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Prokaryotic expression of antibodies and affibodies

Luis Angel Fernández

Recent advances have been made in the development of systems for the display and expression of recombinant antibodies and affibodies in filamentous phages, *Escherichia coli* and other prokaryotic cells. Emphasis has been placed on improving phage and phagemid vectors, alternative systems for expression in different cellular compartments (e.g. the outer membrane, periplasm, cytoplasm and extracellular secretion) and novel multimerization systems for generating bivalent or multivalent binding molecules.

Addresses
Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, 28049 Madrid, Spain
e-mail: lafdez@cnb.uam.es

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Abbreviations

Ab	antibody
Af	affibody
AnkR	ankyrin repeat
CDR	complementarity determining region
Fab	antigen-binding fragment of antibodies
Ig	immunoglobulin
MBP	maltose-binding protein
rAb	recombinant antibody
scFv	single-chain antibody Fv fragment
sdAb	single-domain antibody fragment

Introduction

The possibility of expressing antibodies (Abs) in large amounts and in clonal form in *Escherichia coli* cells has attracted the attention of biotechnologists since the early days of genetic engineering. However, production of complete immunoglobulins (Igs) turned out to be extremely difficult, given their structural complexity. This fact directed interest to the production of small Ab fragments that retain full antigen-binding capacity, a strategy that yielded the first successful reports of active Fab (antigen-binding fragment of antibodies composed of heterodimer V_H–C_{H1}/V_L–C_L) and single-chain Fv (scFv) fragments expressed in the periplasm of *E. coli* in the late 1980s. These studies were followed by the cloning of large repertoires of scFv and Fab genes in phage or phagemid vectors, allowing the display of these recombinant antibodies (rAbs) on the capsid of filamentous phage. Phage display permits the *in vitro* selection of clones with distinct antigen-binding specificities in a process named biopanning, which mimics the clonal expansion of B cells *in vivo* (Figure 1).

These initial findings triggered a research explosion in the field that has continued up to now. The generation of large combinatorial libraries of Fabs and scFvs, the engineering of selected clones to improve their binding and stability properties, and the design of new systems for their expression in different bacterial hosts, cellular compartments and protein formats (e.g. bivalent and multivalent molecules, diabodies, etc.) have been the major areas of investigation. The search for even smaller rAb fragments has led to the use of single-domain antibodies (sdAbs), based on natural V domains from heavy-chain-only Abs (e.g. V_{HH} camelbodies) or engineered V_H or V_L domains with autonomous antigen-binding activity. Other antigen-binding fragments have been constructed using the rational design of binding capacities in small protein scaffolds, not based on Ig domains, and these are generally referred to as affibodies (Afs).

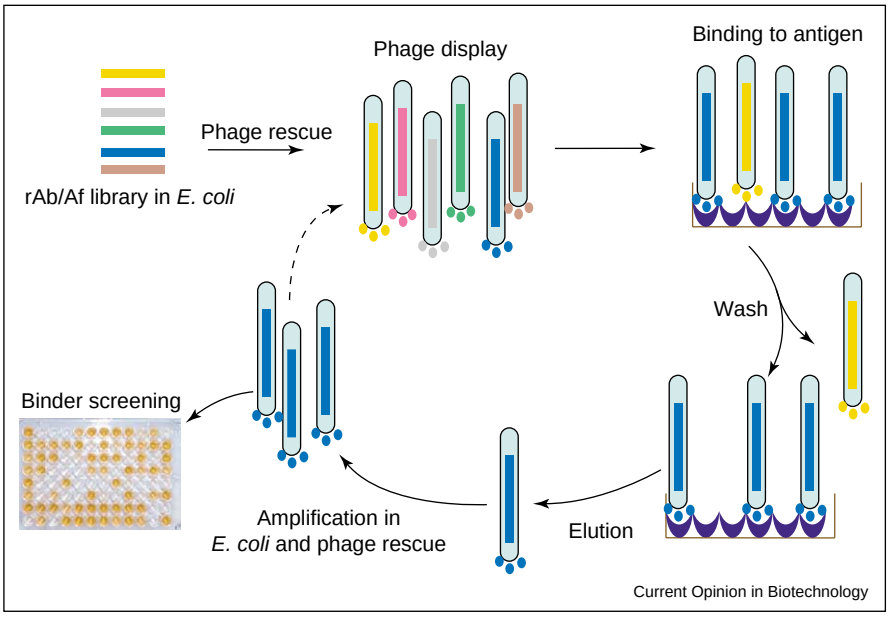
This review deals with the more recent developments in rAb and Af expression and display systems in prokaryotic cells. Given space limitations, structural studies addressing the interaction of rAbs and Afs with proteins and haptens will not be discussed [1–6]. The reader is also referred to general reviews for comprehensive coverage of this technology [7–14].

Phage display

rAbs and Afs are generally displayed in filamentous phages (e.g. M13) as fusions to the minor coat protein pIII (~3 to 5 copies/virion), which is essential for phage infection and packaging. Hence, vectors for phage display are either directly derived from complete phage genomes or are phagemids (plasmids with phage packaging signals) encoding pIII. Alternative phage-display systems have been reported, like those based on the minor coat protein pIX [15•], but have not been extensively used.

Choosing between a phage and a phagemid vector is relevant for biopanning and for the affinity of the selected clones. A recent study using a non-immune human scFv library [16•] has clearly shown that phage vectors allow higher display levels and make biopanning more efficient (i.e. greater numbers of binders are isolated in fewer rounds). The reason for these differences stems from the need for a helper phage (e.g. VCS-M13 or

Figure 1



The selection of specific rAb and Af binders using phage display. Gene libraries of rAbs or Afs, cloned in phage or phagemid vectors, can be rescued in phage particles displaying the corresponding rAb or Af on their capsids. This allows the specific binding of phage particles displaying rAb and Af binders to a given immobilized antigen, for example, on a solid surface (other strategies have also been developed [77]). Elution of bound phages, and their amplification by re-infecting *E. coli* cells, permits their clonal expansion. After a few rounds of panning (including phage binding, elution and amplification), the binding activity of individual clones can be screened by using enzyme-linked immunosorbent assays on microtiter plates.

M13KO7) for rescue of phagemid vectors (Figure 2). Wild-type pIII, encoded by the helper phage, is packaged more efficiently than scFv–pIII fusions encoded by phagemids. As a result, phagemid virions contain none or a single copy of the scFv–pIII hybrid, whereas several copies can be packaged in phage virions.

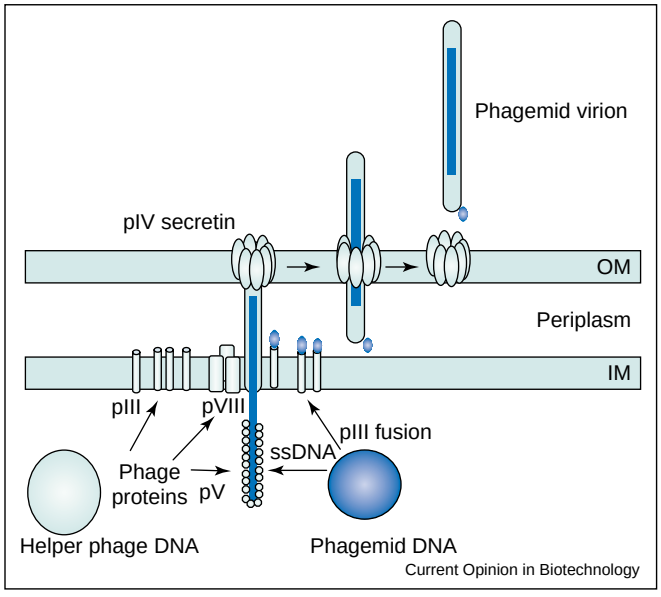
In some situations, such as panning against rare targets on cell surfaces, phage multivalency is desirable. Other factors, such as culture conditions, *E. coli* strain, and the signal peptide present in the vector, also have an important influence on the display levels of pIII fusions [17•,18]. In the case of Fabs, multivalency is difficult to achieve even with phage vectors owing to their larger size (proteolysis of the Fab fusion generates wild-type pIII that is packaged in the virion). A new vector system allows the display of bivalent Fabs fused to leucine zippers on phagemid virions [19].

By contrast, monovalency of phagemid vectors benefits the affinity of the selected scFv clones. On average, scFvs isolated from phagemids have five- to tenfold higher affinities than those from phage vectors, in which avidity effects allow the selection of low-affinity clones [16•]. Panning with the antigen in solution can minimize the problems of low affinity associated with scFvs displayed on phage vectors [20].

A novel phagemid vector has been developed that enables the removal of phage particles not displaying an scFv–pIII fusion before panning [21••]. The method is based on the production of fusions between scFv, a cellulose-binding domain (CBD) and pIII. Phage particles displaying scFv–CBD fused to pIII are captured on cellulose filters whereas 'bald' phages are removed by washing.

An interesting option that combines the advantages of phage and phagemids systems is the use of mutant helper phages lacking pIII. The pIII mutant helper phages produce multivalent phagemid particles that can be used for the initial round of biopanning, whereas rescue with standard helper phages produces monovalent (high-affinity) particles for the following rounds [22••]. Two improved mutant helper phages with partial deletions or amber stop codons in gene 3 have been reported (Hyperphage and Phaberge) [17•,22••]. Compared with other M13–pIII mutants [23,24], the new helpers appear to be more stable and produce higher titers of rescued phagemids. These mutant helper phages can also be extremely useful for the selective infection of phage (SIP) [25]. SIP phagemid vectors contain an N-terminal truncation of pIII that produces non-infective particles unless the displayed rAbs or Afs interact with the desired antigen (provided in *trans* and fused to the N-terminal domain of pIII) [26].

Figure 2



Phagemid packaging into filamentous phage particles. A simplified scheme depicting the packaging of phagemid vector DNA into filamentous phage particles. Phagemid DNA (navy blue circle) is packed as single-stranded DNA (ssDNA, navy blue solid line) into capsids of filamentous phage (light blue rod) composed of the major coat protein pVIII and some minor coat proteins like pIII. Capsid proteins (e.g. pVIII, pIII) and other phage proteins required for packaging, such as pV (a ssDNA-binding protein) and pIV an outer membrane (OM) secretin, are encoded by the DNA of a standard helper phage (light blue circle). Wild-type pIII and pIII fusions, encoded by phagemids, insert into the inner membrane (IM) before assembly into the terminal tip of a filamentous virion (3–5 copies/virion). Usually, wild-type pIII is packaged more efficiently than the pIII fusion, which are found in a single copy per virion unless a M13ΔpIII helper phage is used for rescue.

Bacterial display

The display of rAbs and Afs on the surface of bacteria is not only an alternative expression system for the screening of binders from libraries, but opens new potential applications — like the generation of whole-cell affinity sorbents, the delivery of passive immunity to mucosal body surfaces, and the targeting of bacteria to certain antigens or tissues. In *E. coli*, initial reports of the surface display of scFvs were achieved using lipoproteins [27] and lipoprotein–porin fusions (Lpp–OmpA′) [28]. The major disadvantage of these expression systems is their toxicity for *E. coli* and the absence of a *bona fide* secretion of the scFvs, which become surface-exposed mostly because of the leakiness of the bacterial outer membrane after induction.

Recently, bacterial autotransporters have been proved as an effective system for the surface display of single Ig domains and stable scFvs in *E. coli* [29••]. Autotransporters comprise a large family of proteins secreted by Gram-negative bacteria. They are characterized by a C-terminal domain that inserts into the outer membrane and assembles into an oligomeric complex with a 2 nm hydrophilic pore through which the N-domain of the protein is translocated [30]. By substituting the natural protease N-domain of the IgA protease from *Neisseria gonorroheae* (an autotransporter) with different scFvs, V_{HH} domains, and strings of two or three V_{HH} domains, it has been shown that stable scFvs and V_{HH} can be displayed on the surface of *E. coli* cells with efficiencies close to 100% [29••]. Using this expression system it was also shown that *E. coli* cells displaying an scFv against enteric coronaviruses were able to act as delivery vehicles of passive immunity capable of neutralizing the infection of mammalian cells cultured *in vitro* [31].

A proof-of-principle of the delivery of passive immunity by commensal bacteria colonizing mucosal surfaces was obtained using an *in vivo* animal model and the food-grade Gram-positive *Lactobacillus zeae* displaying an scFv against the SAI/II adhesion molecule of *Streptococcus mutans*, the major pathogen involved in the development of dental caries [32••]. Efficient scFv display in *L. zeae* cells was achieved by fusion to the last 244 amino acid fragment of proteinase P. Rats that were orally treated with *L. zeae* cells displaying this scFv showed increased resistance to the development of dental caries.

Additional work has highlighted the potential use of Gram-positive microorganisms for the surface display of rAb and Af libraries. Afs based on the scaffold of protein A of *Staphylococcus aureus* have been displayed on *Staphylococcus carnosus* cells. Mixing an *S. carnosus* cell population displaying different Afs with fluorescence-labeled antigens allowed the selection of Afs with given

specificities by fluorescence-activated cell sorting [33]. The display system is based on the C-terminal cell-wall anchor domain of protein A of *S. aureus* [34].

Expression systems for rAbs

Figure 3 summarizes various strategies for targeting the expression of active rAbs to distinct compartments of *E. coli* (e.g. extracellular medium, outer membrane, periplasm and cytoplasm). Expression of rAbs is typically achieved by fusion to N-terminal signal peptides, which target the protein to the periplasmic space of *E. coli* where chaperones such as Skp, FkpA, DsbA and DsbC assist the folding of the Ig domains and form the correct disulfide bridges to stabilize the structure [35,36,37••]. Production yields in the periplasm usually range from 0.1–10 mg/L of induced culture ($OD_{600nm} = 1$). *E. coli* host strains lacking the major periplasmic proteases (*DegP* and *Prc*) have been shown to increase the yield of Fabs produced in the periplasm two- to threefold [38]. In exceptional cases, much higher periplasmic yields of sdAbs have been reported (100 mg/L) [39].

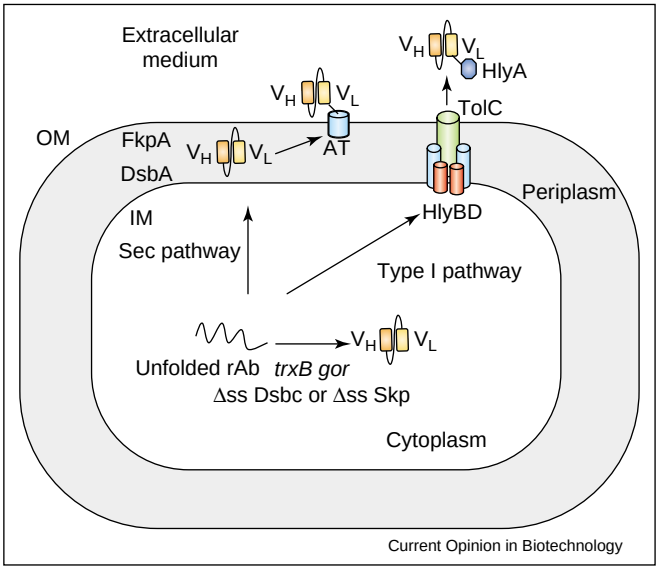
Periplasmic overexpression may render an important fraction of the produced rAb insoluble. Fusion of scFvs to the periplasmic chaperones DsbC or DsbG, and co-expression of DsbC in *trans*, have been shown to increase significantly the fraction of soluble and functional scFv in the periplasm of *E. coli* [40].

Higher levels of rAbs can be produced in the cytoplasm of *E. coli* using common overexpression systems (e.g. T7 promoter vectors) and shake flask cultures (yields >50 mg/L/OD_{600nm}). However, these cytoplasmic rAbs are reduced (i.e. disulfide bonds are not formed in the cytoplasm of wild-type *E. coli* strains), unfolded, and form inclusion bodies that need to be solubilized under strong denaturing conditions (e.g. 8 M urea). The rAbs purified from these inclusion bodies can be refolded *in vitro* by dialysis of the denaturing agent in the presence of a redox pair (e.g. reduced and oxidized glutathione 1:1). The efficiency of refolding is highly variable depending on the specific clone, although excellent results have been reported for some scFvs [41•,42•].

An scFv can also be expressed in active (but not oxidized) form in the cytoplasm of wild-type *E. coli* cells by grafting its complementarity determining region (CDR) to frameworks regions derived from a highly stable scFv. In some cases, this process has been shown to maintain the unaltered specificity and affinity of the original scFv clone [43••].

An alternative to the above approaches is the use of *E. coli* strains that promote the correct folding and oxidation of rAb in the cytoplasm *in vivo*. As oxidized scFvs seem to refold *in vitro* with higher efficiency [44••], these strains may also be useful for the refolding of scFvs from

Figure 3



Targeting of functional rAbs to various *E. coli* compartments. rAbs (illustrated here as an scFv molecule for simplicity) can be expressed in *E. coli* using the classical Sec pathway that targets the protein to the periplasmic space. Periplasmic chaperones (e.g. DsbA and FkpA) fold the Ig domains or rAbs using this expression strategy. When fused to a C-terminal autotransporter domain (AT), these rAbs can be redirected to the outer membrane (OM) and become exposed at the surface of the bacteria. Alternatively, rAbs devoid of Sec signal peptides can be expressed in a functional form in the cytoplasm of *E. coli trxB gor* mutant strains. Cytoplasmic co-expression of signal sequence-less (Δss) derivatives of periplasmic chaperones (e.g. DsbC or Skp) assist in many cases the folding of the cytoplasmic rAbs. In addition, rAbs can be secreted toward the extracellular medium, directly from the cytoplasm, by fusion with a C-terminal secretion signal from *E. coli* α-hemolysin (HlyA) and its type I transporter (TolC-HlyB-HlyD). See text for details. IM, inner membrane.

inclusion bodies. Functional (correctly folded and oxidized) and soluble Fab and scFv molecules have been produced in the cytoplasm of *E. coli* cells carrying mutations in the genes coding for thiorredoxin reductase (*trxB*) and glutathione oxidoreductase (*gor*), with yields similar or even higher than those obtained in the periplasm [37••,45••,46•]. *E. coli trxB gor* mutant cells have an oxidizing cytoplasm capable of forming disulfide bridges in proteins [47,48]. At induction temperatures above 30°C efficient folding of Fabs and scFvs in *E. coli trxB gor* mutants appears to require cytoplasmic co-expression of the periplasmic chaperones DsbC or Skp (devoid of their N-terminal signal peptides) [37••,45••]. Interestingly, *in vivo* biotinylation of an scFv expressed in the absence of chaperones in the cytoplasm of *E. coli trxB gor* cells was only efficient at temperatures below 30°C [49•]. Biotinylated rAbs can be bound to avidin- and streptavidin-containing resins (e.g. streptavidin magnetic beads) and immunoconjugates (e.g. streptavidin-peroxidase) for purification, immunoprecipitation and detection purposes.

Protein chaperones are not always needed. Two Fabs were shown to accumulate at high levels in active form in the cytoplasm of *E. coli trxB gor* cells (10–30 mg/L/OD_{600nm}) in the absence of chaperones [46•]. Similarly, a catalytic scFv fused to the C terminus of NusA was produced in a folded form in the cytoplasm of *E. coli trxB gor* cells without co-expression of chaperones [50]. Significantly, the unfused scFv aggregates in inclusion bodies in the cytoplasm of *E. coli trxB gor* cells and was rapidly degraded in wild-type *E. coli* cells, either alone or fused to NusA. N-terminal fusions to maltose-binding protein were also shown to improve the expression of scFvs in the cytoplasm of wild-type *E. coli* cells (but in a reduced and only partially folded form) [51]. Taken together, the above data indicate that folding and oxidation of rAbs generally requires the cytoplasm of *E. coli trxB gor* cells and the activity of chaperones (e.g. DsbC) or other solubilizing factors (e.g. N-terminal fusions), although particular clones may fold efficiently in their absence.

An alternative to the periplasmic and cytoplasmic expression of rAbs is their secretion to culture supernants using the α-hemolysin (HlyA) system of *E. coli* [52••,53]. HlyA is a monomeric toxin that is secreted directly from the cytoplasm into the extracellular medium across a three-component protein channel (TolC/HlyB/HlyD) connecting the inner and outer membrane [54,55]. ScFvs and sdAbs, devoid of N-terminal signal peptide and fused to the C-terminal domain of HlyA, have been secreted into the culture medium by *E. coli* cells expressing TolC/HlyB/HlyD. These rAbs accumulated as the sole polypeptide in the culture medium at concentrations similar to those obtained by their periplasmic expression (0.5–2 mg/L). The mechanism of folding and oxidation of rAbs secreted by the HlyA pathway is unclear, but appears to be intimately associated with the TolC/HlyB/HlyD channel and is independent of periplasmic chaperones (e.g. DsbA, DsbC) [56]. As different rAbs have distinct folding requirements, it remains to be determined to what extent this periplasmic-independent pathway is compatible with the folding of the diverse sequences found in rAb libraries.

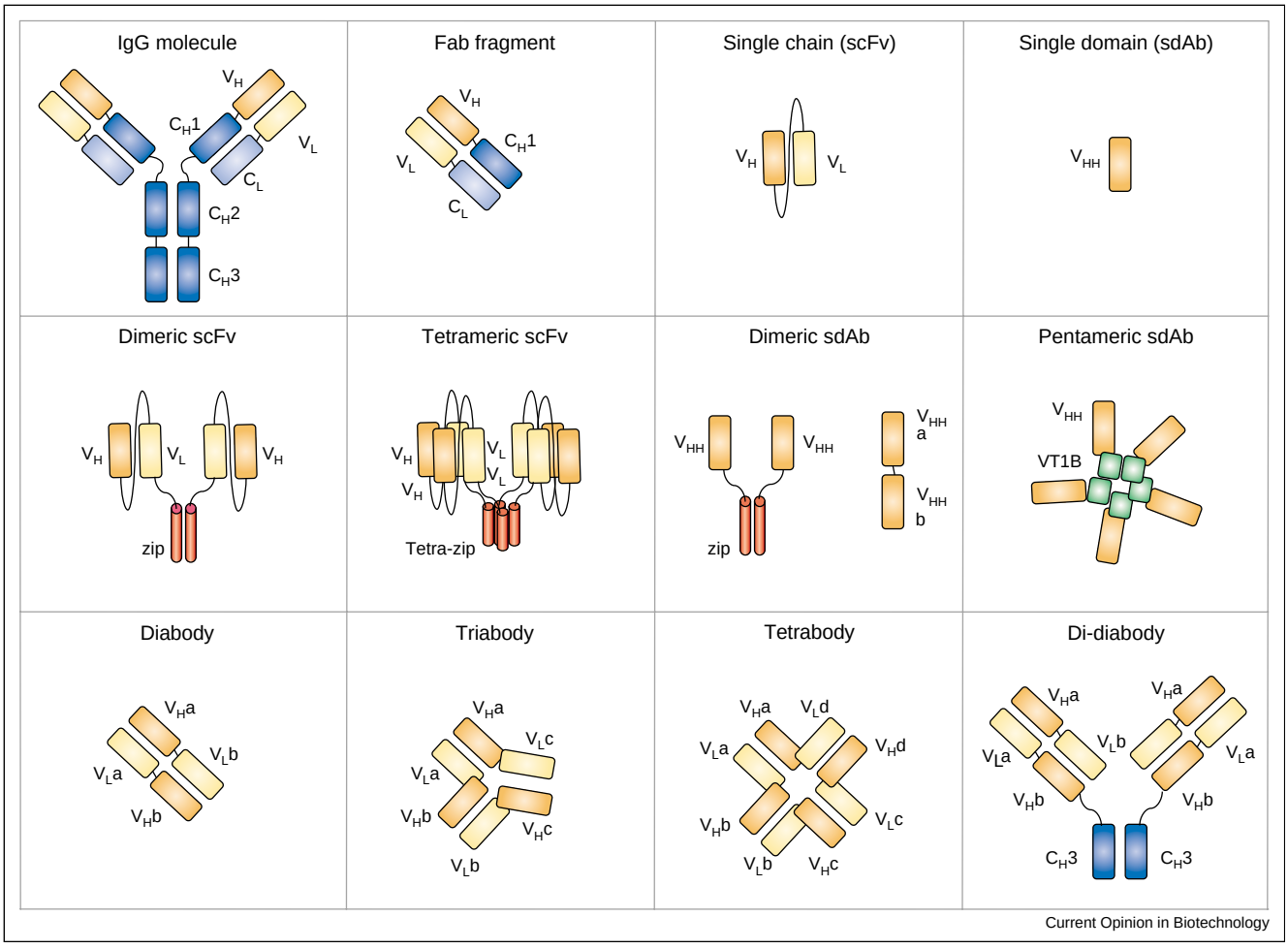
Oligomerization strategies

The oligomerization of rAbs to make bivalent and multivalent molecules of high functional affinity (avidity) has been elicited using a variety of approaches. Earliest reports employed dimerizing and oligomerizing protein motifs (e.g. amphipathic helices) with and without stabilizing disulfide bonds (reviewed in [13]). Alternatively, shortening the length of the linker peptide connecting the V_H–V_L domains in scFvs generates diabodies (scFv dimers), triabodies and tetrabodies, with mono- bi- tri- or tetra-antigen specificities (reviewed in [57]). Bivalent dimeric and bi-specific camel sdAbs have also been produced by fusing two V_{HH} domains with a natural hinge peptide [58]. Figure 4 summarizes the structure of various rAbs (Fabs, scFvs and sdAbs) and some of the oligomeric forms that are discussed here.

In general, rAbs of high avidity can be obtained with the above techniques, although they suffer some problems related to aggregation, unintended swapping between V domains, and instability in the bloodstream. Therefore, current studies are focusing on the major factors influencing their stability and expression [59,60], while others search for new methodologies to produce oligomeric rAbs of high-avidity. For instance, novel 'di-diabody' molecules of high stability have been obtained in the periplasm by the interaction of two bi-specific diabody molecules with the constant C_H3 domain from a natural IgG [61]. Stable dimers of V_{HH} have been secreted to the supernatants of *E. coli* cultures using a modified HlyA signal containing the leucine zipper of GCN4 [52••]. Another report used the B subunit of the Verotoxin 1 (VT1B), a Shiga-like $A:B_5$ toxin from *E. coli* O157:H7, to assemble stable and soluble pentamers of sdAb with high avidity in the periplasm [39,62••]. The sdAb molecules were derived from a naïve llama V_{HH} library and the small VT1B monomer (7.7 kDa) was fused to the N termini of the sdAbs, which did not interfere with their binding properties.

A novel multimerization system, based on the interaction between the prokaryotic ribonuclease Barnase (110 amino acids) and its inhibitor Barstar (89 amino acids), has revealed important advantages [44••]. Barnase and Barstar interact with extremely high affinity ($K_D \sim 10^{-14}$ M), thus making complexes of remarkable stability. These polypeptides are soluble, fold independently of its interacting pair, and can be secreted to the periplasm. Fusing these proteins to the C terminus of scFvs has allowed the production of stable mono- di- and trivalent complexes

Figure 4



Structures of common recombinant antibodies. Schematic drawing showing the domain structure of various rAbs (Fabs, scFvs and sdAbs) and some of their oligomeric formats discussed in the text. The structure of a natural IgG molecule is also given for reference. Constant domains (C) are in blue and variable domains (V) in yellow; Ig domains are depicted as rectangles for both the heavy (H) and light (L) chains. Amphipathic α helices with dimerization (zip) and tetramerization (tetra-zip) capacity are shown in red. The pentameric B subunit of Verotoxin 1 (VT1B) is shown as a green square.

of high avidity and with long half-lives *in vivo*. Of specific note using this approach, homogeneous bi-specific dimers can be produced *in vitro* during the process of large-scale protein purification by in-column refolding.

Protein modifications

Various modifications have been engineered into rAbs and Afs to assist downstream processing. For instance, scFvs fused to two chitin-binding domains can be immobilized on inexpensive chitin beads directly from crude protein extracts and used in immunoaffinity chromatography to purify proteins recognized by their scFv moiety [63•]. Fusion of one or two Afs (based on the 58 amino acid three-helix bundle domain of staphylococcal protein A) to β-galactosidase (a homotetramer in the cytoplasm of *E. coli*) resulted in immunoconjugates that exhibited improved binding properties [64•,65]. The $(Af_2$-β-galactosidase$)_4$ complex was produced at high yields in shake flask cultures of *E. coli* (~400 mg/L) and could be used directly in enzyme-linked immunosorbent assays and immunohistochemistry [64•].

Phage display vectors have also been modified to produce scFvs with an extra cysteine residue near their C termini [66]. The free thiol group of this cysteine allows the specific chemical crosslinking of polyethylene glycol (PEG) maleimide to the rAb. The covalent attachment of PEG (PEGylation) can extend plasma half-life and increase the solubility of rAb and other proteins [67]. Another important protein modification is biotinylation. scFvs can be biotinylated *in vivo* in the cytoplasm of *E. coli trxB gor* cells with very high efficiencies (>90% of the total scFv produced) [49•]. This has been achieved by fusion of the scFv to the 89 amino acid C-domain of the

biotin carboxyl carrier protein, a substrate of the endogenous *E. coli* biotin ligase. Unlike most chemical methods, *in vivo* biotinylation guarantees site-specific conjugation of a single biotin molecule per scFv polypeptide.

Novel protein scaffolds

New protein libraries based on single Ig domains and other stable proteins scaffolds have been described recently. For instance, a library of sdAbs based on heavy-chain-only Abs of the isotype novel antigen receptor (IgNAR) was obtained from nurse sharks (*Ginglymostoma cirratum*) immunized with hen egg-white lysozyme (HEL). From this library, sdAb binders of high stability and with high affinity to HEL have been selected by phage display [68].

Two Af libraries have been constructed based on ankyrin repeats (AnkR) as protein scaffolds [69••,70]. AnkR proteins are composed of several 33 amino acid repeats stacked in a row, each repeat comprising a β-turn followed by two antiparallel α helices and a C-terminal loop [71]. The AnkR proteins are found in organisms from all phyla and can be located in the cytoplasm, anchored to membranes or secreted to the extracellular space. The strategy to generate the AnkR Af libraries was based on the design of a synthetic AnkR consensus sequence, derived from natural AnkR sequences, with randomized amino acid positions at the β-turn and the short hinge connecting the two α helices. This AnkR module was repeated two or three times, depending on the specific library, and the fusion proteins were flanked at their N and C termini with capping AnkR of defined sequence [69••]. The AnkR Afs were highly stable, soluble and accumulated in the cytoplasm of *E. coli* at high concentrations (yields averaged 200 mg/L of culture in shake flasks). More importantly, high-affinity binders (K_D in the nanomolar range) against different protein targets (e.g. MBP) were selected from these libraries using ribosomal display (a powerful technique for the selection of binders directly from an *in vitro* transcription-translation reaction [72,73]). The same report also showed a co-crystal of MBP with its binder AnkR Af, revealing the interaction surface between these two molecules. The three-dimensional X-ray structure of the MBP–Af complex showed the randomized amino acids of the AnkRs forming a concave surface contacting MBP and covering ~600 $Å^2$ of its surface, which is only slightly smaller than the surface covered in antigen–antibody complexes (777 ± 135 $Å^2$) [74]. Given the extraordinary properties of AnkR Afs (e.g. high solubility, affinity, flexible modularity, absence of disulfide bonds, etc.), these Afs may be suited not only for *in vitro* applications (e.g. affinity purification, protein co-crystallization and protein chips), but might also have a role as intracellular inhibitors of specific processes *in vivo*.

Finally, a recent report [75] described the generation of a new Af library based on a stable variant of green fluorescent protein (GFP), although this has not been confirmed [76].

Conclusions

Although additional work is still needed to increase the functional expression of rAbs and Afs in various cellular compartments of *E. coli*, this organism is clearly the best choice for these technologies given its high transformation efficiencies and the panoply of vector systems to display, express and modify rAbs and Afs. Future research is likely to improve functional expression, select new stable protein scaffolds with desired properties for specialized applications, integrate affinity maturation and display systems, and expand the use of these technologies to other prokaryotic microorganisms.

Update

A recent study evaluates the influence of different vector and culture conditions for efficient Fab phage display and expression in *E. coli* [78]. The S-layer protein of *Bacillus sphaericus* CCM 2177 has been fused at its C terminus to a V_{HH} camel antibody against prostate cancer specific antigen (PSA). The hybrid S-layer protein retained the ability to self-assemble with the V_{HH} moiety exposed to the outer surface of the protein lattice. After recrystallization on gold chips, this protein lattice was used as a sensing layer in surface plasmon resonance to detect PSA [79].

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