# Strategies and Applications of Antigen-Binding Fragment (Fab) Production in *Escherichia coli*

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**Abstract**

With the advancement of genetic engineering, monoclonal antibodies (mAbs) have made far-reaching progress in the treatment of various human diseases. However, due to the high cost of production, the increasing demands for antibody-based therapies have not been fully met. Currently, mAb-derived alternatives, such as antigen-binding fragments (Fab), single-chain variable fragments, bispecifics, nanobodies, and conjugated mAbs have emerged as promising new therapeutic modalities. They can be readily prepared in bacterial systems with well-established fermentation technology and ease of manipulation, leading to the reduction of overall cost. This review aims to shed light on the strategies to improve the expression, purification, and yield of Fab fragments in *Escherichia coli* expression systems, as well as current advances in the applications of Fab fragments.

**Keywords**
- mAbs
- Fab
- *E. coli* expression systems
- applications

## Introduction

Since the first therapeutic monoclonal antibody (mAb) drug was approved by the Food and Drug Administration (FDA), the past 30 years had witnessed substantial strides in the development of antibody therapeutics. During this period, more than 80 mAb therapeutics have been approved for the market with the advances in technology.[1] Among the marketed antibodies, the majority are fully human or humanized immunoglobulins (Igs), followed by a small fraction of antibody fragment-based therapeutics. Notably, full-length antibodies have a long circulating half-life, which makes them unsuitable for some diagnostic and therapeutic applications.[2] With the conjugation of some anticancer products to the effector molecules (such as radiolabels, chemicals, or toxins), antibody–drug conjugates (ADCs) are expected to expand into fields other than malignant tumors and autoimmune diseases, and will dominate the field of drug discovery in the near future.[3] However, the growing needs of the market require pure molecules in large amounts, which is challenging to the production cost, the productivity of expression systems, as well as the quality of expressed recombinant antibody fragments.

To overcome the present problems, engineering of antibody-based fragments has represented a new direction to produce mAb alternatives with functional properties of conventional antibodies, for example, antigen-binding fragment (Fab), Fab dimers, single-chain variable fragments (scFvs), diabody (VHH), and ScFab (single-chain Fab) (►**Fig. 1**).[4] As shown in ►**Fig. 1**, IgG molecule (~150 kDa) is composed of two identical heavy chains (~50 kDa) and two identical light chains (~50 kDa). Fab is a fragment of Ig molecule with a heavy chain shortened by constant domains CH2 and CH3 while the Fab of heavy chain is called VHH antibody (or nanobody)[5,6]; scFvs are fragments of variable regions of the heavy and light chains, which are connected by

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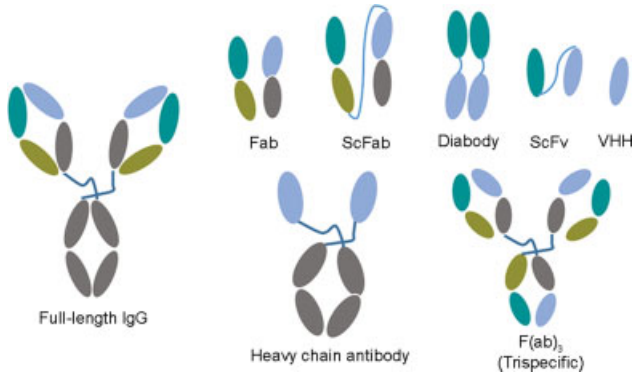


**Fig. 1** The structure of IgG and mAb-derived alternatives. IgG, immunoglobulin G; mAb, monoclonal antibody.

a peptide linker[7]; ScFab is the fragment in which the carboxy-terminus of the constant light chain is fused to the amino-terminus of the variable heavy chain via a flexible linker.[8] Among those alternatives, Fab would be the most suitable fragment for clinical applications, and the reasons may be attributed to the following aspects: (1) Fab fragments have better tissue penetration capacity and faster clearance than the larger intact mAbs, and thus may represent a better way to selectively deliver radioisotopes, toxic drugs, and toxins to the target site; (2) Fab fragments lack Fc domain, which renders them the ability to bind antigen monovalently without mediating antibody effector functions and reducing immunogenicity; and (3) Fab is more stable than scFv and can be expressed in *Escherichia coli*, which has been recognized as being easier production, lower costs, as well as compatibility with phage display.[9]

Fab was generated by enzyme digestion.[5] Papain and aspartic acid protease pepsin are two enzymes that are frequently used to digest IgG1 to produce the majority of Fab, as well as $F(ab')_2$ fragments, respectively. IgG-degrading enzymes *Streptococcus pyogenes* (IdeS protease) and endoproteinase Lys-C are shown to be attractive alternatives to generate $F(ab')_2$ and Fab fragments with better fragment yields and less nonspecific cleavage.[10] However, enzyme digestion was usually accompanied with inefficient proteolysis that is hard to control, and the production of heterogeneous Fab cannot be genetically manipulated.[11] Considering this, current efforts are focusing on the directed generation of Fab fragments as recombinant proteins that can be expressed in a wide range of expression systems such as bacteria,[12] yeast,[13] insects,[14] and mammalian cells.[15] An *E. coli* expression system has various advantages over other systems including its low cost, rapid growth on inexpensive substrates, high biomass, readily scalable cultivation, and easy genetic manipulation for therapeutic protein production.[16]

However, the expression level of Fab fragments in *E. coli* is low. Fab fragments contain disulfide bonds (four intrachain and one interchain) required for structural and functional integrity. Incorrectly folded Fab fragments can inevitably be generated.[5] Apart from incorrect folding, low expression levels of Fab fragments in *E. coli* can be ascribed to toxicity effects, intracellular degradation, aggregation, and inefficient translocation. According to the literature, the typical yield of Fab proteins in *E. coli* flask cultures ranges from 0.1 to 10 mg/L and high-density *E. coli* cultures grown in laboratory fermenters can yield up to 1 g/L.[17] In most cases, the Fab expression yield can be further optimized from undetectable level to a relatively high yield through genetic engineering.

In this review, we discuss several factors, such as different vector elements, bacterial strains, and cultivation conditions, to mitigate potential problems, and explore the influence of gene sequence, secretion signal sequences, and chaperone co-expressions on expression yields of Fab fragments. Furthermore, the development and the prospects of Fab fragments are examined in detail.

## Expression of Fab Fragment in *E. coli*

The cytoplasm of *E. coil* is a reducing environment maintained by the thioredoxin–thioredoxin reductase (trxB) system and the glutaredoxin–glutaredoxin reductase (gor) system.[18] In fact, the formation of disulfide bond rarely occurs in the cytoplasm. Cysteine residues are part of catalytic sites in many enzymes, where the formation of disulfide bond could lead to protein inactivation, misfolding, and aggregation.[19] However, the formation of disulfide bond can be specifically achieved in prokaryotic periplasm and eukaryotic endoplasmic reticulum, during which proteins need to be directed to these compartments with the help of signal peptides or leader peptides.[16] When the gene (*trxB*) is inactivated by mutation, and structural disulfide bonds within a protein is then formed in the cytoplasm, followed by the possible production of Fab fragments in the cytoplasm. Thus, Fab fragments can be expressed in the cytoplasm of *E. coli*, either as soluble proteins or inclusion bodies (IBs), and can also be secreted to the periplasm or to the culture medium through signal peptides. The expression of Fab fragment in *E. coli* is cost-effective, and through various optimization methods, the expression, solubility, and stability of Fab fragment can be improved within a short time.

### Expression of Fab in the Cytoplasm

Whether a product remains soluble or as aggregates in the cytoplasm depends on intrinsic properties of the peptide or protein sequence, promoter strength, and fermentation parameters, such as temperature and growth rate.[20] When proteins are expressed at high levels in *E. coli*, it is common to find that most of the proteins are insoluble, which called IBs.[21] A possible explanation for the formation of IB is the lack of required cell machinery and overexpression of heterologous proteins in the cytoplasm when a protein is partially folded or misfolded.[22]

To enhance the production of soluble functional antibody Fab fragments in an oxidizing bacterial cytoplasm, different methods have been described. For instance, Venturi et al reported an approach of utilizing redox-mutant strains with increased oxidizing capacity to permit the correct folding of Fab fragments.[11] When an oxidizing cytoplasmic environment was successfully provided by thioredoxin- and glutathione reductase-deficient *E. coli* strain FA113, the expression of (soluble) Fab will be increased by 50- to 250-

fold in comparison to other reported overexpression strategies. Some other approaches include co-expression of different folding enzymes, such as peptidyl-prolyl *cis*/*trans*-isomerases and periplasmic dithiol-disulfide oxidoreductases.[23] Gaciarz et al also suggested a system called CyDisCo used for efficient production of disulfide bond containing proteins in the cytoplasm of *E. coli*.[16] CyDisCo is based on co-expression of a sulfhydryl oxidase and a protein disulfide isomerase, which catalyze the formation and isomerization of the disulfide bond. The results indicated that more than 90% of the tested scFv and Fab fragments could be produced and correctly folded in the biologically active form within the cytoplasm.[16] Furthermore, the addition of certain fusion partners, such as maltose-binding protein and small ubiquitin-related modifier (SUMO), is also reported to improve stability, solubility, and the expression yield of recombinant proteins.[16] In 2017, Rezaie et al applied a dual SUMO fusion strategy to successfully obtain a Fab fragment with correct folding (12 mg/L).[24] Moreover, the fusion of leucine zipper (LZ) pairs LZA and LZB or c-Jun and c-Fos to the C-terminus of heavy and light chains, respectively, enhanced correct pairing of the heavy and light chains that led to the production of active Fab as Zipbody in the *E. coli* expression system.[25]

However, Fab fragments are more commonly expressed as IBs in the cytoplasm of *E. coli*. Although protein expression in the form of IBs is often undesirable, their formation is advantageous because of the high purity of the target protein in the aggregate fraction and the increased protection from proteolytic degradation when compared with the soluble counterpart.[26,27] IBs should be isolated and solubilized, followed by refolding and purification of solubilized proteins to achieve functional activity. Among these steps, solubilization and refolding are the crucial processes for high recovery. IBs can be traditionally solubilized using high concentrations of denaturants like urea and guanidine hydrochloride (GdnHCl),[28] or with the advanced methods employing detergents, organic solvents, and low concentrations of chaotropes.[29] Refolding is identified as formation of disulfide bonds in the presence of optimal concentrations of both oxidizing and reducing agents, and usually performed using pairs of redox agents, such as reduced/oxidized glutathione (GSH/GSSG), DTT/GSSG, cysteine/cystine, and cysteamine/cystamine with a molar ratio of reduced to oxidized compounds of 1:1 to 10:1.[30] Furthermore, the optimal conditions of refolding can be acquired by the addition of additives to buffers to reduce protein aggregation and misfolding. The most commonly used additives with low molecular weights are *L*-arginine, low-concentration (1–2 mol/L) urea, and detergents.[31] Buchner and Rudolph designed a renaturation procedure that allowed the production of microbially expressed Fab fragments at the yields up to 40% of the total amount of recombinant protein, by which the obtained recombinant Fabs were identical to the parental murine Fab in functional and physicochemical parameters.[32] IBs offer an attractive manufacturing option. Currently, there are two marketed Fab fragments, which are expressed as IBs in the cytoplasm of *E. coli*: ranibizumab (Lucentis) and certolizumab pegol (Cimzia).

## Expression of Fab in the Periplasm

It is clear that extracellular protein expression is preferred over conventional cytoplasmic expression with a simpler downstream purification process and more efficient protein folding.[33] Unfortunately, the yields of Fab fragments in periplasm are often relatively low, because of hurdles like inefficient translocation across the inner membrane, protein folding, proteolysis, and toxicity.[34] ►**Fig. 2** depicts some factors that may help solve the problem as mentioned, and the corresponding approaches may include optimization of secretion signal sequences; co-expression of molecular

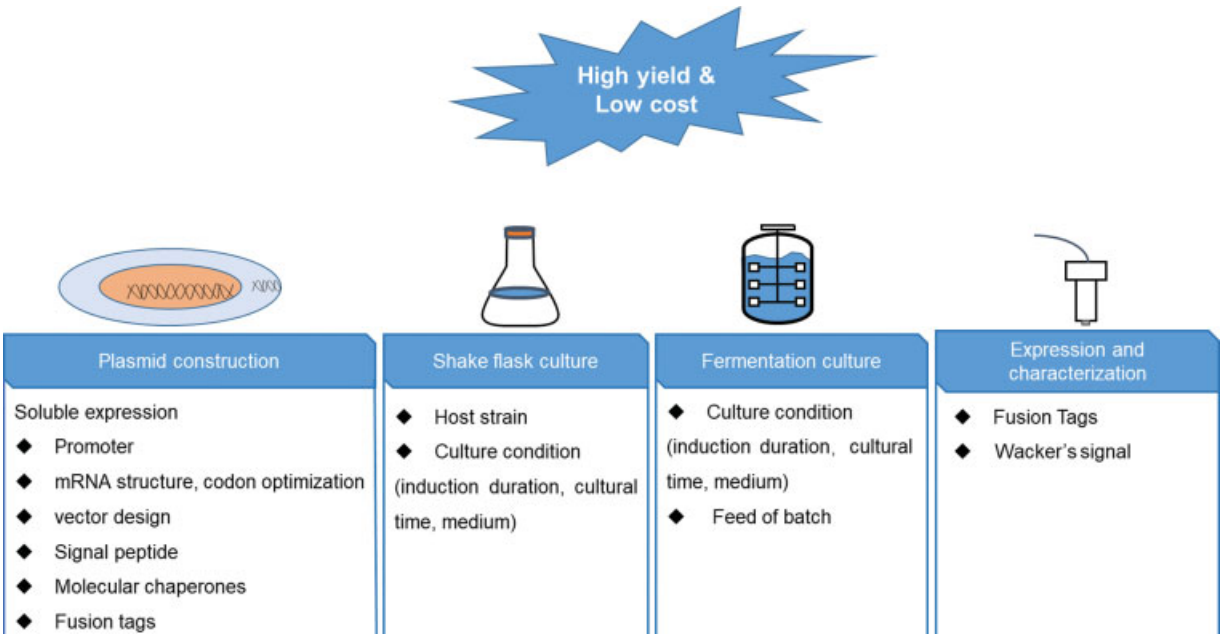


**Fig. 2** A proposed technology in *E. coli* to generate cost-effective Fab fragment.

chaperones for proper disulfide bridging; host bacteria mutants; and incorporation of expression augmenting genetic elements into the vector.[35] Toxicity of some Fab fragments can be greatly reduced by modulating transcriptional and translational rates, typically by inducing protein expression at low temperature, by modification of culture medium,[36] or by using more tightly controlled promoters.

There is no general rule to optimize culture parameters. It should be evaluated on a case-by-case basis, since not all factors play an equally important role in improving the expression of a specific protein. For example, to boost bacterial expression, Lin et al combined several methods such as design of a new expression vector, optimization of Fab light-chain codons, and co-expression of chaperon protein, which eventually increased the production of Fab in *E. coli* by 100-fold.[37]

## Signal Peptide

The periplasm of *E. coli* is a redox environment enriched with foldases including disulfide isomerases (Dsb proteins) and peptidyl-prolyl isomerases, which facilitate correct disulfide bond formation of the proteins of interest.[38] Routing to the periplasmic space is achieved by fusing the recombinant protein to a proper signal peptide. The translocation mechanism depends on the type of signal peptide. *Escherichia coli* secretary pathway (SEC) and the signal recognition particle (SRP)-dependent translocation machinery are responsible for transporting/targeting most of the proteins into the periplasm, while the TAT (twin-arginine translocation) pathway enables translocation of folded proteins only in *E. coli* mutants with oxidative cytoplasm.[13] In addition, signal peptides play a crucial role in controlling secretion efficiency. Among the common leader peptides, four use the SEC pathway (PelB, OmpA, PhoA, and pIII) and three use the SRP pathway (DsbA, TorT, and TolB). In 2008, Thie et al compared SRP and SEC pathway leader peptides for antibody fragment production in *E. coli* and concluded that both pathways were compatible with antibody phage display and production of soluble antibody fragments.[39] Additionally, Nagano et al revealed that alteration of the Ser to Ala at -3 and -1 in the C-region of the signal peptide could significantly improve the Fab secretion in *E. coli*.[40] Their modified signal peptide could be applied to enhance periplasmic expression of antibody fragments in *E. coli*.

## Vector Engineering

Vector design plays a key role for the successful expression of Fab in *E. coli*.[36] Currently, three different vector cassettes are utilized to express Fab molecules in *E. coli*: (1) VL-CL and VH-CH1 genes driven by two promoters in two separate expression cassettes[41]; (2) one dicistronic expression cassette with two translation initiation sites with VH-CH1 before VL-CL[42]; and (3) one dicistronic expression cassette with two translation initiation sites with VL-CL before VH-CH1.[43] Corisdeo and Wang compared three expression cassettes to produce a human Fab against tetanus toxoid and showed that the third vector enabled the largest increase (eightfold) in the production levels of secreted and correctly assembled Fab. For basic research purposes, the second (2) design is predominant.[36]

## Codon Usage

It is well known that codon usage plays a significant role in gene expression levels and protein folding. In the past decade, considerable progress in the speed and cost of gene synthesis facilitated the complete redesign of entire gene sequences, thus markedly improving the likelihood of successful production of heterologous proteins for academic as well as biopharmaceutical use.[44] It is believed that codon usage alters protein folding and modulates the efficiency of translation initiation.[45] Studies suggested that rare codons had a bigger effect on increasing protein expression than more common codons.[46] Previous studies showed a striking abundance of nonoptimal codons in the signal sequences of secretory proteins. Accordingly, alteration of synonymous codon in the signal sequence also affects protein yield by slowing the rate of translation across the N-terminal signal sequence to facilitate proper folding of the secreted protein or by facilitating the binding to the SRP, consequently leading to more efficient translocation.[47,48] Optimization of codon usage of signal sequences was successfully employed in improving the expression levels of antibody fragments. Kulmala et al established synonymous PelB signal sequence libraries attached to light and heavy chains of a Fab fragment, and discovered that codon usage of the fifth leucine position of the light-chain PelB affects the expression levels of Fab fragment.[49]

## Molecular Chaperones

Co-expression of molecular chaperons can decrease accumulation of IBs and can assist proteins in adopting the correct conformation. These chaperons are foldases tailored to help trigger protein folding by interacting with the expressed proteins and form a protein–chaperon complex.[50] It is well documented that co-expression with molecular chaperons improves solubility and proper folding of various recombinant proteins and antibody fragments.[38] DnaK chaperones act in an ATP-dependent manner and cooperate with DnaJ and GrpE co-chaperones to mediate protein folding under both normal and stressful growth conditions.[51] Dsb (disulfide bond) enzymes A–G are found within the periplasm and are involved in the catalysis of disulfide bonds.[34] DsbA typically catalyzes disulfide bond formation in folding proteins while DsbC functions as a disulfide isomerase. The coexistence of these two proteins as the thiol-disulfide oxidoreductases allows the establishment of disulfide bridges. The DsbA mutations could stabilize the α-helical structure of the hydrophobic core and enhance binding to SRP, resulting in a higher expression.[8] In addition to Dsb proteins, periplasmic peptidyl-prolyl isomerases FkpA and SurA are also involved in the protein-folding process. FkpA can alleviate the stress response in *E. coli* cells during accumulation of misfolded proteins, suppress the formation of IBs, and promote proper folding.[52] In addition, SurA maintains outer membrane integrity and also catalyzes the folding of other outer membrane proteins.[53]

### Host Bacteria

Humanized Fab fragments, produced in the periplasm of *E. coli*, are subject to degradation caused by host-cell proteases. To increase Fab yield and reduce proteolysis, *E. coli* strains deficient in periplasmic proteases, but still able to maintain high cell density, were developed. These strains lack the protease activity of Tsp, protease III, and DegP, and incorporate suppressor mutations in the *spr* gene to restore the wild-type phenotype of the cells, thereby increasing the productivity by twofold.[34] *E. coli* BL21 (DE3) and K12 hosts are extensively used for the production of various forms of biopharmaceuticals. BL21 cells are deficient in the Lon protease, which degrades many foreign proteins. Several commercialized K12 host bacteria strains exist, such as *E. coli* W3110, Rosetta-gami 2, SHuffle T7, and MG1655, some of which carry additional modifications for improved folding. Rosetta-gami 2 contains *trxB* and *gor* gene mutations. Shuffle T7 contains *trxB* and *gor* gene mutations as well as constitutive DsbC expression, and MG1655 generally shows a high growth rate and productivity.[33] *Escherichia coli* strain FA113 also lacks the activity of thioredoxin and glutathione reductase, and its oxidizing cytoplasmic environment enabled Venturi et al to express two Fab fragments in the cytoplasm with very high yields at 10–30 mg/L.[11] Nonetheless, it is difficult to predict which kind of host bacteria would generate the best yields. Therefore, perhaps due to their well-documented use, *E. coli* W3110 and BL21(DE3) remain prevalent strains in the expression of Fab fragments.

### Culture Condition and Feed Batch

Control of the growth temperature during fermentation allows the stable expression of heterologous proteins.[54] Previous studies demonstrated that low-temperature fermentation (below 30°C) is advantageous for Fab expression in *E. coli* on account of the reduced translation rate and protein aggregation. At low temperatures, the rate of synthesis of the target protein is slow and the rate of transport to the periplasm is correspondingly reduced, and subsequently reduces the burden on bacteria. The composition of the medium also has an important impact on soluble expression. Medium rich in metal and salt ions is more suitable to Fab-soluble expression. Metal ions assist in the formation of disulfide-bonded enzymes, and increase soluble expression. Evidence suggests that addition of $Mg^{2+}$ to the medium can improve the soluble expression by 50%.[55] For large-scale production, the optimization of fermentation conditions is crucial to improve yields. Kim et al reported that increased nitrogen supply together with low temperature significantly improved the yield of biologically active form of Fab by 59.7%.[56] An et al developed a fed-batch fermentation process using glycerol as a carbon source, which achieved soluble biologically active protein at 80 mg/L.[57] In ►**Table 1**, we list studies that successfully achieved expression of Fab in *E. coli.*

### Leakage into the Extracellular Medium

Fab fragments that are directed toward the periplasmic space could, depending on the growth conditions, preferentially localize to the culture growth medium.[58] Bäcklund et al investigated the leakage of proteins from periplasm to the medium.[59] They suggested that a higher feed rate will not lead to higher amounts of product in the periplasm, since there is a comparatively higher leakage. Therefore, it is necessary to determine the dependency of Fab leakage on cultivation conditions, a practical way to manipulate Fab localization. Ukkonen et al pointed out that the yield and leakage of Fab fragments are dependent on combination of several variables, including expression strain, culture medium, and aeration rate. Studies of the association between the temperature and aeration showed that low temperature (30° C) decreased the leakage to the culture supernatant from 70 to 40%.[60]

Moreover, a promoter engineering strategy was also applied to reduce Fab′ fragment leakage. Schofield et al reduced Fab′ leakage by converting the promoter from Ptac to Ptic using site-directed mutagenesis.[61] Balancing promoter strength with the overall metabolic burden of cellular "housekeeping" processes could also address translocon overload, caused by periplasmic translocation of exogenous proteins. Wacker's ESETEC secretion technology was an efficient technology for the expression and secretion of Fab exceeding 4.0 g/L, which was approximately 40-fold higher than secretion into the periplasm.[35] Wacker's ESETEC platform contained two steps: briefly, the recombinant product was first translocated into the periplasmic space via the Sec pathway of *E. coli*, where it folded in a correct conformation, and then it was selectively transported across the outer membrane into the fermentation broth.

Fab-based fusion proteins, such as immunocytokines and immunotoxins, have also been studied. Fab and fusion protein are either expressed separately and then connected by a linker, or the Fab and protein are genetically fused together as a whole molecule.[62] Holzer et al expressed Fab IL-8 (CXCL-8) fusion protein in the periplasm of *E. coli* with the aid of the pel B leader sequence, which showed specific binding to IL-8 receptors and also displayed chemotactic activity.[63] Moreover, Nelke et al fused a single-chain-encoded B cell activating factor (scBaff) trimer to Fab against TNFRSF members, and proved that the fusion proteins remained intact in the antibody–scBaff fusion proteins.[64] Bauss et al developed RG7787, a humanized SS1 Fab fragment fused with 24-kD fragment of Pseudomonas exotoxin A (PE24), with inhibitory effect on proliferation of lung cancer cell lines with picomolar potency both *in vitro* and *in vivo*.[65]

## Purification of Fab Fragments Expressed in *E. coli*

Fab fragments are classically generated by digesting immunoglobulin with papain, and purified by protein L affinity chromatography with a strong affinity to the variable region of kappa Lc of IgG molecule.[61] However, its applicability is limited to the kappa light-chain Fab subclass. Furthermore, protein L requires extreme pH for elution and its performance is often not comparable with protein A in the binding affinity, selectivity, and capacity.[66] Also, several other chromatographic techniques, including antigen affinity, ion-

**Table 1** Summary of studies that report expression of Fab in *E. coli*, including the parameters that were critical for achieving the documented yield

| Parameter | Target | Vector | *E. coli* strain | Condition | Yield | Reference |
|---|---|---|---|---|---|---|
| Promoter | | | | | | |
| T7 | Thrombopoietin mimic | pET28a modified | Not mentioned | 22°C,16–20 h, 0.02 mmol/L IPTG | 3.5 mg/L | 37 |
| Ptic | Not mentioned | pTTODA33 modified | *E. coli* W3110 | Fed-batch fermentation | Not mentioned | 61 |
| phoA | VEGF-A | pBR322 vector | *E. coli* W3110 | Fed-batch fermentation | 332 mg/L | 56 |
| Arabinose | Matrix metalloproteinase-14 | Self-constructed | BL21 | 30°C, overnight, 0.2% arabinose (w/v) | Not mentioned | 90 |
| Signal peptide | | | | | | |
| STII | VEGF-A | pBR322 vector | *E. coli* W3110 | Fed-batch fermentation | 332 mg/L | 56 |
| PelB | HER2 | pFLAG-based vector | *E. coli* W3110 | 20°C, overnight, 0.1 mmol/L IPTG | Not mentioned | 40 |
| OmpA | HER2 | pFLAG-based vector | *E. coli* W3110 | 20°C, overnight, 0.1 mmol/L IPTG | Not mentioned | 40 |
| Chaperone | | | | | | |
| CyDisCo | HER2 | pET23 modified | K12 *E. coli* modified | 30°C, 6 h, 0.5 mmol/L IPTG | 3–50 mg/mL | 16 |
| DsbC | Not mentioned | pKO3 modified | W3110 mutated | 30°C, overnight, 200 μmol/L IPTG | 2.4 g/L | 34 |
| DsbA | Not mentioned | pET30a | BL21 (DE3) | Fed-batch fermentation | 0–7.4 g/g (cell dry mass) | 91 |
| Skp | Digoxin | pET25b | Origami (DE3) | 30°C, 3 h, 1 mmol/L IPTG | 0.8 mg/L | 42 |
| DnaK/DnaJ/ GrpE | TNF-α | pET-22b modified | Shuffle | 25°C, 8 h, 0.1 mmol/L IPTG | 0.98 μg/mL | 92 |
| Fusion tags | | | | | | |
| SUMO | VEGF | pET28 modified | Shuffle | 30°C, 6 h, 0.5 mmol/L IPTG | 12 mg/L | 24 |
| Leucine zipper-peptide | *E. coli* O157 | pET22b | Shuffle | 16°C, 24 h, 1 mmol/L IPTG | 2.5 mg/mL | 25 |
| Medium | | | | | | |
| Enbase | VEGF | pET28 modified | Shuffle | 30°C, 6 h, 0.5 mmol/L IPTG | 12 mg/L | 24 |
| Luria–Bertani medium | EBOLA virus | pD444-SR | BL21 (DE3) pLysS strain | 30°C, overnight, 1 mmol/L IPTG | 5–10 mg/L | 93 |

Abbreviations: IPTG, Isopropyl ß-D-1-thiogalactopyranoside; SUMO, small ubiquitin-related modifier; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

exchange, size-exclusion chromatography, and hydrophobic interaction, have been adopted for the purification of Fab fragments.[67]

Affinity-based chromatography is the most widely applied technique to purify Fab fragments. Affinity chromatography can separate antibodies on the basis of their specific and reversible interaction with immobilized target antigens or tags.[68] There are four main steps in affinity-based purification, including equilibration, binding, washing, and elution. At present, immobilized metal affinity chromatography offers a series of advantages over other affinity ligands, such as ligand stability, low cost, high loading capacity, mild elution conditions, and easy regeneration in large-scale purification. The separation mechanism could be roughly described as the following: initially, the recombinant antibodies with specific group tags (e.g., histidine tag) are bound onto the resin, then nontagged proteins and most impurities are washed away, and finally the protein of interest is eluted by changing the pH or by addition of a competitive molecule.[4,69]

Some novel affinity-based chromatography strategies are applied for antibody purification. Alves et al described an

affinity chromatography method that utilized unconventional nucleotide-binding site (NBS) present on the antibody Fab arms as a target for selective purification of antibodies from complex mixtures.[70] The affinity column was prepared by coupling indole butyric acid, which had a monovalent affinity for the NBS, and could consistently yield >95% antibody recovery with >98% purity during purifications performed with the chimeric mAb rituximab. Spooner et al tested the ability of a novel anti-CH1 resin, CaptureSelect IgG-CH1, which can effectively purify correctly assembled Fab and remove the light-chain dimer as a generic one-step process for both kappa and lambda type Fab.[71] In addition, ion exchange chromatography is a technique used for purification of charged biomolecules on the basis of reversible interactions between oppositely charged biomolecules and chromatography resins,[72] and based on the great capacity of cation exchangers for the capturing and concentrating target proteins in the early stage of purification process, the ion-exchange chromatography method has also been used for the purification of the fragment.[73,74] Typically, a combination of multiple chromatographic steps is needed when designing the optimal purification strategies of industrially manufactured Fab.

## Application of Fab Fragment

Among the seven marketed Fab fragment drugs, only ranibizumab and certolizumab pegol are generated in *E. coli* as IBs, and the remaining Fabs are produced by cleavage of IgG with papain. ►**Table 2** lists the monoclonal Fab antibody fragments approved by FDA. Ranibizumab is a recombinant, humanized, affinity-matured, mAb Fab fragment against all isoforms of vascular endothelial growth factor-A (VEGF-A), which was developed specifically for intraocular use.[75] Numerous randomized and controlled clinical trials showed that this medication was effective in improving both vision and anatomical outcomes. Because Fab fragments had considerably shorter serum half-life than full-length antibodies, and Nakamura et al suggested a strategy to prolonging circulating half-life by conjugation of polyethylene glycol (PEG) to Fab.[76] Certolizumab pegol is a PEGylated Fab fragment of anti-tumor necrosis factor-α for the treatment of psoriasis, Crohn's disease, and rheumatoid arthritis.[77–79] ►**Table 2** shows the Fab fragments approved by FDA and ►**Table 3** lists some monoclonal Fab antibody fragments that are being studied in clinics.

Conjugating with a functional molecule including fluorophore, enzyme, toxin, or drug represents a promising strategy to achieving additional functionalities of antibody fragments, such as serving as detecting agents or ADC.[80] ADCs are composed of a targeting moiety represented by a mAb or antibody fragment and a cytotoxic compound that is either chemically conjugated or genetically fused. Due to a better distribution or penetration *in vivo* as well as reduced immunogenicity, the ADC may lead to increased therapeutic effects.[81] To enhance killing activity of gelonin on cancer cells, Kornberger and Skerra designed a novel antibody–toxin conjugate, Fab-gelonin, in which anti-HER2 Fab was fused with the extended SrtA recognition motif at the C-terminus of its heavy chain to prevent interference with antigen binding, while the toxin was fused through a $Gly_2$ sequence at its N-terminus.[82] Huang et al applied bis(vinylsulfonyl) piperazines as efficient linkers to selectively re-bridge disulfides of the Fab regions and produced highly homogeneous conjugates loaded with two drugs, with superior efficacy and reduced toxicity in comparison to the FDA-approved drug: Trastuzumab emtansine (also known as T-DM1).[83] Wissler et al illustrated the utility of site-specific Fab conjugation as a general strategy for making sensitive positron emission tomography imaging probes, which allowed for effective

**Table 2** Monoclonal Fab antibody fragments approved by FDA

| Fab generic name (brand name) | Description | Preparation | Indication | Company | Year of approval |
|---|---|---|---|---|---|
| Abciximab (ReoPro) | Anti-integrins $\alpha IIb\beta_3$ chimeric Fab | Papain | Prevention: restenosis | Centocor, Inc. | 1994 |
| Arcitumomab (CEA-Scan) | Fab′ labeled with 99mTc against CEA | Papain | Diagnosis: colon cancer | N/A | 1996 |
| Crotalidae Polyvalent Immune Fab (CroFab) | Polyclonal sheep Fab | Papain | Therapy: snake bites | N/A | 2000 |
| Digoxin Immune Fab (DigFab) | Polyclonal sheep Fab | Papain | Therapy: Digoxin overdose | N/A | 2001 |
| Ranibizumab (Lucentis) | Anti-VEGF-A humanized Fab | *E. coli* | Therapy: macular degeneration | Genentech | 2006 |
| Certolizumab pegol (Cimzia) | Anti-TNF-α PEGylated humanized Fab | *E. coli* | Therapy: Crohn's disease | UCB, Inc. | 2008 |
| Idarucizumab (Praxbind) | Anticoagulant, humanized Fab | Papain | Therapy: antidote to dabigatran etexilate (Pradaxa) | Boehringer Ingelheim | 2015 |

Abbreviation: CEA, carcinoembryonic antigen.

**Table 3** Monoclonal Fab antibody fragments studied in clinic (Sources: clinicaltrials.gov, drugbank)

| Fab generic name (brand name) | Description | Clinical Indication | Development status (NCT number) |
|---|---|---|---|
| Certolizumab pegol | Anti-TNF-α PEGylated humanized Fab | Juvenile idiopathicarthritis | Phase III [NCT01550003] |
| | | Interstitial cystitis | Phase III [NCT01550003] |
| Abciximab (ReoPro) | Anti-integrins $\alpha IIb\beta_3$ chimeric Fab | Acute myocardialinfarction | Phase III [NCT00299377] |
| | | Acute coronary syndrome (ACS) | Phase IV [NCT00133003] |
| Abrezekimab | Anti-human-IL-13 humanized Fab | Asthma | Phase I [NCT02473939] |
| Dapirolizumab pegol | Anti-CD40L PEGylated humanized Fab | Systemic lupus erythematosus | Phase II [NCT02804763] |
| Glenzocimab | Anti-human platelet glycoprotein VI | Acute ischemic stroke | Phase I/II [NCT03803007] |
| Naptumomabestafenatox | Anti-5T4 antibody fused with a staphylococcal superantigen | Nonsmall cell lung carcinoma | Phase I [NCT00056537] |
| Onartuzumab | Anti-Met humanized Fab | Solid tumors | Phase III [NCT02488330] |

visualization and mapping of biodistribution of programed death ligand 1 (PD-L1).[84] In another interesting design, two Fabs were linked by PEG to obtain Fab-PEG-Fab, which was used as an anti-VEGF agent and displayed antiangiogenic properties comparable to or better than bevacizumab (a monoclonal IgG1 antibody that targets VEGF), thus showing a great potential in therapeutic indications.[85]

## Conclusions

Among seven marketed Fab fragment drugs, only ranibizumab and certolizumab pegol are generated in *E. coli* as IBs. Low expression yields of Fab fragments in *E. coli* remains a bottleneck to the acquisition of commercially useful products. This article reviewed the production of Fab fragments in *E. coli* with several advantages over other platforms, such as low cost, well-understood cell biology, and easy manipulation. A series of optimizations at the molecular level have been attempted to enhance the production of Fab, including co-expression of chaperones to obtain correctly folded protein, optimization of secretion signal sequences, modification of host bacteria with gene deletion, or improving the culture conditions. Although an individual strategy may not be applicable to every protein production, each one is worth pursuing due to the possibility of greatly improved yield.

In addition, new antibody–enzyme fusion proteins and bifunctional antibodies may point to a promising direction to optimize the *E. coli* expression system.[86] For example, Rithisan et al reported efficient cytoplasmic soluble expression of a Fab fused with *E. coli* alkaline phosphatase (AP), N-terminal Ser-Lys-Ile-Lys (SKIK) tag and LZ, which proved to enhance the association of the light chain and the heavy chain of Fab.[87] The SKIK-Fab-LZ-AP fusion was well expressed remaining the ability of antigen binding and AP activity. Kang et al demonstrated that removal of cysteine residues responsible for an interchain disulfide bond in a Fab molecule optimizes the periplasmic expression of a Fab-effector fusion protein in *E. coli*.[88] Du et al generated bifunctional antibodies by grafting full-length proteins into constant region loops of a Fab, which showed that the fusion proteins retained antigen-binding activity of the parent antibody with an additional activity associated with the protein insert.[89] At the same time, other production methods, such as yeast, transgenic plant, and cell-free expression systems, provide new alternatives to facilitate generation of Fab antibodies.

In conclusion, antibody drug diversification is expected to expand the range of innovative antibody-based therapeutics, including bispecific antibodies, antibody conjugates, and nanobodies. With the deeper understanding of the renaturation process, soluble protein expression, as well as the further development of genetic engineering, it is conceivable to produce antibody fragments with high yields and low costs. As one of the first small-molecule antibodies studied, Fab fragments are expected to play an increasingly important role in diagnosis and treatment of diseases.

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Conflict of Interest
The authors declared no conflict of interest.

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