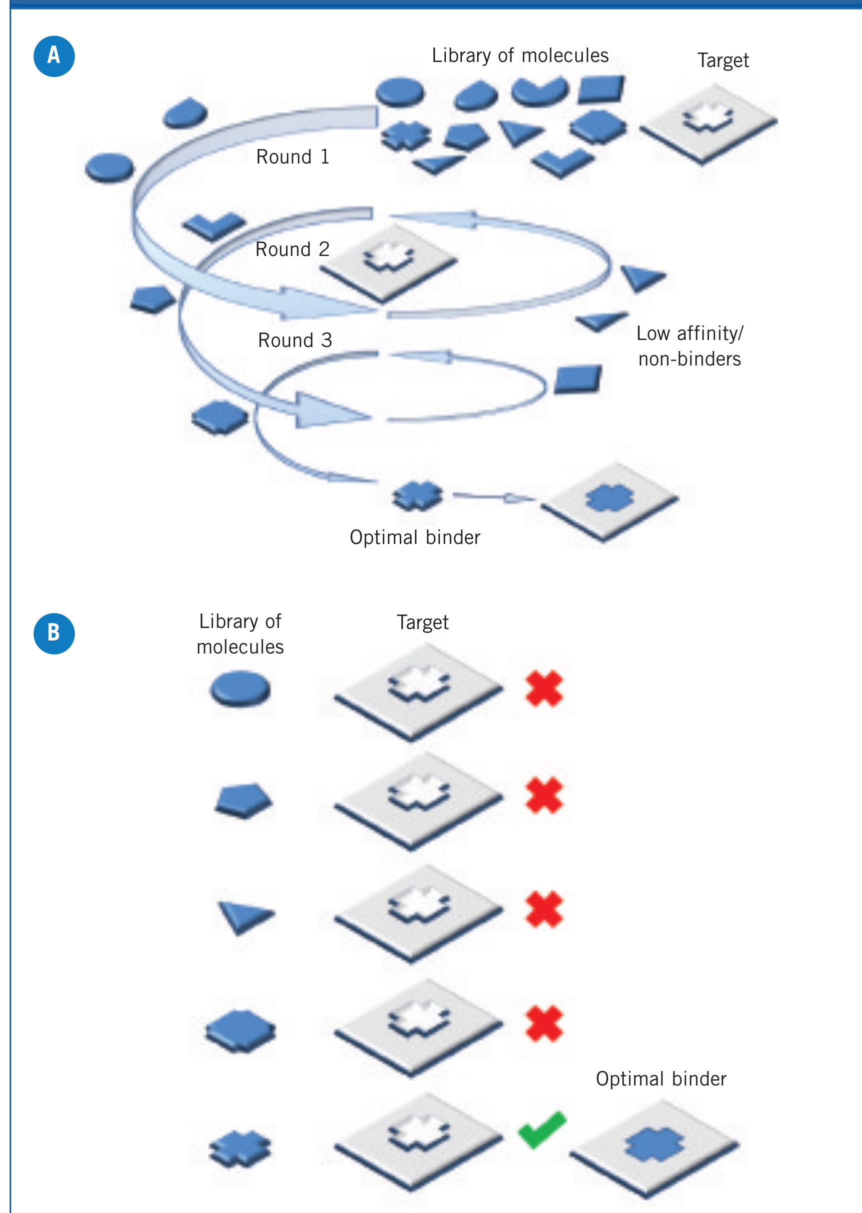
The Evolution of Molecular Display

Chris Ullman of Isogenica Ltd gives an insight into molecular display methods and how they are rejuvenating protein and peptide discovery, enabling the selection of novel molecules with greater therapeutic potential

Twenty-five years have passed since the publication of George Smith's seminal paper in *Science* describing the display of a foreign peptide on the coat of filamentous phage. Expression and specific recovery of the engineered phage using a 'panning' process was termed 'phage display' and gave rise to a more general methodology of molecular display. Since then, phage display has become an essential component of the protein engineer's toolkit where diverse pools (or 'libraries') of peptides and proteins containing hundreds of millions of mutations can be rapidly created and the best candidates selected; a process akin to Darwinian evolution or, more precisely, 'molecular evolution'. The versatility and flexibility of phage display has been a major factor in its adoption in many laboratories. Thousands of papers have been published describing its application, and its contribution to the study of ligand-receptor and protein-DNA interactions cannot be underestimated. However, phage display has its limitations, particularly in terms of speed, library size, expressed protein size and the intractability of some targets. Realisation of these limits has spawned a number of new methods that use the same principles but exploit the cellular machinery in a cell-free environment. These technologies interrogate greater sequence space leading to the rapid discovery of even better peptides and proteins. This article briefly reviews the impact of phage display and assesses the performance of the next generation of display technologies.

Figure 1: A) molecular display compared to B) a high-throughput screening approach. Many more molecules can be screened in parallel using a molecular display



Phage display was invented approximately one year before the first high-throughput combinatorial chemistry approach was described. Both techniques synthesise chemical compounds as ensembles, or libraries, which are screened for desirable properties. However, phage display harnesses the power of a natural system to synthesise peptides and proteins. Cycles of selection and enrichment, prior to screening, allow the interrogation of massive libraries (approximately 10^{10} members) which dwarf the capacity of high-throughput screening (HTS) techniques (see Figure 1); even at a rate of 100,000 compounds per day, it would take HTS hundreds of years to screen similarly sized libraries (1,2). The key to the effective functioning of phage display, and all molecular display technologies, is the formation of a robust link between the encoding nucleic acid (genotype) and the expressed protein (phenotype), such that when the expressed protein is selected, its sequence can simply be translated from its accompanying nucleic acid code.

PHAGE DISPLAY

Phages are viruses that infect bacterial cells. The rod-shaped filamentous phages are composed of a proteinaceous tube containing a single stranded DNA genome. The coat proteins each have been

adapted to display foreign peptides, thus demonstrating the versatility and the adaptability of the virus, but the most common fusions are with the abundant P8 protein or P3 (a minor coat protein expressed at the tip of the phage and essential for infection). The peptides that are displayed as fusions with phage coat proteins are panned for improved or altered characteristics. The panning procedure applies specific pressure that drives the selection of the fittest candidates in the gene pool towards a desired outcome.

In practice, the selection pressure is usually a target molecule which is immobilised or captured onto a surface. The protein library is exposed to the target and a subpopulation of the proteins binds. Diversity is important as most of the phages are lost from the system under stringent washing conditions, leaving bound peptides and their associated phage particles to be enriched by infection and re-growth in bacteria (see Figure 2). Those that are best adapted for survival will eventually dominate the selection environment. Panning conditions are important for the selection of the best clones. Conditions ideally need to be balanced so that stringency is sufficiently high enough to apply pressure for the selection of optimal peptides without diminishing the yield. Not all foreign proteins are amenable to display, and the expression of larger proteins has been less successful due to folding and secretion problems (1,3-5).

THE IMPACT OF PHAGE DISPLAY ON DRUG DEVELOPMENT

Short peptides of random sequence are the simplest combinatorial phage libraries (1). These have successfully produced hits for diverse applications. Peptides that have been selected by phage display to agonise or antagonise protein interactions are now reaching the market or the latter stages of development (see Table 1). Technological advances include the selection of tissue-specific peptides by *in vivo* panning experiments (biopanning) in a human patient and similar biopanning experiments have also shown success for peptide translocation across skin in rats (6,7).

Phage display has revolutionised the ability to engineer larger protein biologics, in particular antibodies. However, as whole antibodies could not be functionally expressed in bacteria, segments had to be amplified from variable region genes and then engineered into single-chain variable fragment antibody (scFv) libraries. It was not until 1990 that these fragment libraries were first displayed on the surface of phage fused to P3. Further work led to the cloning of antigen specific 'immune' libraries from immunised mice and humans, 'naïve' libraries from healthy individuals and 'synthetic' antibody libraries constructed from synthetic oligonucleotides. These advances enabled phage display to compete with hybridoma technology and offered credible alternatives to immunisation. The speed at which hits with novel specificity could be generated, the lack of bias introduced by immune tolerance, and the ability to mimic the natural processes of somatic hypermutation and V-gene rearrangement in human framework sequences *in vitro*, all provided key advantages over hybridomas. To date, the most successful antibody libraries have been those based upon the natural variable domains of the antibody in which rational optimisation of the framework regions and diversification of the complementarity-determining regions (CDRs) has been achieved.

Figure 2: Stages of the selection cycle ('panning' process) of phage display. A) The bacteria are first transformed with the DNA library to produce phages. B) The phages are then selected against the target where specific clones bind. C) The bound clones are then eluted and the cycle is repeated following bacterial infection

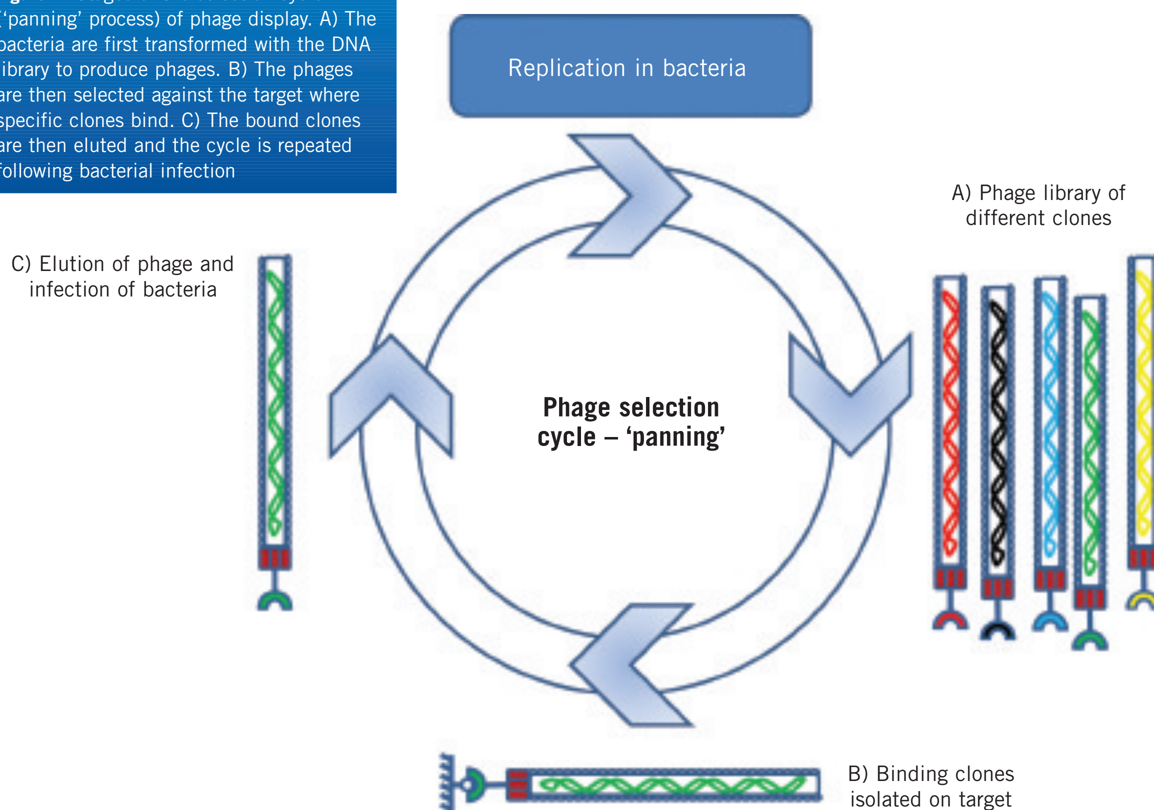


Table 1: Examples of peptides and proteins selected using phage display that have reached the clinical stages of development (adapted and updated from Rothe *et al*, 2006 (8))

Name	Format	Target	Indication	Stage	Company
Humira (adalimumab)	IgG1	TNF	Autoimmune diseases	Market	Abbott
DX-88 (Ecallantide)	Kunitz domain	Kallikrein	Herediatary angioedema	Market	Dyax
Nplate	Peptibody (Peptide-Fc)	Thrombopoietin	Chronic immune thrombocytopenic purpura	Market	Amgen
ABthrax (raxibacumab)	IgG1	B anthracis	Anthrax	Pending approval	HGSI
ABT-874	IgG1	IL-12	Crohn's disease, Psoriasis	Phase II, III	Abbott
LymphoStat-B (belimumab)	IgG1	Blys	Rhuematoid arthritis, SLE	Phase II, III	GSK/HGSI
IMC-11F8 (necitumumab)	IgG1	EGFR	Non-small cell lung cancer, colorectal cancer, solid tumours	Phase I, II, III	Eli Lilly & Co/BMS
HGS-ETR1 (mapatumumab)	IgG1	TRAIL-R1	Solid and haematological malignancies	Phase I, II	HGSI
IMC-1121B	IgG1	VEGFR2	Cancer	Phase II, III	Eli Lilly & Co
GC-1008	IgG	TGFß	Pulmonary fibrosis, solid tumours	Phase I, II	AZ
iCo-008 (CAT-213) (bertilimumab)	IgG4	Eotaxin1	Vernal keratoconjunctivitis	Phase II	iCo Therapeutics Inc
IMC-A12	IgG1	Insulin-like growth factor receptor	Cancer	Phase I, II	Eli Lilly & Co
MT201 (adecatumumab)	IgG	EpCAM	Metastatic breast cancer, colorectal cancer	PC, Phase I, II	Micromet/Merck Serono
MOR103	IgG	GM-CSF	Rheumatoid arthritis	Phase II	Galapagos/Morphosys
Nanobody	VHH (camelid)	vWF	Thrombophilia	Phase II	Ablynx
AMG-386	Peptibody (Peptide-Fc)	Angiopoietin-1 and -2	Cancer	PC, Phase I, II	Amgen
DX-890/EPI-hNE4	Kunitz domain	Neutrophil Elastase	Acute respiratory distress syndrome	Phase II	Debiopharm
MEDI-547	IgG	EphA2	Solid tumours	Phase I	MedImmune
MT203	IgG1	GM-CSF	Inflammatory and autoimmune diseases	Phase I	Micromet
CAT-354	IgG4	IL-13	Asthma	Phase I	MedImmune
IMCEB10	IgG	FLT3	Leukemia	PC, Phase I	Eli Lilly & Co
Hematide	Peptide	Erythropoietin	Anemia	Phase I	Affymax
POT-4	Peptide	C3 convertase	Age-related macular regeneration	Phase I	Potentia Pharmaceuticals Inc

Humira (adalimumab), a TNFα inhibitor for the treatment of rheumatoid arthritis, was the first antibody derived from phage display to be approved and there is a growing pipeline (see Table 1) (3,8). Closely related techniques are yeast and bacterial display which enable the display of scFvs or scFvs and Fabs, respectively, upon the surface of cells (4).

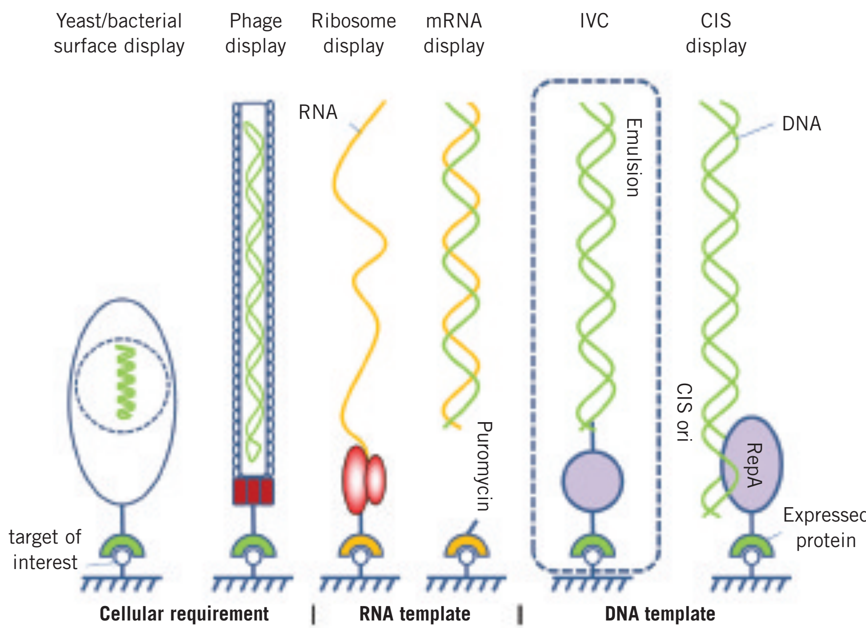
Phage display has contributed greatly to the understanding and application of protein-DNA interactions, and in particular zinc fingers. The zinc finger is the most abundant DNA-binding domain, small in size and possessing a compact structure that coordinates a zinc ion. Their small structure, tolerance to engineering and modular nature has enabled multiple domains to be expressed on phage and a code for DNA recognition to be determined. These results have opened the door for possible site-specific control and correction of deleterious genes in humans (9,10).

However, despite success, phage display is limited by a laborious and inefficient process known as transformation, which is necessary for phage production in *E. coli*. Often, it takes months to construct libraries of 10^{10} members.

THE DEVELOPMENT OF ACELLULAR *IN VITRO* DISPLAY TECHNOLOGIES

Acellular *in vitro* molecular display technologies, which avoid the need for transformation, enable more of the sequence landscape to be displayed, ultimately leading to greater diversity and an increased probability of higher affinity hits. These systems use the transcription and translation machinery extracted from prokaryotic or eukaryotic cells, thereby enabling theoretical library sizes up to 10^{14} to be investigated. They also do not rely on a secretory pathway for display, can select for a wider variety of antimicrobial

Figure 3: Details of the key features of the different display technologies



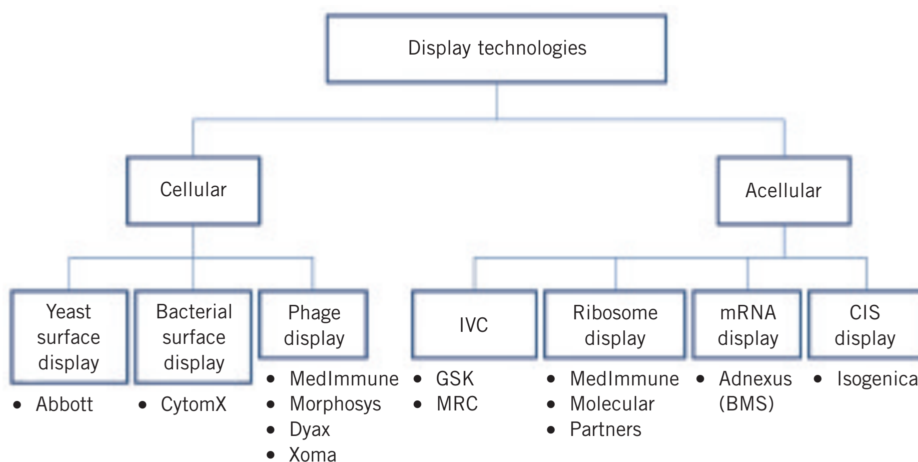
peptides and have less non-specific interactions. The distinguishing features of these technologies are shown in Figure 3.

The first cell-free, *in vitro* molecular display technique – ribosome display – was originally developed in an *E. coli* lysate for the display of peptides. The ribosomes were stalled on the mRNA template and the nascent peptide remained in a complex, which could then be disrupted by EDTA. The released RNA was subsequently amplified by an RT-PCR step. Later, scFvs were successfully displayed on ribosomes using both bacterial and eukaryotic systems (8,11,12). However, the system is very sensitive to RNase degradation and mechanisms have been employed to reduce this effect (13). Ribosome display methods have also been used in the selection of peptides containing non-natural amino acids, and non-antibody structures such as ankyrin domains (14-16).

A related technique, mRNA (or *in vitro* virus) display differentiates itself from ribosome display by the formation of a covalent link between the template and the expressed protein via puromycin. Puromycin is carried on a DNA primer appended to the mRNA template and mimics amino-acyl tRNA, binding covalently to the nascent peptide as a result of the peptidyl transferase activity of the ribosome (17). The DNA primer is then used in a reverse transcription step to stabilise the RNA template in a RNA/DNA hybrid. Coupling efficiency between protein and mRNA has been reported to be 10 to 40 per cent, but seven panning rounds were typically needed in the selections (18). The mRNA display technique has been used for a number of different applications including the incorporation of non-natural amino acids (19).

DNA based systems have advantages of speed and stability over RNA templates as the DNA template is less sensitive to degradation, therefore libraries can be generated quickly by standard PCR procedures. One system, CIS display, harnesses the ability of a DNA-binding protein, RepA, that exclusively binds back to its encoding DNA (termed *cis*-activity). Coupling of the protein and DNA is non-covalent and is approximately 40 per cent efficient, supporting effective library sizes over 10^{13}, with enrichment factors between 10^3 and 10^5 fold per round. CIS display was the first published example of recovery of a specific binder from a 1 in 10^{10} dilution, therefore demonstrating potential for unprecedented library sizes (20). This technique has been used to select in the presence of proteases (21), and to select from scaffold and scFv libraries (unpublished data). Another system using a *cis*-acting DNA binding protein that links covalently to its template has been used for scFvs to select for tetanus toxin binders from an immune human library with enrichment between 14- and 300-fold (22).

Figure 4: Molecular display technologies and ownership of key IP regarding use of the platforms or the use of the libraries (please note the list is not exhaustive)



In vitro compartmentalisation (IVC) provides an alternative way of linking phenotype and genotype and mimics the natural compartments of living organisms, entrapping DNA and ITT components in water-in-oil emulsions by directly mixing mineral containing surfactants in water; each droplet having approximately one gene. The method is particularly advantageous for the direct selection of enzymes; however, efficiencies may be reduced by the incomplete separation of DNA molecules into individual droplets or by fusion of the compartments. IVC has been

further adapted for use with microfluidics (8,23-25).

THE FUTURE OF DISPLAY TECHNOLOGIES

With over 25 years of development behind phage display methods, it's utility in the discovery of novel pharmaceutical biomolecules and in the understanding of protein function cannot be underestimated. It has been the pathfinder of molecular display, leading to a new generation of selection technologies (see Figure 4, page 76). These new technologies are becoming more commonplace and the improvements that they offer are gradually being realised. Amongst these improvements, rapid library design and selection and interrogation of larger library diversity have rejuvenated peptide and protein discovery. The ease in which new protein designs can be validated and optimised allows greater exploration of the structural space which, in turn, will facilitate a greater understanding of the molecular interactions of the proteome and enable the selection of novel molecules with greater therapeutic potential.

References

1. Smith GP and Petrenko VA, Phage Display, *Chem Rev* 97: pp391-410, 1997
2. Houghten RA, Dooley CT and Appel JR, *In vitro* and direct *in vivo* testing of mixture-based combinatorial libraries for the identification of highly active and specific opiate ligands, *AAPS J* 8: ppE371-382, 2006
3. Hoogenboom HR, Selecting and screening recombinant antibody libraries, *Nat Biotechnol* 23: pp1,105-1,116, 2005
4. Levin AM and Weiss GA, Optimizing the affinity and specificity of proteins with molecular display, *Mol Biosyst* 2: pp49-57, 2006
5. Fernandez-Gacio A, Uguen M and Fastrez J, Phage display as a tool for the directed evolution of enzymes, *Trends Biotechnol* 21: pp408-414, 2003
6. Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M *et al*, Steps toward mapping the human vasculature by phage display, *Nat Med* 8: pp121-127, 2002
7. Chen Y, Shen Y, Guo X, Zhang C, Yang W *et al*, Transdermal protein delivery by a coadministered peptide identified via phage display, *Nat Biotechnol* 24: pp455-460, 2006
8. Rothe A, Hosse RJ and Power BE, *In vitro* display technologies reveal novel biopharmaceutics, *FASEB J* 20: pp1,599-1,610, 2006
9. Isalan M and Choo Y, Engineering nucleic acid-binding proteins by phage display, *Methods Mol Biol* 148: pp417-429, 2001
10. Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM *et al*, Highly efficient endogenous human gene correction using designed zinc-finger nucleases, *Nature* 435: pp646-651, 2005
11. He M and Taussig MJ, Ribosome display: cell-free protein display technology, *Brief Funct Genomic Proteomic* 1: pp204-212, 2002
12. Zahnd C, Amstutz P and Pluckthun A, Ribosome display: selecting and evolving proteins *in vitro* that specifically bind to a target, *Nat Meth* 4: pp269-279, 2007
13. Villemagne D, Jackson R and Douthwaite JA, Highly efficient ribosome display selection by use of purified components for *in vitro* translation, *J Immunol Methods* 313: pp140-148, 2006
14. Nakajima E, Goto Y, Sako Y, Murakami H and Suga H, Ribosomal synthesis of peptides with C-terminal lactams, thiolactones, and alkylamides, *Chembiochem* 10: pp1,186-1,192, 2009
15. Bonetto S, Spadola L, Buchanan AG, Jermutus L and Lund J, Identification of cyclic peptides able to mimic the functional epitope of IgG1-Fc for human Fc gammaRI, *FASEB J* 23: pp575-585, 2009
16. Amstutz P, Koch H, Binz HK, Deuber SA and Pluckthun A, Rapid selection of specific MAP kinase-binders from designed ankyrin repeat protein libraries, *Protein Eng Des Sel* 19: pp219-229, 2006
17. Hosse RJ, Rothe A and Power BE, A new generation of protein display scaffolds for molecular recognition, *Protein Sci* 15: pp14-27, 2006
18. Takahashi TT, Austin RJ and Roberts RW, mRNA display: ligand discovery, interaction analysis and beyond, *Trends in Biochemical Sciences* 28: pp159-165, 2003
19. Millward SW, Fiacco S, Austin RJ and Roberts RW, Design of cyclic peptides that bind protein surfaces with antibody-like affinity, *ACS Chem Biol* 2: pp625-634, 2007
20. Odegrip R, Coomber D, Eldridge B, Hederer R, Kuhlman PA *et al*, CIS display: *In vitro* selection of peptides from libraries of protein-DNA complexes, *Proc Natl Acad Sci USA* 101: pp2,806-2,810, 2004
21. Eldridge B, Cooley RN, Odegrip R, McGregor DP, Fitzgerald KJ *et al*, An *in vitro* selection strategy for conferring protease resistance to ligand binding peptides, *Protein Eng Des Sel* 22: pp691-698, 2009
22. Reiersen H, Lobersli I, Loset GA, Hvattum E, Simonsen B *et al*, Covalent antibody display: an *in vitro* antibody-DNA library selection system, *Nucleic Acids Res* 33: pE10, 2005
23. Tawfik DS and Griffiths AD, Man-made cell-like compartments for molecular evolution, *Nat Biotechnol* 16: pp652-656, 1998
24. Sepp A, Tawfik DS and Griffiths AD, Microbead display by *in vitro* compartmentalisation: selection for binding using flow cytometry, *FEBS Lett* 532: pp455-458, 2002
25. Griffiths AD and Tawfik DS, Directed evolution of an extremely fast phosphotriesterase by *in vitro* compartmentalization, *EMBO J* 22: pp24-35, 2003

About the author

Chris Ullman is Chief Scientific Officer at Isogenica Ltd, a small biotech company that is using its CIS display technology to engineer peptides and proteins. Chris has been at Isogenica since 2002, where he has been involved in the development of CIS display. Prior to joining Isogenica, Chris was Team Leader for Gendaq Ltd (later acquired by Sangamo Biosciences Inc), developing customised transcription factors. The company was spun-out from Sir Aaron Klug's Laboratory at the LMB, Cambridge.

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