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Prokaryotic expression of antibodies

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Summary

Maximizing the expression yields of recombinant whole antibodies and antibody fragments such as Fabs, single-chain Fvs and single-domain antibodies is highly desirable since it leads to lower production costs. Various eukaryotic and prokaryotic expression systems have been exploited to accommodate antibody expression but *Escherichia coli* systems have enjoyed popularity, in particular with respect to antibody fragments, because of their low cost and convenience. In many instances, product yields have been less than adequate and intrinsic and extrinsic variables have been investigated in an effort to improve yields. This review deals with various aspects of antibody expression in *E. coli* with a particular focus on single-domain antibodies.

I. Introduction

Recombinant antibodies (rAbs) have been expressed in various formats and are being increasingly used in or developed for cancer therapy [1–9]. Antibody therapeutics for cancer are already a multi-billion dollar a year market and a large number of monoclonal antibodies are at various stages of clinical trials. Antibody engineering techniques, based largely on bacterial expression of antibodies, are a major driving force in the development of these drugs.

In terms of combining site topology, rAbs can be grouped as classical, or conventional antibodies, and fragments thereof, where the antibody combining site is formed by the association of V_H and V_L domains, or single-domain antibodies (sdAbs), where the combining site resides in a single domain as in V_HHs, V_Hs or V_Ls, (Figure 1). Antibody fragments and their manifold derivatives have been engineered for a variety of specific applications.

The antibody formats commonly selected for prokaryotic expression have been antigen binding fragments (Fabs) and single-chain variable regions (scFvs) from conventional antibodies (Figure 1). There is extensive literature on rAbs [6,10–14] and on the expression [15–17] and design of antibody fragments in terms of scFv domain order [18–20], scFv linker length [17,21–23], site directed mutagenesis of the variable domains [24–28], expression in various organisms from bacteria [29,30] to transgenic animals [31,32] and targeting the antibodies to different subcellular compartments [33–35]. These efforts have been directed towards designing antibody molecules with (i) high affinity, specificity and solubility, (ii) genetic stability of the construct within the expression host and the stability of protein products *per se* and (iii) optimal and economical expression system(s) with high protein yields and feasible purification strategies and downstream processing [12,31,36–43]. Antibody expression is influenced by intrinsic factors such as antibody gene sequence, transcription and translation efficiencies and spontaneous protein folding and extrinsic factors or physiological effects such as translocation inside the cell, processing, assisted protein folding, protein degradation and toxicity to *E. coli* [40,43–45].

It is well established that reducing the complexity and the size of the antibody molecule generally avoids many problems related to *in vivo* expression yield, correct folding, good solubility, thermal stability

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and conformational stability [3,13,46]. In this regard, single variable domains of antibodies (V_H or V_L) are preferable to Fabs or scFvs. However, these molecules do not exist naturally as single entities and associate with each other to make a functional unit. Camelidae, wobbegongs and nurse sharks make substantial amounts of their immunoglobulins (Igs) as antibodies which lack light chains (Figure 1; see [47] for the Igs from wobbegongs and nurse sharks), which means that single variable domains can function in terms of antigen binding [48–51], and can be cloned and expressed as sdAbs [47,52–54]. It is now widely accepted that the discovery and engineering of heavy chain antibodies (i.e., camelid IgGs which lack light chains) has greatly improved our knowledge of domain function in antibodies and has opened new perspectives in antibody design and application. Recent scientific literature definitively shows that sdAbs, regardless of their origin, and their derivatives will occupy an important place in the future development of antibody-based reagents for analytical, diagnostic and therapeutic applications [1,3,7,55–61].

II. rAb expression systems

Diverse prokaryotic and eukaryotic expression systems have been developed for rAb expression. These have included bacterial [29,30,62–67], yeast and filamentous fungus [40,68], eukaryotic alga [69], insect cell [70], plant [71], mammalian cell [72,73] and transgenic animal systems [32]. While in many instances rAbs can be expressed in several different expression systems, there is sometimes less flexibility in terms of choice of expression system due to structural requirements on the part of the rAb. For example, a requirement for certain therapeutic IgGs to have appropriate glycosylation necessitates their expression in mammalian cells [74]. Also, due to their complex structure, whole antibodies have been preferably expressed in eukaryotic systems which have the appropriate cellular machinery for efficient folding and assembly. Recently, however, a fully active non-glycosylated IgG with the ability to bind neonatal receptors was expressed at high level in *E. coli* [75]. Fabs, scFvs and sdAbs have a much simpler structure and do not require glycosylation. Thus, bacterial expression, and almost exclusively *E. coli* expression, has been the method of choice for expression of these molecules.

III. *E. coli* expression strategies

Incentives for the use of *E. coli* expression systems include simple fermentation conditions, ease of genetic manipulation, ease of scale-up, relatively short duration between transformation and protein purification, no concerns about viruses that are harmful to humans and relatively low capital costs for fermentation. However, *E. coli* expression has its own drawbacks including inefficient production of *bona fide*, complex, multi-domain molecules such as IgGs (see above) and the possibility of bacterial endotoxin contamination of purified products.

Two basic strategies have been applied to express various formats of antibody fragments, including Fabs, scFvs and sdAbs, in *E. coli*. The two approaches involve directing the antibody product to either the reducing environment of the cytoplasm or the oxidizing environment of the periplasmic space between the cytoplasmic and outer membranes or the culture medium.

III.A. Cytoplasmic expression

The cytoplasmic approach benefits from a high expression level of antibodies using a strong promoter (e.g., T7 promoter). The expressed proteins generally accumulate in the cytoplasm as inclusion bodies (up to 0.5 g/L in shake-flask cultures and 3.1 g/L in fermentors) due to their foreign nature, high expression rate and lack of disulfide bonds because of the reducing environment of the cytoplasm, and thus, need to be converted to active species by *in vitro* renaturation [76–80]. However, in rather infrequent instances, where rAbs are stable without the conserved disulfide bonds [81,82] and, thus, their folding is independent of the redox conditions of the cytoplasm, functional expression can be achieved without resorting to *in vitro* renaturation. Such disulfide-less rAbs are also important tools for intrabody technology where antibody fragments fold in the reducing environment of the cytoplasm [83,84]. An advantage of the cytoplasmic expression approach is that inclusion bodies can easily be recovered from other cellular components because of their large size and high density following lysis of bacterial cells. Moreover, this approach is useful for producing antibody-based fusion proteins such as immunotoxins that might be toxic for bacterial cells or rAbs that are unstable due to intracellular degradation when expressed in a soluble or secreted form. However,

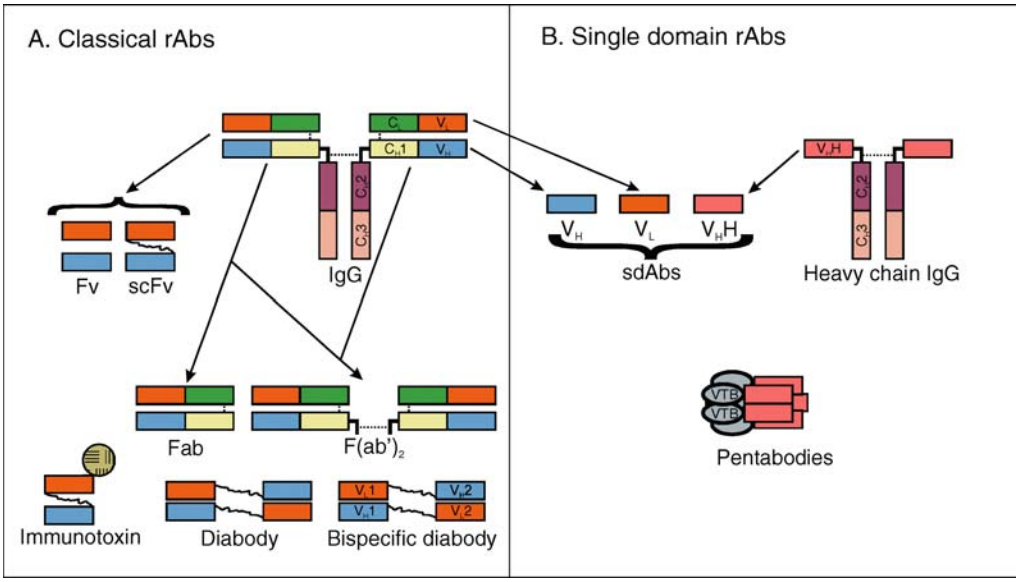

Figure 1. Diagramatic representation of a conventional immunoglobulin G(IgG), a camelid heavy chain antibody and antigen binding fragments derived from each antibody type. The figure only depicts those antigen binding fragments which are explicitly mentioned in the text. For a more complete graphic list of antigen binding fragments the reader can refer to many of the reviews cited in the text on the subject of the recombinant antibodies. For simplicity the interdomain noncovalent interactions in classical rAbs and camelid heavy chain IgG (HC IgG) are not depicted. The dotted lines and the lines connecting the V_Ls to the V_Hs represent disulfide linkages and linkers, respectively. In immunotoxin an scFv is attached to a toxin. In Pentabodies, VTB represents the verotoxin B subunit.

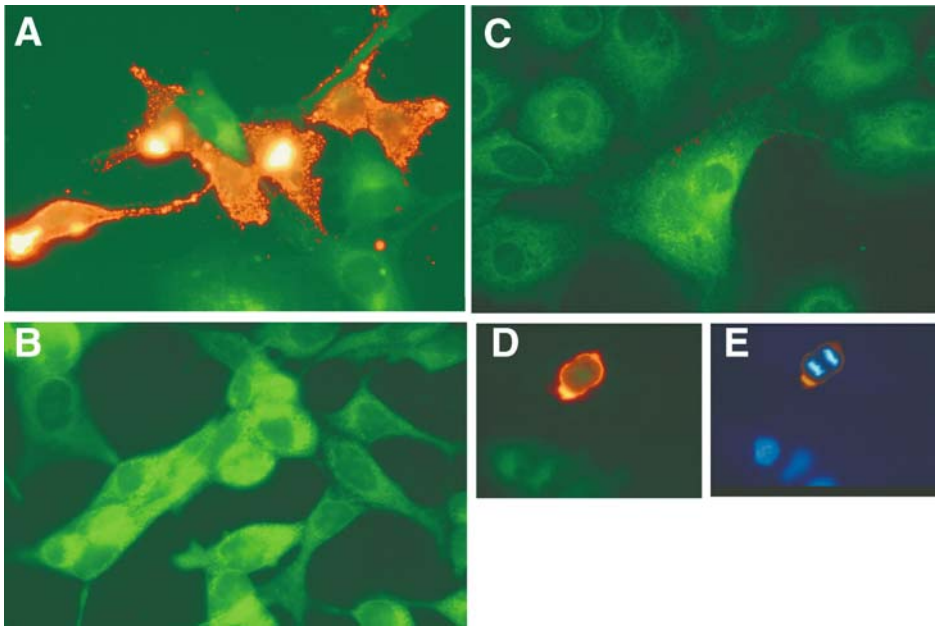

Figure 3. Immunocytochemical staining of (A) A549 lung cancer cells, (B) LNCaP prostate cancer cells and (C) normal epithelial cells by AFAI, an sdAb isolated by panning a phage antibody library against A549 cells. The AFAI sdAb appears to bind to an antigen that is present on A549 cells during cell division (D and E). Cells were stained with phage-displayed AFAI as the first anibody, anti-M13 (phage) IgG as the second antibody and Alexa Fluor® 546 (red) labelled goat anti-mouse IgG as the third antibody. Endoplasmic reticulum was stained with $(DiOC_5)_3$ (green).

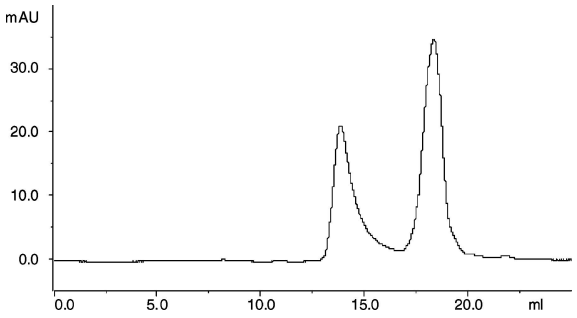

Figure 2. Superdex 200 size exclusion chromatogram showing the separation of monomeric (first peak) and pentameric (second peak) llama V_HH.

correct *in vitro* refolding and purification of functional product is a complex and time-consuming process, requiring expertise and involving many steps. Problems and limitations commonly encountered with this approach, for antibodies in particular, are (i) difficulties in predicting the tendency of different sequences to form inclusion bodies and their susceptibility to proteases, (ii) the need for genetic manipulations at the mRNA 5'-end to avoid possible hairpin structure formation, (iii) refolding efficiency which is highly variable depending on the specific antibody fragment with yields varying from 10–40% for Fab and Fv fragments and (iv) the need for separation of correctly folded protein from the incorrectly folded protein [6,12,24,85–87].

Improvements have been made to the functional expression in the cytoplasm by using *E. coli* cells carrying mutations in the genes coding for thioredoxin reductase and gluthathione oxidoreductase [16]. These mutant cells have an oxidizing cytoplasm capable of forming disulfide bridges in proteins. As an alternative, the functional production yields of antibody fragments in the cytoplasm can also be significantly improved by co-expression with chaperones and foldases or by a fusion protein strategy (see below).

III.B. Antibody secretion to the periplasm or culture medium

The periplasmic strategy imitates the natural folding process and secretion of Igs in eukaryotes. In bacteria, there is secretory machinery which directs proteins carrying specific signal sequences such as pelB, phoA and ompA to the periplasmic space [88]. The periplasmic space is a more oxidizing environment than the cytoplasm and is equipped with a number of proteins important for folding and assembly of recombinant proteins, such as those that catalyze disulfide bond formation and rearrangement (DsbA, PDI and DsbC) or chaperones such as SKp or FkpA [86,89,90]. Antibody fragments expressed in the periplamic space have been shown to be correctly folded with yields of 0.1–100 mg/L in shake flasks [29,30,91,92] and 1–2 g/L in fermentors [37]. Moreover, extraction of periplasmically expressed proteins can easily be performed by a simple osmotic shock procedure and purification of antibodies from periplasmic extracts is less problematic than purification from cell lysates since there are fewer contaminating bacterial proteins in periplasmic extracts. However, it should be borne in mind that the *E. coli* machinery for protein folding and export to the periplasm has limited capacity and that high expression of recombinant proteins often results in the accumulation of insoluble product in the periplasm. It is now recognized that aggregation *in vivo* is a function of the solubility and stability of the folding intermediates in the periplasmic environment and not of the fully folded protein [12]. Moreover, secretion of recombinant protein interferes with the normal function of the secretory machinery of the cell and, therefore, can be toxic to the host cell, leading to induction of periplasmic proteases, enhanced outer membrane permeability and reduced levels of folding catalysts [6,13,40].

As an alternative to secretion to the periplasm, Fernandez et al. [93] have described an expression strategy in which antibody fragments are secreted into the culture medium using the *E. coli* α-hemolysin (HlyA) system. This pathway is comprised of a three-component protein channel (TolC/HlyB/HlyD) connecting the inner and outer membrane [94,95]. The monomeric toxin HlyA is secreted directly from the cytoplasm to the extracellular medium. Antibody fragments are fused to the C-terminal domain of HlyA and are secreted into the culture medium by *E. coli* cells expressing TolC/HlyB/HlyD. Although the yields for secreted antibody fragments are similar to those obtained with periplasmic expression, further studies are needed to investigate the pros and cons of this methodology as an alternative to the aforementioned strategies for large scale of production of antibodies.

III.C. Factors influencing expression

Protein production is a multifaceted phenomenon involving complex processes such as, transcription,

mRNA processing, translation, post-translational modification, protein folding and assembly and protein export. Factors that affect any of these processes can influence product yield. These factors include intrinsic features of the gene encoding the antibody, intrinsic features of the antibody structure and its amino acid sequence and extrinsic or physiological factors. For any given antibody molecule, a review of the factors which are important for *in vivo* expression of correctly folded antibodies will help pinpoint aspects of expression that can be improved in order to arrive at an optimized expression strategy.

Expression of correctly folded protein is largely dependent on the intrinsic properties of the expression plasmid and the nucleotide sequence of the gene encoding a particular antibody construct and its sequence. Factors such as codon features, plasmid copy number [13], the presence of tightly repressible promoters [96,97], upstream elements such as leader sequence and amount and stability of mRNA [13] all have an influence on product yield.

Expression of functional protein in *E. coli* is extremely amino acid sequence dependent. While one rAb may express very well in a functional form, another may do so very poorly [24,27,28]. The complexity and nature of antibody fragments affects expression profiles. For Fabs, factors such as transfer to the periplasm, assembly rate, balance between the synthesis of two chains and the order of Fd ($V_H + C_H1$) and light chain genes are important [98]. For scFv molecules, the length and amino acid composition of the linker can have great impact on expression [21,23,43,99,100]. Obviously, the smaller is the size of a protein, the less is the impact of the various intrinsic and extrinsic factors on its expression. This has been one of the driving forces behind attempts to reduce the size of the antigen binding unit to a minimum. In this regard, naturally occurring heavy chain antibodies from Camelidae have introduced ideal solutions to problems in antibody expression, engineering and application. We will discuss the advantages and applications sdAbs derived from heavy chain antibodies elsewhere in this review.

A set of the factors involving physiological effects that influence *in vivo* expression relate to complex post-translational events such as translocation inside the cell (kinetics of membrane transport), processing, assisted protein folding and assembly (association of V_H and V_L in scFvs or Fd and light chain in Fabs), protein degradation and toxic effects. In addition, protein folding is also influenced by bacterial culture temperature [6,101], the redox potential of the host environment and the presence of folding catalysts, chaperones and other accessory molecules [44]. The nature of the *E. coli* host as well as culture conditions such as type of nutrients and the amount of inducers have been shown to influence production yield of antibody fragments [102–104].

IV. Optimization of rAb expression in *E. coli*

With the growing success of protein drugs, monoclonal antibodies in particular, and the large number of protein drugs in clinical trials protein production capacity is of fundamental concern. One of the ways to address this challenge is to optimize product yields and significant improvements have been made in optimizing various expression systems for higher protein production. Routine production of monoclonal antibodies exceeding 1 g/L in Chinese hamster ovary cells has been reported [74]. An anti-carcinoembryonic antigen scFv has been produced by the yeast *Pichia pastoris* at 1.2 g/L [105]. Antibody expression levels of 1–25 g/L in mouse and goat milk has been reported [32]. A bispecific diabody and a Fab' were expressed in functional form in *E. coli* at 1–2 g/L [37,106]. However, further improvements are needed if therapeutic rAbs are to be produced in the required amounts without very large increases in manufacturing infrastructure. Total world production of therapeutic antibodies in 2002 was reported to be about 1,000 kg. It is estimated this number will increase several fold in the near future and will be in the vicinity of 20,000 kg per year for one therapeutic antibody alone [69].

Approaches taken in efforts to improve the expression of antibodies, namely, an evolutionary approach, a rational approach and an approach in which the antibody fragment is co-expressed with chaperone(s) and/or foldase(s) or fused to a second protein with chaperone-like activities are described. Similar mutagenesis techniques are employed for the evolutionary and rational approaches.

IV.A. Mutagenesis techniques

A variety of synonymous, codon-based mutagenesis approaches have been employed for improving protein expression yields. One approach has been to optimize codon usage by replacing host organism rare codons with preferable codons that presumably

have higher corresponding tRNA concentrations [107–110]. In another approach codon pair usage are optimized by mutating "slow pair" codons to "fast pair" codons [111,112]. To our knowledge, the above two approaches have been applied only to expression of rAbs in non-*E. coli* hosts [28,100,113] and molecules other than antibodies in *E. coli* hosts [114–116], but the concept should be applicable to enhancing rAb expression in *E. coli*. Codon/codon pair optimization appears to enhance protein yields by increasing translation efficiency.

A wobble-base codon mutagenesis approach was employed by Stemmer et al. [117] to increase the yield of an Fv in a dicistronic expression format. The leader peptide codons of the V_H cistron were mutated at the wobble base positions and a library of 10^7 mutants was constructed. Screening by a colony lift method identified an Fv with 4 to 11-fold improvement in expression yield. In a similar approach, several signal peptides selected from a wobble-base library for their ability to increase the expression of alkaline phosphatase reporter increased the expression yield of a scFv and a Fab' [15]. Protein yield improvements have been attributed to the formation of favorable 5'-end mRNA secondary structure which enhances translation initiation. The signal peptide wobble-base codon mutagenesis/alkaline phosphatase reporter system was also applied to improving Fab' expression, significantly increasing the yield to 580 mg/L [98,108]; a good balance of light and Fd chain synthesis was found to be important in obtaining higher yield. In another experiment, the same authors [98] increased the expression of the Fab' by 3-fold with a codon usage optimization approach different from above; the 5' codons of the light chain were replaced not with the average preferred codons but with the preferred codons used at the 5' end of *E. coli* periplasmic genes [15]. Balanced light and Fd chain synthesis, possibly caused by changes in the secondary structure of the RNA in and around the mutated area may have led to a more favorable secretion/folding process, with the net result being an increase in functional protein expression.

IV.B. Evolutionary approaches

This strategy is based on the construction of recombinant libraries of antibody-expressing clones with directed or random mutagenesis generated by molecular biology techniques. Thereafter, techniques such as genetic screening, phage display, yeast display or ribosome display are employed to isolate protein variants with exhibit enhanced expression. Many high expressing rAbs have been isolated by an indirect selection, i.e., based on affinity and stability selection criteria not expression.

Martineau et al. [79] applied an evolutionary/genetic selection approach to an scFv for improving its functional expression in the cytoplasm of *E. coli*. An scFv with the ability to bind and activate a mutant β-galactosidase was used as an starting point to construct a library by random mutagenesis, and the library was co-expressed with β-galactosidase in the cytoplasm of a lac^- bacterium. Mutants with improved expression were selected by plating on limiting lactose. Following several cycles of library construction and selection an scFv with 50-fold higher functional expression in the cytoplasm was isolated. Although this selection approach is not a general one it may be used as a basis for developing a more general approach.

A phage display approach in a phagemid/helper phage format and based on affinity selection can be used to select for high-expressing rAbs [118]. In this system well-expressing rAbs would be displayed more efficiently due to their better folding properties, and thus will be favored during binding selection. From a mutant phage display library constructed for the purpose of obtaining higher affinity binders, Deng et al. [119], in addition to obtaining variants with improved binding, isolated two mutants with protein expression yields of 50 and 120 mg/L compared to 10–15 mg/L for the wild type. The affinities of these two binders, however, were comparable to that for the wild type. In a similar experiment, Jackson et al. [120] observed that that the enrichment of clones following several rounds of panning of a mutant phage display library was based on not only affinity, which was the intended selection pressure, but also expression levels.

Evolutionary approaches based on stability selections can also be used to select for high-expressing rAbs. This is because a positive correlation has been found between stability and the expression level of rAbs. Jespers et al. [59] reported the isolation of several aggregation resistant human V_H domains from a synthetic phage display library by heat denaturation. Compared to the parent wild type clone, which was highly prone to aggregation, these V_Hs had much better expression, as high as 10-fold. In another report, Jung et al. [26] applied a temperature stress-guided

selection strategy and identified mutants from a phage display library which, compared to the wild type, had improved thermostability, 20-fold better affinity and 3-fold better expression yield. Using a ribosome display approach, Jermutus et al. [121] performed selections under decreasing redox potential and isolated mutant scFvs which were stable in the absence of their disulfide linkage. In addition to higher stability, the mutants had significantly higher expression than the wild type in *E. coli*, as high as 4-fold. Shusta et al. [122] have shown a positive correlation between yeast surface display level and protein thermostability and expression. Based on this fact, Graff et al. [123], panned an anti-carcinoembryonic antigen scFv mutants yeast display library for enhanced display level and under stability pressure. They successfully isolated several scFvs with increased expression level, as high as 100-fold compared to the wild type.

IV.C. Rational approaches

This approach is based on the biophysical properties of individual antibodies, detailed structural comparisons of antibodies and the effects of mutations obtained in evolutionary experiments on antibody properties [83]. CDR grafting is a typical example of this approach and involves grafting the contact residues from a non-soluble scFv onto the scaffold of a well-expressed and stable scFv, leading to soluble expression and good thermodynamic stability of the hybrid molecule [124]. A second example of this approach involves the isolation of naturally occurring Abs without cysteines or making cysteine-free scFvs by valine-alanine scanning in both V_H and V_L [79,84]. In another approach, V_L and V_H framework region amino acid replacements, that improve expression level (up to 700 mg/L) and limit cell lysis were made [25]; for example, at V_H position 6, a glutamic acid to glutamine mutation results in 30-fold higher expression of soluble scFv since the amino acid at this position promotes correct folding by interacting with a folding intermediate [125]. As examples for sdAbs, functional expression of several human V_Hs were drastically increased by amino acid substitution at a few key solubility positions [126–128]. Other rational approaches which have resulted in improved expression yields include (i) chain shuffling in which hyperstable V_H or V_L domains are paired with a library of randomized V_L or V_H for subsequent panning [129] and (ii) replacement of hydrophobic patches at the antibody variable/constant domain interface [27].

IV.D. Coexpression with chaperones and foldases

Proper and efficient folding of antibodies *in vivo* involves molecular chaperones and foldases such as protein disulfide isomerase and peptidyl-proline *cis-trans* isomerase [130–132]. The functional yield of antibodies by an *in vitro* renaturation approach and by *E. coli* cell-free translation systems is also enhanced in the presence of molecular chaperones and foldases [85,133–136]. Chaperone overexpression in *E. coli* has been implicated as a means of preventing denaturation and misfolding of overexpressed heterologous proteins [137,138] and expression of many heterologous proteins with simultaneous overexpression of molecular chaperones has resulted in increased soluble functional protein yields in *E. coli* [139–142]. Chaperones and foldases are known to enhance expression yields by facilitating folding, preventing aggregation, reactivating aggregates and reducing protein degradation [136,142–144]. Attempts have also been made to enhance *in vivo* rAb production yield in *E. coli* by co-overexpression of foldases and chaperones.

Knappik et al. [19] tested the effect of overexpression of periplasmic *E. coli* protein disulfide isomerase DsbA and *E. coli* proline *cis-trans* isomerase PPIase A on the functional co-expression of Fv, Fab and scFv forms of the antibody McPC603 whose functional expression yield was limited by the periplasmic folding process. Overexpression of PPIase marginally increased functional expression in all instances, except for a scFv in the V_L/V_H orientation (scFv-L), by 1.8 fold. No further yield increase was observed when scFv-L was co-expressed with both PPIase and DsbA. Co-expressing the Fab with DsbA in a different expression format did not increase its functional expression. Aggregation which precedes or is independent of isomerization may limit protein folding. A lack of DsbA contribution to soluble yield was also observed for another scFv [135] and may reflect its weak isomerization activity.

In the search of factors that enhance the phage display of proteins as well as the functional expression of proteins in the *E. coli* periplasm, Bothmann and Plückthun [89] identified a periplasmic factor (Skp), by a phage display technique and from an *E. coli* protein library, which increased the phage display of

several scFvs. Co-expression of several scFvs with Skp increased their functional periplasmic expression yields significantly. A correlation was observed between scFv expression properties and the effect of Skp on functional expression—the better a given scFv was in terms of functional expression, the less the effect of the Skp co-expression on scFv functional folding. Skp co-expression was also shown to increase soluble functional expression of anti-atrazine and anti-diuron scFvs in the *E. coli* periplasm. This paralleled a concomitant decrease in the production of aggregated species [145]. As observed by others [89], the functional expression level of the scFvs correlated with the expression level of Skp.

Employing the same selection approach [89], Bothmann and Plückthun [90] identified a periplasmic PPIase, FkpA, which when co-expressed with several scFvs increased their functional periplasmic expression by as much as 10-fold with the FkpA effect being independent of its PPIase activity [90,144]. FkpA assists folding by suppressing aggregation early during folding events and reactivating inactive proteins in later folding events [144]. As observed for Skp, the beneficial effect of FkpA was greater for scFvs with poor folding properties and had no effect on a scFv with good folding characteristics. Combined co-expression of FkpA and Skp had the same effect as FkpA alone [90].

Co-expression of PPIase PhFKBP with anti-hen egg lysozyme Fab which essentially expresses as inactive aggregates in the *E. coli* cytoplasm improved soluble expression of the Fab [143]. Improved soluble functional expression was attributed to suppression of aggregation and the chaperone-like folding activities of PhFKBP and not its PPIase activity. Consistent with these results, co-expression of periplasmic PpiA and SurA PPIase did not increase functional expression yields of scFvs [90].

Co-expression of an anti-CEA scFv-human interleukin 2 fusion with GroES/EL resulted in a 2-fold increase in both its soluble cytoplasmic expression and activity following *in vitro* refolding [104]. Levy et al. [146] studied the effect of cytoplasmic co-expression of GroEL/ES, trigger factor, DnaK/J, DsbC and Skp on the functional expression of 26–10 anti-digoxin Fab in *E. coli*. With the exception of DnaK/J, which had a negative effect, all increased the functional Fab expression in the cytoplasm, and experiments with GroEL/ES showed that the increase in soluble Fab production was accompanied by a reduction in Fab aggregation. Skp had the largest beneficial effect on expression (eight-fold) followed closely by trigger factor. The beneficial effect of trigger factor on soluble expression of recombinant proteins was also demonstrated in another study [147]. In contrast to Levy et al. [146], who did not observe a beneficial effect with DnaK/J, Nishihara et al. [147] showed that co-expression of DnaK/J-GrpE significantly increased soluble protein production. As another contradictory result, DnaK, but not GroEL/ES, was beneficial in terms of increasing functional yields of a scFv [136]. These contradictory results point to the complexity of chaperone and foldase action. Chaperones and foldases have differential folding effects depending on the substrate structure, show synergistic effects in combinations and may be more effective in certain combinations than others [135,136,141,147]. It also appears that the effect of chaperones and foldases are concentration and time dependent [19,85,134,145,148]. Thus, for a given chaperone, its expression and/or its timing may be more optimal in one expression system under study than another, hence better or positive expression effects. A further complication is that while a higher expression of chaperones and folding catalysts correlates with higher functional yields of target proteins, an "excessive" or non-stoichiometric expression may have negative effect [146] due to, e.g., growth inhibition effects [149,150] or deleterious effects relating to plasmid stability and induction [44,151, 152].

IV.E. Fusion protein strategy

Heterologous expression of rAbs, and other proteins, in *E. coli* has been increased or made possible by fusing them at the gene level to proteins such as *E. coli* MBP, glutathione-S-transferase and Staphylococcus protein A (see [153] and below for a more complete list of proteins). Apparently, enhancement of expression is mediated by the chaperone-like activity of the certain proteins by preventing or significantly diminishing rAb inclusion body formation. Unless it is disruptive to the activity of the rAb, the protein is typically fused upstream of the rAb since this orientation has been more consistent in terms of increasing expression yields. The fusion protein often has ligand binding activity which facilitates the purification of the fused rAb by one-step affinity chromatography with a ligand-functionalized

column [154–156]. The fused protein can also be exploited as a tag for detecting a rAb [155].

Enhancing protein yield by the fusion protein strategy has been successfully carried out for both cytoplasmic and periplasmic expression of rAbs. Zheng et al. [157] reported that a catalytic scFv was expressed mainly as inclusion bodies in the cytoplasm, even at 22°C, with the amount of soluble product being too low to purify. However, when, the scFv was fused to the C-terminus of the highly soluble *E. coli* N utilization substance protein A (NusA) [158], the level of soluble expression increased dramatically to 3 mg/L. Fusion to NusA promotes functional disulfide bond formation [157]. When fused to the C-terminus of the *E. coli* MBP, several scFvs expressed much better than their unfused counterparts in the reducing environment of the *E. coli* cytoplasm [154]. A notable example was scFv 4-4-20/212 which expressed virtually entirely in an insoluble form in *E. coli* in an unfused format but gave 50 mg/L of soluble, active product when fused to MBP. In another report, Hayhurst [159] improved periplasmic expression of an anti-atrazine scFv and an anti-diuron scFv by fusion to the MBP (N-terminal fusion) and/or human IgG kappa light chain constant domain, Cκ, (C-terminal fusion). The anti-atrazine construct MBP-scAb (scAb = scFv-Cκ) produced significantly more protein than scAb-MBP, underlying the importance of MBP fusion order. C-terminal fusion of Cκ has been shown to increase cytoplasmic expression of an scFv in mammalian cells by stabilizing the construct, i.e., preventing degradation [160]. Ideno et al. [161] fused the C-termini of an anti-hen egg lysozyme scFv and Fab, which were mostly expressed as inclusion bodies in their unfused states, to an archaeal FK506 binding protein (FKBP)-type peptidyl-prolyl *cis-trans* isomerase (PPIase). The fused versions were mostly expressed as soluble proteins in the cytoplasm of *E. coli*. The increased rAb expression was attributed to the fusion protein's chaperone-like activity, not to its PPIase activity.

The fusion protein approach to improving yield has its limitations. Any one fusion partner may not increase soluble expression of a particular rAb and other fusion partners may need to be tried [161,162]. Another drawback to this approach is the frequent requirement for downstream experiments to separate the fusion partner from the rAb, especially when the fused rAb is not as active as the unfused one. It is also possible that, while a rAb fusion may have good solubility and stability, the rAb may not in the absence of the fusion partner.

V. Single-domain antibodies

The term sdAb was originally introduced by Ward and co-workers [163] to describe murine V_H domains that were screened for binding to lysozyme. These murine V_H domains had good affinities for antigen but poor solubilities due to the absence of a V_L partner, demonstrating that additional domain engineering is required to generate fully functional sdAbs from conventional antibodies. The discovery of camelid heavy chain antibodies in 1993 [49] opened up new possibilities for the engineering of sdAbs. Following the discovery of these unique antibodies, functional V_HH domains specific for lysozyme and tetanus toxoid were isolated from a phage library constructed from the antibody repertoire of an immunized camel [52,53]. The discovery of heavy chain antibodies also opened up new opportunities for the generation of functional murine [164] and human V_H and V_L domains [3,126,128,165,166].

V.A. Heavy chain antibodies

Camelidae heavy chain antibodies are homodimers where each monomer unit is comprised of a single variable domain (V_HH), a long or short hinge region and two constant domains corresponding to C_H2 and C_H3 of conventional antibodies (Figure 1) [49–51,167]. The lack of the first constant domain (C_H1) in heavy chain antibodies is structurally related to the absence of a light chain, as C_H1 anchors the constant domain of the light chain [168]. Genomic studies have shown that the DNA encoding C_H1 is spliced out during mRNA processing [169,170]. Crystal and solution structures of several V_HHs have shown that the general 'immunoglobulin domain fold' of conventional antibody variable domains is kept intact in V_HHs [53,171–173]. However, there are structural differences and amino acid replacements which seem to be specific for V_HHs. New canonical structures for the CDR1 and CDR2 loops result in a much larger structural repertoire [167,174,175]. Camelid V_HH repertoires contain unusually long average CDR3s with camel V_HH CDR3s being long compared to V_Hs (average length of 16–18 amino acids) and llama V_HH CDR3s covering a wide range of lengths [50,51,176]. It has been shown that in at least some V_HH structures the longer CDR3 folds over the "former" V_L interface [53,177]. Framework 2 amino acid substitutions in V_HHs relative to V_Hs, namely, V37F/Y, G44E/Q, L45R/C and W47G/S/L/F

[167,176,178], veneer the otherwise hydrophobic region interacting with the V_L domain in conventional antibodies. Indeed, the solubility of a human V_H was improved by incorporation of the V_HH FR2 amino acid substitutions [126]. However, the total expression level and the original antigen-binding properties were sacrificed, emphasizing that residue interrelationships are involved in V_H folding, expression and antigen binding. Recent structural studies comparing a V_HH and its humanized derivative showed that Glu 44 and Arg 45 are the key elements in making the domain soluble whereas Tyr 37 and Arg 45 are important in V_L domain pairing [179]. From these studies it was concluded that in addition to the V_HH-specific amino acid replacements, other mutations and veneering are required to make a functional, stable and soluble human or mouse V_H [7,179].

A class of antibodies, termed IgNARs, discovered in nurse sharks and webbegongs are related to camelid heavy chain antibodies in terms of overall format [48,54]. The variable domains of these antibodies (i.e., V_Hs) have been cloned, expressed in bacteria and their structures in complex with lysozyme have been solved [47,180]. These antigen-binding units are comparable, although somewhat smaller, in size to V_HHs. However, their primary structure and fold is quite divergent from that of human, mouse or Camelidae V_Hs or V_HHs.

V.B. Advantages of sdAbs

The fact that sdAbs are much less complex than conventional antibody fragments offers significant advantages in terms of antibody engineering and production in good yield. Only a small set of primers is needed for amplification of sdAbs and construction of sdAb phage libraries is relatively straightforward since assembly reactions are not required as for scFvs [7,52,167]. Because they are single domains of relatively small size, they are efficiently expressed in *E. coli* with yields of up to 80 mg/L in shake-flask cultures [52,92] and in yeast and fungal species with yields of up to 100 mg/L in shake-flask cultures and kilogram quantities from large fermentors (1.5×10^4 litre fed-batch fermentation) [181,182] as soluble, non-aggregating protein. This in marked contrast to the expression of conventional antibody fragments such as Fabs or scFvs for which much lower yields of soluble product are generally obtained. While the complexity and multidomain nature of Fabs and scFvs relative to sdAbs is a major reason for the difference in yield, residues at the interface of variable and constant domains [27] and the expression efficiency and balance of light and Fd chains can also affect Fab yield [98]. For scFvs, yield can be affected by domain orientation [19], sequence and length of the linker joining the two domains [21,100] and susceptibility of the linker to proteases [183,184].

sdAbs have excellent physical properties and are superior to conventional antibody fragments in this regard. sdAbs have high thermal and conformational stability. It has been shown that the V_HH domains can withstand prolonged incubation at temperatures above 90°C, a property that is attributed to a reversible unfolding behaviour [46,185,186]. By contrast, conventional human and mouse V_Hs often aggregate irreversibly on thermal denaturation. Melting points ranging from 60°C to 78°C have been reported for camel and llama V_HHs [46,57]. Also, it has been shown that V_HHs are resistant to proteases [187] and to harsh conditions such as the presence of nonionic and anionic surfactants, high urea concentrations and extreme pH [7,56].

Because of their small size and long protruding CDR3s V_HHs can access epitopes that are inaccessible to conventional antibody fragments, such as clefts and cavities which are often the hallmarks of enzyme-substrate and receptor-ligand interactions [53,188]. It has also been shown that V_HHs can access inaccessible and conserved epitopes on the surface of trypanosomes [189]. They may also be efficient reagents for targeting tumors where penetration into poorly vascularized tissue is crucial to the success of a drug [3]. The ability of sdAbs to transmigrate across an *in vitro* model of the human blood brain barrier may be related to their small size. Molecules of this nature have great potential for the delivery of therapeutics across the blood brain barrier in the development of treatments for neurological diseases [61].

V.C. Engineering sdAbs for improved function

The small size of sdAbs may limit their application in instances where a prolonged serum half-life is desirable. Two solutions to this shortcoming have been successfully applied for other antibody fragments such as scFvs and Fabs, namely, covalent attachment of polyethylene glycol (PEG) to the antibody fragment (PEGylation) [3,190] and physical linkage of the antibody fragment to a naturally existing serum protein with extended half-life such as serum albumin or the Fc region of antibodies [3,191].

Due to their small size, high stability and good expression yield and the fact that they show little tendency to aggregate, sdAbs are ideal antibody fragments for constructing multispecific and multivalent antibody reagents [7,187,192]. Zhang et al. [187] have described a method for producing pentameric sdAbs in good yield in *E. coli.* Like their monomeric counterparts, the so-called pentabodies have excellent biophysical properties [187] and do not aggregate (Figure 2). Pentamerization has the effect of enhancing sdAb binding to immobilized antigen by several orders of magnitude [187].

A pentavalent sdAb approach to tumor antigen discovery has been successfully applied to the identification of a novel carcinoembryonic antigen-related cell adhesion molecule 6 (CEA6) epitope on lung adenocarcinoma [193]. An sdAb specific for non-small cell lung carcinoma was obtained by panning a non-immune llama sdAb library [92] against the A549 cell line. A pentameric form of the sdAb was used to identify the antigen recognized by the sdAb as a form of CEA6 by a 2-D gel electrophoresis/Western blotting approach. Phage-displayed sdAb serves as an excellent immunocytochemical reagent [187], (Figure 3) as does the pentabody form of the sdAb (MacKenzie et al., unpublished results). The pentabody strongly stains a sub-population of A549 cells, weakly stains some other cancer cell lines and does not stain normal epithelial cells (Figure 3(A)–(C)). While not conclusive, there is evidence that the antigen recognized by the sdAb is transiently expressed during cell division (Figure 3(D)–(E)). These results show that a phage sdAb library/pentabody approach may provide a useful tumor marker discovery platform and demonstrate that pentabodies are very useful reagents for immunostaining and proteomics.

VI. Conclusions

The bacterial expression of antibody fragments has been a primary deriving force behind the rapid expansion and major successes of antibody engineering in the past two decades. Antibody library screening by phage display, which is now a relatively routine procedure for the *de novo* isolation of monoclonal antibody fragments and for improving antibody properties by evolutionary approaches, is contingent upon expression of properly folded antibodies in *E. coli*. Antibody engineering and bacterial expression provide a convenient means of generating antigen binding fragments for evaluation, isolation and production of antibody in this manner alleviates any concerns about the use of animals for such purposes.

As applications for monoclonal antibodies and their derivatives continue to increase, it is likely that bacterial expression will play a more significant role in their manufacture. However, is unlikely that bacterial expression of whole antibodies will become practical because of the difficulties in expressing such large molecules in bacteria and the requirements for glycosylation. While much has been learned about the factors influencing antibody yield in *E. coli*, it is not possible to identify general conditions that will give good expression of a particular antibody format. Antibody sequence remains the key factor in determining yield and various engineering and expression strategies may have to be investigated in order to achieve acceptable product yield.

In recent years it has become clear that bacterial expression of sdAbs is much less problematic than the expression of antigen binding fragments from conventional antibodies. While sequence greatly influences sdAb expression, these molecules generally express, as functional correctly folded protein at levels that are at least 10-fold higher than those obtained for more complex antibody fragments. In addition, sdAb-based fusion proteins also tend to express at high levels in *E. coli*. It is likely that there is a bright future for the bacterial expression of sdAbs and sdAb-based molecules.

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