Advances in Antibody Phage Display – A review

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ABSTRACT

Antibody phage technology greatly facilitates the isolation of good-quality monoclonal antibodies to virtually any target antigen. Large combinatorial phage display libraries of human antibodies are routinely being used for the identification of antibody candidates for clinical applications. However, preclinical studies in rodents would benefit from the availability of good-quality single-pot mouse synthetic naïve antibody libraries, which at present are not available. Such libraries would be particularly useful for the generation of murine antibodies against self or highly conserved antigens or in case of highly toxic or deadly pathogenic antigens which do not allow animal immunization.

Keywords: Antibodies, Phage display, Immunoglobulin, Single-chain variable fragment (scFv) and the Fab fragment.

BACKGROUND

Antibodies are a class of glycoproteins belonging to the immunoglobulin superfamily found in blood or other bodily fluids of vertebrates. Five classes of immunoglobulins are known - IgM, IgD, IgG IgA and IgE – that differ for their constant region. IgGs are the most relevant class of antibodies for pharmaceutical applications.

The classic structure of an IgG consists of two identical light chains (25 kDa) and two identical heavy chains (50 kDa) covalently linked by a disulphide bridge between the two heavy chains and between heavy and light chains. Each heavy and light chain contains a variable domain (VH and VL respectively) of around 110 amino acids where the variability on the antibody structure is located. Within each variable domain, three hypervariable loops confer the binding properties and are defined as complementarity determining regions (CDRs).

Mechanisms of action

In the last decades several monoclonal antibodies have been approved from FDA for the treatment of cancer and other diseases. Most monoclonal antibodies on the market are naked IgGs. Naked IgGs have three main mechanisms of action which allow them to operate *in vivo*: neutralization, antibody-dependent cellular toxicity and complement activation.

The neutralization activity consists in the binding of the antibody to the target antigen and thereby blocking its biological function. Therapeutic applications include the neutralization of toxins (antiserum) or signaling molecules such as cytokines (e.g. Adalimumab**,** Remicade, Ustekinumab) or growth factors (e.g. Bevacizumab, Lucentis).

IgGs could also give rise to antibody-dependent cellular toxicity (ADCC). ADCC is the mechanism of cell-mediated immunity whereby the binding of antibodies on a target antigen expressed on a cell induces the lyses of the target cell. Classical ADCC is mediated by natural killer (NK) cells. NK cells expose on the cell membrane the FcãRIII, which recognizes the Fc portion of IgG. Once FcãRIII receptors bind to the Fc portion of antibodies bound to a cell surface antigen, NK cells release cytotoxic granules containing perforin and granzymes that enter the target cell and promote cell death by triggering apoptosis. ADCC is an important mechanism of action of therapeutic monoclonal antibodies, including trastuzumab and rituximab.

Antibodies (mainly IgM, IgG1 and IgG3) are able to trigger the classical pathway of complement activation: the starting event is the binding of C1q to the Fc region of antibodies bound to multiple sites of a cell surface (typically a pathogen). Subsequent activation of the complement cascade ultimately leads to three possible events: (i) direct killing of the cell via lysis (by creating pores on the membrane), (ii) opsonization and engulfment of the cell by phagocytes and (iii) uptake of the complement-coated-antigen by antigen presenting cells and its presentation to the adaptive immune system.

Antibodies and their derivatives can also be used as vehicle to concentrate a therapeutic agent specifically at the site of disease sparing healthy tissues. A targeted therapy is the ideal approach for the therapy of cancer where often potent drugs cannot be used at therapeutic relevant regimens because of their severe side effects. The targeted delivery of drugs by means of antibodies promises to enhance their therapeutic index and limit their side effects.

For tumor targeting applications the use of antibody fragments (*i.e.* scFv, diabody, or mini-antibody format), is preferable to full IgGs as they allow a better extravasation from the blood circulation and a superior tissue penetration. In comparison to naked IgG, antibody fragments *per se* do not mediate any therapeutic activity and need to be linked to bioactive agents such as cytokines, toxins, procoagulant factors, drugs with a cleavable linker, radionuclides and photosensitizers.

Therapeutic applications of Antibodies

Since their introduction, monoclonal antibodies have had a progressively increasing impact on different areas of medical diagnostics and therapeutics. Monoclonal antibodies have been approved for the treatment of cancer, inflammatory diseases, cardiovascular diseases, macular degeneration, transplant rejection, multiple sclerosis, and viral infection. Most of these antibodies have the neutralization of their target or the induction of ADCC as mechanism of action. In the future, we may also see approval of antibody derivatives linked to a bioactive agent. These "immuno-derivatives" may offer the possibility to deliver a potent therapeutic agent that acts selectively at the site of the disease, sparing healthy tissues, and may considerably enhance the therapeutic index of the bioactive agent. For the therapy of cancer the U.S. FDA approved a number of monoclonal antibodies, some of which have become blockbusters in spite of their generally modest clinical benefit.

Bevacizumab is a humanized monoclonal antibody that targets and blocks the VEGF-A and was approved for the first-line treatment of metastatic colorectal cancer and as second line for other solid cancers. The neutralization of VEGF-A seemed to be a promising strategy as this molecule is supposed to be a key factor for the neoangiogenesis process required for tumor growth. Clinical studies showed that bevacizumab combined with chemotherapy can prolong life of patients of 4-5 months compared to patients treated with standard chemotherapy (Hurwitz, Fehrenbacher *et al*. 2004) Trastuzumab is a humanized monoclonal antibody approved for the therapy of metastatic breast cancer. The antibody targets the extracellular domain of the human epidermal growth factor receptor 2 (HER2), a protein over-expressed in ~20% of women with breast cancer. ADCC and arrest of cell proliferation by disrupture of receptor dimerization or downstream signaling pathways may be possible mechanisms of action *in vivo*, though they have been only demonstrated *in vitro* (Sarup, Johnson *et al*. 1991; Cooley, Burns *et al*. 1999).

Antibody phage display technology

Phage display is a powerful methodology that allows the selection of a particular phenotype (e.g., a ligand specific to a desired antigen) from repertoires of proteins displayed on bacteriophage. Phage display was first described in 1985 by Smith (Smith 1985), who presented the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power and applications of the methodology were further improved by the groups of Winter (McCafferty, Griffiths *et al.* 1990) and Wells (Lowman, Bass *et al.* 1991) who demonstrated the display of functional folded proteins on the phage surface (an antibody fragment and a hormone, respectively). Phage technology is based on the fact that a polypeptide (capable of performing afunction, classically the specific binding to an antigen) can be displayed on the phage surface by inserting the gene coding for the polypeptide into the phage genome. Thus the phage particle physically links genotype to phenotype. It is possible to create large repertoires of phage (phage display libraries) in which the proteins displayed on each phage are slightly different

from each other. If one is able to purify from this large phage repertoire a phage particle by virtue of the phenotype (e.g., the binding specificity) displayed on its surface, one also isolates the genetic information coding for the binding protein, and can amplify the corresponding phage by means of bacterial infection. As an example, one can consider the selection of a binding specificity from repertoires of binders.

The library on phage is panned against an antigen of interest. Unbound phage is discarded whereas specifically binding phage are collected and amplified in bacteria. Several rounds of selection can be performed (in general 2-4 rounds using antibody phage libraries). As a result, even very rare phenotypes present in large repertoires can be selected and amplified from a background of phage carrying undesired phenotypes. The possibility to amplify the selected phage in bacteria during biopanning experiments allows the enrichment of the pool of phage with the desired phenotype. Filamentous phage infects strains of *E.coli* that harbour the F conjugative episome by attaching to the tip of the F pilus and translocating the phage genome (a circular single-strand DNA molecule) into the bacterial cytoplasm. The genome is replicated involving both phage- and host-derived proteins, and packaged into elongated "filamentous" viral particles of roughly 6 nm in diameter and 900 nm in length.

Filamentous phage particles are covered by several thousand copies of a small major coat protein (pVIII). Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX are present at the opposite extremity. The minor coat protein pIII (the product of gene III), is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus. Peptides and proteins have been displayed on phage as fusions with the coat proteins pIII (Smith 1985; Parmley and Smith 1988) or pVIII (Greenwood, Willis *et al*. 1991). Display of proteins encoded by a cDNA library as carboxy terminal fusion with the minor coat protein pVI has also been described (Greenwood, Willis *et al*. 1991).The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pVIII or pIII fusions and an antibiotic resistance gene). Phage vectors carry all the genetic information necessary for the phage life cycle.

With pIII fusions in phage vectors, each pIII coat protein displayed on phage is fused with the heterologous polypeptide. Using phage vectors, most peptides and folded proteins can be displayed as pIII fusions, while only short peptides of 6-7 residues containing no cysteine give rise to functional phage when displayed as pVIII fusions (Iannolo, Minenkova *et al*. 1995). Phagemids, a more popular type of vector for phage display, are plasmids that carry the gene III with appropriate cloning sites and a packaging signal (Hoogenboom, Griffiths *et al*. 1991). For the production of functional phage particles, phagemid containing bacteria have to be super-infected with helper phage particles, which contain a complete phage genome.

Antibody phage display libraries

Antibody phage display technology is the display and use of repertoires of antibody fragments on the surface of bacteriophage. The filamentous phage surface constitutes a physical link between genotype and phenotype of the antibody, in the same way that surface immunoglobulins are linked to the B cells *in vivo*. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains of a full immunoglobulin are connected on the same polypeptide chain by a flexible polypeptide spacer or as Fab fragments where the association of the variable domains is stabilized by the first constant domain of the heavy chain and the first constant domain of the light chain (Better, Chang *et al*. 1988; Cabilly 1989). scFv fragments have a molecular weight of about 25 kDa and are not glycosylated. In a scFv fragment the order of the V domains may vary, with the V_H domain at the N-terminus or at the C-terminus (Bird, Hardman *et al*. 1988; Huston, Levinson *et al*. 1988), whereby the linker length has to be adjusted for optimal spatial arrangement of the two V domains (Huston and Haber 1996). The most common format VH-(Gly4Ser)3-VL has been also used for the construction of various phage libraries (Clackson, Hoogenboom *et al*. 1991; Marks, Hoogenboom *et al*. 1991; Hoogenboom and Winter 1992)

By definition the term "antibody phage display library" refers to a collection of recombinant phage which display an antibody fragment on their surface. The total number of different phage particles displaying each a unique antibody fragment in the repertoire defines the size of the library, which is a critical parameter for the success of antibody phage technology. The larger the library, the greater the chance of finding antibodies that bind to any given epitope, and the higher the affinity (Perelson and Oster 1979). The second key parameter which defines library performance is the diversity carried by the amino acid sequences of the antibodies in the library. As in the immune system, the antibodies of a phage display library may have a common scaffold, while diversity is inserted in the amino acid positions which determine the specificity of binding. CDR3 loops represent the antibody region in which diversity is mainly concentrated in nature.

Thanks to the extensive characterization of the V-genes and their flanking regions, several sets of "universal" PCR primers have been described for the cloning of human (Marks, Hoogenboom *et al*. 1991; Tomlinson, Walter *et al*.1992), murine (also usable for rat) (Orlandi, Gussow *et al*. 1989; Clackson, Hoogenboom *et al*. 1991; Kettleborough, Saldanha *et al*. 1993; Orum, Andersen *et al*. 1993; Ridder, Schmitz *et al*. 1995), rabbit (Ridder, Schmitz *et al*. 1995), and chicken V-genes repertoires (Davies, Smith *et al*. 1995). On the basis of the strategy followed to obtain diversity, antibody phage display libraries can be classified in "Immune repertoires" (antigen-biased), and "Singlepot" libraries (antigen-unbiased).

Immune antibody phage display libraries

Immune antibody phage display libraries (Burton, Barbas *et al*., 1991; Clackson,

Hoogenboom *et al*. 1991) take advantage of the diversity created *in vivo* by the immune system: in this case the source of variable immunoglobulin genes are B cells from an animal immunized with the antigen of interest or an immune patient. The resulting libraries are enriched in antigen-specific immunoglobulin domains, some of which have already been matured by the immune system, and may therefore yield high-affinity antibodies even when the library size is not spectacular (e.g., 10^7 clones). For example, Chester *et al*. (Chester, Robson *et al*. 1994) isolated from a murine immune library a well-behaved scFv fragment specific for the carcinoembryonic antigen (CEA) with a dissociation constant in the low nanomolar range. This scFv (MFE-23) has been shown to selectively target human tumors xenografted in nude mice (Verhaar, Chester *et al*. 1995). There are some disadvantages in isolating antibodies from immune repertoires. When the source of V genes is an immunized animal, the resulting antibodies are not human and therefore potentially immunogenic. Animal immunization and library construction are necessary for each individual antigen, making the whole procedure long and labour intensive. However, the isolation of human anti-tumor antibodies from phage repertoires of antibodies derived from cancer patients immunized with autologous tumor cells (Cai and Garen 1995), or from their tumor-draining lymph nodes (Kettleborough, Ansell *et al*. 1994) is a powerful strategy for the isolation of novel tumorassociated binding specificities. We foresee those immune libraries, obtained by immunization against complex antigen mixtures and analyzed using efficient selection schemes (Hoogenboom 1997) and screening methodologies (e.g., high-throughput immunohistochemistry), will continue to be useful tools for the discovery of novel tumor markers.

Single-pot libraries

Single-pot libraries contain virtually all possible binding specificities and are not biased for a particular antigen. They are cloned once, with the aim to reach a complexity $> 10^8$ clones and, if possible, $> 10^9$-10^{10} clones. The corresponding phage are stored frozen in aliquots (Neri, Pini *et al*. 1998) and can directly be used in panning experiments against a variety of different antigens. Typically, when using pure antigen preparations, specific monoclonal antibodies are almost always isolated in 2-4 rounds of panning (5-10 days of work). In general, bothlibrary design and library size contribute to the performance of the library, and to the quality of the isolated antibodies. Larger libraries have a higher probability of containing high affinity antibodies (Griffiths, Williams *et al*. 1994). It is technically possible to make phage display libraries of complexity $>10^9$ using electroporation, and $>10^{11}$ using combinatorial infection and cre-lox mediated recombination (Waterhouse, Griffiths *et al*. 1993; Griffiths, Williams *et al*. 1994). However, the combinatorial diversity that can in practice be explored in panning experiments is limited by several factors, including the solubility of phage particles (typically d"10^{13} transforming units/ml), the efficiency of antibody display on phage, and the phage recovery yields in biopanning experiments (de Haard, Kazemier *et al*. 1998). Single-pot libraries can be classified as naïve or synthetic.

a. Naïve repertoires

In this case V-genes are isolated from unimmunized animals or human donors, and are combinatorially assembled to create large arrays of antibodies. The murine naïve repertoire has been estimated to contain $<5\times10^8$ different B lymphocytes, while the human repertoire may be a hundred to a thousand times bigger (Winter, Griffiths *et al*. 1994). This array of antibodies may be cloned as a "naïve" repertoire of rearranged genes, by harvesting the V genes from the IgM mRNA of B-cells isolated from peripheral blood lymphocytes (PBLs), bone marrow, or spleen cells.

b. Synthetic repertoires

In synthetic repertoires, variability is entirely created outside the natural host. To construct a synthetic antibody library, V-genes are typically assembled by introducing randomized CDRs into germline V-gene segments (Hoogenboom and Winter 1992). The antibody residues in which synthetic diversity is concentrated are chosen to correspond to regions of natural sequence diversity of the primary antibody repertoire. Since the VH CDR3 is the most diverse loop, in composition, length and structure, it is usually chosen for partial or complete randomization.

The choice of the germline V-genes into which one can insert combinatorial diversity can greatly vary. The variable regions of human antibodies are assembled from 51 different VH germline genes (Chothia, Lesk *et al*. 1992) and 70 different functional VL segments (40 Vκ and 30 Vλ; (Tomlinson, Cox *et al*.1995; Tomlinson, Walter *et al*. 1996; Ignatovich, Tomlinson *et al*. 1997). One can choose to use only one type of scaffold, based on qualities of the scaffold (Pini, Viti *et al*. 1998), or keep one of the heavy or light chains constant and use different scaffolds of the other one (Nissim, Hoogenboom *et al*. 1994), or take full advantage of the diversity of the scaffolds and combine the different heavy and light chains as much as possible (Griffiths, Williams *et al*. 1994). Since not all of the different chain variants are equally well represented in the functional repertoire, there might be a disadvantage using such a great variation of scaffolds. Indeed, there is evidence that only a few germline V-genes

dominate the functional repertoire (Kirkham, Mortari *et al*. 1992; Tomlinson, Cox *et al*. 1995). By using scaffolds that are not often represented among the binders, library diversity would be wasted. To avoid this, one could consider constructing libraries with only one light chain and concentrate combinatorial diversity solely in the heavy chain (Nissim, Hoogenboom *et al*. 1994). This approach has proven to work well in practice and offers the possibility to affinity mature the binders by randomizing the light chain in a second step (Neri, Carnemolla *et al*. 1997).

Murine antibodies and antibody libraries

Mouse monoclonal antibodies are routinely generated by means of hybridoma technology (Kohler and Milstein 1975). B lymphocytes of immunized animals are

fused with immortalized myeloma cells producing hybrid cell lines (hybridomas) that able to secrete monoclonal antibodies and can be screened for the desired binding specificity.

The development of antibody-based therapeutics often requires the development and *in vivo* testing of antibody-based products in syngeneic preclinical settings (*e.g*., murine antibodies in mouse models of pathology). Certain antigens are not immunogenic in rodents (Carnemolla, Leprini *et al*. 1992; Melkko and Neri 2003) (i.e. self proteins and conserved antigens) or cannot be used for immunization (lethal toxins or highly toxic proteins) that would allow the development of a B lymphocytes population specific for the desired antigen necessary to generate hybridomas. Current preclinical therapy studies involving human antibodies and antibody derivative therapeutics in mouse models are limited by the mouse anti-human antibody (MAHA) response. To minimize the MAHA response preclinical studies are limited to use of immunodeficient mouse strains or to short-time therapies. To overcome this problem it would be useful to rely on good-quality mouse antibodies, as mice are often the standard animal model for the *in vivo* testing of novel antibody-based therapeutics.

A mouse synthetic antibody phage display library would thus fulfill the increasing need of mouse monoclonal antibodies that cannot be generated by hybridoma technology. While several antibody libraries from immunized mice have been described so far (Clackson, Hoogenboom *et al*. 1991), there are only few reports of naïve single pot mouse antibody libraries (Gao, Huang *et al*. 1999; Okamoto, Mukai *et al*. 2004; Imai, Mukai *et al*. 2006), which have so far been used only for the isolation of few monoclonal antibodies. Such naïve libraries, based on the combinatorial assembly of VH - VL genes extracted from non immunized mice, have still an unsatisfactory performance towards self antigens and conserved antigens due to immunological tolerance. Therefore a naïve synthetic mouse antibody phage display library may overcome this limitation and allow the isolation of mouse antibodies against self and conserved antigens of pharmaceutical relevance (*i.e*. EDB of fibronectin). At present no library with these prerequisites is available.

Other selection methodologies

Yeast Display

Yeast display of antibody fragments has demonstrated to be an efficient and productive methodology for directed evolution of scFv fragments for increased affinity and thermal stability and for the display of naïve scFv and immune Fab libraries. A major advantage of yeast display is the possibility to characterize the binding properties, such as the affinity and epitope binding characteristics, of a clone without the need of subcloning, expression and purification of the antibody fragment. A further strength of yeast display is the compatibility with Fluorescent-Activated-Cell-Sorting (FACS). By means of FACS, one can isolate yeast clones of interest, based on their ability to bind fluorescently labelled antigen.

In yeast display the á-agglutinin yeast adhesion receptor is used for the display of recombinant proteins on the surface of *Saccharomyces cerevisiae*. In *S. cerevisiae*, the á-agglutinin receptor acts as an adhesion molecule to stabilize cell-cell interactions and facilitate fusion between mating a and á haploid yeast cells. The receptor is composed of two proteins, Aga1 and Aga2: once Aga1 is secreted from the cell it becomes covalently attached to â-glucan in the extracellular matrix of the yeast cell wall. Aga2 binds to Aga1 through two disulfide bonds and after secretion remains attached to the cell through Aga1. The display on the yeast surface of a recombinant protein profit by the association of Aga1 and Aga2. The gene of interest is cloned into a vector as an in frame fusion with the AGA2 gene. Expression of both the Aga2 fusion protein from the vector and the Aga1 protein in the host strain is regulated by a tightly regulated promoter, GAL1. The use of this promoter allows the expansion of a scFv library 10^{10}-fold without any recognizable changes in either the percentage of antibody expression or the frequency of specific clones within the library. Upon induction with galactose, the Aga1 protein and Aga2-scFv fusion protein associate within the secretory pathway, and the epitope tagged scFv antibody is displayed on the cell surface at 10,000-100,000 copies per cell.

scFv antibody expression on the yeast cell surface can be monitored by flow cytometry with fluorescently labelled antibodies recognizing either the C-terminal c-myc or the N-terminal hemagglutinin (HA) epitope tags encoded by the display vector. The extracellular surface display of scFv by *S. cerevisiae* allows the detection of appropriately labelled antigen-antibody interactions by flow cytometry. The binding interactions between antigen and scFv antibody are easily visualized by either direct or indirect fluorescent labelling of the antigen of interest. In a appropriate concentration range the fluorescent signal for antigen binding correlates to the affinity of the clone for this antigen. The use of yeast display of non-immune human scFv libraries is still limited as it has only been available since 2002. However, yeast display has some unique strengths as a platform for affinity reagent discovery and optimization. When screening a non-immune library for specific binders, enrichments of 10^9 can be achieved through multiple rounds of enrichment on a cell sorter, magnetic and/or flow cytometry based.

An additional advantage of yeast display is the ease of discriminating between clones with different affinities for the antigen on a flow cytometer during the selection facilitating the isolation of higher affinity clones from lower affinity clones . Moreover yeast display selections are performed in solution, allowing the investigator to precisely control the concentration of antigen and establish a lower affinity threshold preventing the accumulation of low affinity clones, thereby facilitating clone characterization at the end of the selection. However, in cases where the antigen is not monovalent, strong avidity effects may come into play due to dense display of scFv on the cell wall of the yeast cell. The characterization of binding clones is a time consuming and labor-intensive step in any antibody discovery process. Characterizations usually include: dissociation constant (KD)

determination, determination of off-rate (k_{off}) and of on rate (k_{on}) constants and stability analysis. Yeast display is well suited for these analytical tasks, as the binding properties of multiple individually isolated scFv fragments can be rapidly and quantitatively determined directly on the yeast surface using flow cytometry.

Yeast surface display of scFv antibodies has also been successfully utilized to isolate higher affinity clones from small mutagenic libraries ($1x10^6$ clones) created from a single antigen-binding scFv clone. These libraries are constructed by amplifying the parental scFv gene for affinity maturation using error-prone PCR incorporating three to seven point mutations per scFv. One type of selection of a mutagenized library is based on equilibrium antigen binding at defined concentration, usually at a concentration equal to the K_D of the parental clone. Selecting the brightest antigen-binding fraction of the population will often identify clones with increased affinity. Screening for slower off rates can also be performed. This requires saturating the antigen-binding sites and then allowing dissociation to occur in a large volume of buffer that does not contain antigen ("infinite dilution"). Yeast clones still binding biotinylated antigen are visualized on a flow cytometer and the clones retaining the highest degree of binding are sorted. Selections can also be focused on increasing the k_{on} rate constant of an antigen-scFv interaction by using shorter incubation times with a specific concentration of antigen.

Ribosome Display

The screening methodologies mentioned so far have some restrictions: library size is a limiting factor, due to cell transformation efficiency and the cloning of large libraries (e"10^9) can require a considerable amount of time and work. In order to circumvent these problems, fully in vitro selection techniques have been proposed.

In vitro display technologies combine two important benefits for identifying and optimizing ligands by evolutionary strategies. First, by obviating the need to transform cells in order to generate and select libraries, they allow higher library diversity. Second, by including PCR as an internal step in the procedure, they make PCR-based mutagenesis strategies convenient. The concept underlying ribosome display was first described by Mattheakis *et. al* (Mattheakis, Bhatt *et al*. 1994). The key idea is to translate a library of mRNA molecules with a stoichiometric amount of ribosomes. The functional library size is limited by the quantity of *in vitro* transcription translation mixture used. There are two primary requirements for an efficient ribosome display. Firstly, it is necessary that the ribosome stalls on reaching the 3' end of the mRNA without dissociating. This was achieved by removing translation termination codons from the mRNA (Hanes and Pluckthun 1997). This strategy also allows virtually all the full length translated protein to remain attached to the ribosome (Payvar and Schimke 1979). The second indispensable requirement is the correct folding of the protein while still attached to the ribosome in order to obtain satisfactory results in selections experiments. This can be achieved by introducing an unstructured tether or spacer region to the

C-terminal end of a library of proteins, which is genetically encoded as a 3' end fusion to the DNA library. Another issue is the stability of the ternary complex (mRNA-ribosome-protein). Jermutus and co-workers could show that under appropriate experimental conditions, the complexes are stable for more than 10 days, allowing very extensive off-rate selections One unexpected advantage of these ternary complexes is that proteins displayed on the ribosome seem to be less aggregation-prone, expanding the range of proteins for which this technology can be applied.

After *in vitro* translation the ribosomal complexes are directly used for selection either on a ligand immobilized on a surface or in solution, with the bound ribosomal complexes subsequently being captured with, e.g. magnetic beads. The mRNA incorporated in the bound ribosomal complexes is eluted by addition of EDTA, purified, reverse-transcribed, and amplified by PCR. During the PCR step, the T7 promoter and the Shine-Dalgarno sequence are reintroduced by appropriate primers. Therefore the PCR product can be directly used for further selection cycles.

A large synthetic antibody library, HUCAL-1, of $2x10^9$ independent members (Knappik, Ge *et al*. 2000), was used directly as the starting material for ribosome display selections (Hanes, Schaffitzel *et al*. 2000). This naïve library was applied for six rounds of selection using insulin as antigen. In three independent experiments, different scFv families with different framework combinations were isolated. Since the library was completely synthetic (Knappik, Ge *et al*. 2000), the starting scFv sequences were known and any mutation could be directly identified as being generated during the ribosome display procedure by non proof-reading polymerases in the PCR steps. In summary, this procedure mimics to a certain degree the process of somatic hypermutation of antibodies during secondary immunization.

Ribosome display has been shown to work especially well for affinity maturation of scFv fragments. Two studies, here described, have been reported in which a given antibody was evolved to higher affinity. In both cases, off-rate selection combined with error-prone PCR was used.

An antibody fragment specific to fluorescein was evolved (Jermutus, Honegger *et al*. 2001) using selections in which the antibody-antigen complex needed to last up to 10 days, resulting in final dissociation constants of about 100 pM. The evolved scFv fragments all contained between 4 and 11 mutations, with the majority unlikely to be in contact with the antigen. In another study the dissociation constant of a scFv fragment specific to a peptide from the transcription factor GCN4 was improved from 40 to 5 pM (Zahnd, Spinelli *et al*. 2004). In both cases libraries were generated with error prone PCR and DNA shuffling, and selected for decreased off-rates. scFv antibody fragments have also been evolved for stability by ribosome display (Jermutus, Honegger *et al*. 2001). In a recent work, ribosome display was used in order to generate the tightest peptide-binding antibody reported to date. A single-chain Fv antibody fragment, showing a binding affinity of 1 pM to a peptide derived from the unstructured region of bovine PrP, was obtained by applying several rounds

of directed evolution and off-rate selection with ribosome display using an antibody library generated from a single PrP binder with error-prone PCR and DNA-shuffling (Luginbuhl, Kanyo *et al*. 2006).

Bacterial Surface Display

The display of recombinant peptides on the surface of bacteria such as *E. coli* was first described more than a decade ago. A number of peptides have been successfully displayed on bacterial surfaces (Georgiou *et al*., 1993) including antigenic determinants, heterologous enzymes, antibody fragments and peptide libraries, with sizes ranging from a few amino acids (Agterberg *et al*., 1987) to proteins upto 50 kDa (Suzuki *et al*., 1995). Samuelson *et al*., (2002) have reviewed the various classes of proteins that have been displayed on both Gram positive and Gram negative bacteria.

The major obstacle for the export of foreign peptides to the surface of a gram negative bacterial cell is the presence of an extensive network of macromolecules found within the bilayered cell envelope. To address this problem, one needs to select an appropriate leader sequence and a surface localized carrier protein. The former facilitates the translocation of the peptide through the cell membrane and periplasm before reaching the outer membrane, while the latter anchors and displays the target peptide on the outer surface without perturbing membrane integrity while maintaining the function of the displayed peptide. If fused to the highly expressed outer membrane protein OmpA on the E .coli surface, as many as 50,000 to 100,000 copies of the antibody fragment per cell maybe displayed (Francisco *et al*., 1993). Peptidoglycan (PAL) has been able to facilitate the transport and achoring of an scFv through both the inner and outer membranes without any adverse effect on cell growth and viability. The antibody fragment being displayed on the E.coli surface was able to fold properly and bind to its antigen with high affinity. In the presence of fluoresently labelled antigen, cells displaying specific antibody fragments can be isolated using fluorescence assisted cell soring (FACS) flow cytometry. With the ability to isolate one single bacterium, the system was able to attain a 106 fold enrichment of cells expressing scFvs with desired specificity and affinity (Francisco *et al*., 1993). Dhillon and colleagues (1999) displayed an atrazine specific scFv on the surface of E coli thorugh PAL. The fusion protein had no observable effect on cell growth, in contrast to soluble expression of the antibody fragments, which caused cell lysis within four hours.

Patel *et al*., (2001) developed a continuous affinity based selection for enrichment of E coli displaying a starch binding domain (LamB), which is a cell surface protein. In their experiment, a population of E coli cells were mixed with those carrying plasmid engineered to express the starch binding domain on their surfaces in a ratio of 2 X 108 to 1 and allowed to grow in an affinity column with immobilized starch. With fresh medium continuously feeding into the affinity column fermenter, only cells expressing the starch-binding domain are retained inside the column. Cells

with the desired phenotype were simultaneously enriched and replicated inside the fermenter. After 42 h of continuous culture, a 55 million fold enrichment of cells expressing the starch binding domain was observed. This continuous affinity culture and selection method may be an alternative to FACS by eliminating the use of specialized equipment and at the same time increasing efficiency.

Expression of scFvs on the surfaces of Gram positive bacteria has also been investigated. Single chain Fv was functionally displayed on the surfaces of Staphylococcus xylosus and Staphylococcus carnosus through the C-terminal of protein A. There are three advantages of Gram positive bacteria over Gram negative bacteria. First, larger molecules maybe displayed on Grma positive bacteria than Gram negative bacteria. Second, the single layered cell wall of Gram positive bacteria may have better efficiency of fusion protein secretion and folding than the double layered Gram negative bacterial cell surface. Third, the rigid Gram positive cell wall may allow the cells to withstand harsh conditions during affinity selections or other whole cell application such as bioremediation.

CONCLUSION

Yeast display was shown to sample the immune antibody repertoire considerably more fully than phage display, selecting all the scFv identified by phage display and twice as many novel antibodies. Selection of scFv by phage display correlates to binding phage-scFv to antigen as measured by phage ELISA, which is directly related to the level of properly folded scFv expressed from *E. coli*. Increased access to immune repertoires may increase the rescue of novel antibodies of therapeutic or analytical value that often form a minor part of a typical antibody response. Both phage and yeast display technologies have made the discovery and *in vitro* optimization of antibodies routine, greatly expanding the use of antibodies as therapeutic agents. However, these technologies are limited to identification of antibodies against known antigens. A great advance would be to select cognate antibody-antigen pairs in a single process with no prior knowledge of either protein. With this, a catalog of all the antibody-antigen pairs could be created. Exploiting both phage and yeast display, we have taken the first steps to create a technology to select libraries against libraries to identify cognate antibody-antigen pairs.

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