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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

A general platform for efficient extracellular expression and purification of Fab from *Escherichia coli*

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Abstract

Antigen-binding fragments (Fabs) are an important part of monoclonal antibody (mAb) therapeutics and can be cost-effectively produced using an *Escherichia coli* (*E. coli*) expression system. However, Fabs tend to form undesirable aggregates when expressed in the cytoplasm of *E. coli*, substantially reducing the yield of correctly folded proteins. To solve this problem, in this study, we used five Fab fragments targeting IGF1R, Her2, VEGF, RANKL, and PD-1 to develop a novel system employing the alkaline phosphatase (phoA) promoter and the heat-stable enterotoxin II (STII) leader sequence to facilitate the efficient expression and extracellular secretion of Fabs. Following phosphate starvation, all five Fab fragments were expressed in BL21(DE3), were largely secreted into the culture medium, and then, were further purified by affinity chromatography specific to the constant region of the light chain. The purified Fab products were evaluated and were found to have high purity, antigen-binding affinity, and in vitro bioactivity. The mechanism experiments revealed that (1) BL21(DE3) had significantly higher productivity than the K-12 strains investigated; (2) the secretion ability of the PhoA promoter was superior to that of the T7 promoter; and (3) signal peptide, STII, showed higher extracellular secretion efficiency than pelB. Our findings strongly suggested that the phoA-STII-facilitated extracellular production platform is highly promising for application in the manufacturing of Fab fragments for both academic and industrial purposes.

Keywords Fab · phoA · STII · Extracellular production · *E. coli*

Introduction

Since the first approved monoclonal antibody (mAb) drug, muromonab-CD3 (Orthoclone OKT3), was commercialized in 1986, the biopharmaceutical market has witnessed a rapid expansion of mAb therapeutics (Chames et al. 2009; Douthwaite et al. 2017; Reichert 2017). Full-length mAb is a heterotetramer of two identical γ heavy chains and two identical κ or λ light chains and can be cleaved by proteolytic digestion into antigen-binding fragments (Fab) and crystallizable fragments (Fc) (Nelson and Reichert 2009; Weisser and Hall 2009). N-terminal Fab fragments contain the variable fragments which determine antibody specificity, diversity, and antigen affinity (Weisser and Hall 2009). Compared with full-length mAb, Fabs are much easier to clear from circulation as they lack the Fc and glycosylated fragment that protects against proteolytic digestion (Rezaie et al. 2017). Additionally, Fab fragments are smaller, which makes them capable of penetrating tissues and tumors more rapidly and are therefore of particular value in treatment of diseases such as wet macular degeneration (Nelson and Reichert 2009). Due to their small size and simpler conformational structure, Fabs can be produced cost-effectively in *E. coli* expression systems (Humphreys et al. 2002; Rezaie et al. 2017).

When expressed in *E. coli*, Fab fragments are likely to form undesirable aggregates in the cytoplasm because the

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cytoplasm typically lacks an oxidative environment and factors necessary to form the correct disulfide bonds. Consequently, the correctly folded target proteins have to be recovered from inclusion bodies (IBs) using complicated and time-consuming denaturation and refolding procedures (Nelson and Reichert 2009). One approach for solving this problem is using secretory expression because extracellular protein expression has been shown to have significant advantages over conventional cytoplasmic expression, with a simpler downstream purification process and more efficient protein folding (Gupta and Shukla 2017; Jalalirad 2013; Zhou et al. 2018). One challenge that Fab expression in *E. coli* faces is the correct assembly of the light chain and heavy chains. It was reported that the balance between light chain and heavy chain expression could be controlled by varying the codon usage of the signal peptide, thus enhancing the yield of Fab fusion proteins in the periplasm of *E. coli* (Humphreys et al. 2002). Variant signal peptides have been used to optimize Fab expression by increasing the solubility of Fab fragments to achieve high-level production (Humphreys et al. 2002). The type II secretion pathway involves the most widely used signal sequences in *E. coli* and includes the SecB-dependent pathway, the signal recognition particle (SRP) pathway, and the twin-arginine translocation (TAT) pathway (Linton et al. 2012; Zhou et al. 2018). It was reported that different signal peptides, such as pectate lyase B (pelB; SecB pathway) and trimethylamine N-oxide reductase (TorA; TAT pathway), could vary in their abilities to export a certain target protein to the periplasm or the extracellular medium (Linton et al. 2012). Some signal peptides, such as heat-stable enterotoxin II (STII), can enhance both the expression and the secretion to the periplasm in the secretory production of a single-chain variable fragment (Sun et al. 2012). However, a general system for robust, reliable, and reproducible extracellular expression of Fabs has not yet been developed.

In this study, we employed the alkaline phosphatase (phoA) promoter in combination with the STII signal peptide to construct a novel strategy for facilitating the secretory expression of Fabs. Transcription via the phoA promoter can be efficiently induced by lowering the phosphate concentration of the medium; therefore, it is comparatively more cost-effective than using *lac*-related promoters. Indeed, the phoA promoter has successfully been used for the secretory expression of exogenous proteins such as human epidermal growth factor (Wang et al. 2005). The phoA-STII combination was used for the periplasmic expression of anti-VEGF Fab. But here, we developed a different strategy which can be utilized universally for the extracellular expression and purification of Fabs. Using five Fab fragments with different types of IgG1/IgG2 or human/humanized structures, the feasibility of the new phoA-STII system was evaluated with respect to the secretory expression and extracellular secretion of target proteins. Moreover, the crucial parameters contributing to the excretion of Fabs were also investigated to demonstrate that the phoA-STII system is a promising strategy for the production of Fab fragments.

Materials and methods

Bacterial strains and chemicals

Escherichia coli DH5α, BL21 (DE3), Rosetta-gami 2 (DE3) were obtained from Weidi Biotechnology (Shanghai, China), and SHuffle® T7 K-12 were obtained from New England Biolabs (Ipswich, MA, USA). PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) was purchased from MilliporeSigma (St. Louis, MO, USA). Capto L and KappaSelect resins were purchased from GE Healthcare (Piscataway, NJ, USA). All chemicals and reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China) unless specially described.

Construction of Fabs expressing plasmids

The DNA sequences encoding VH and VL of five Fabs as listed in Table 1 were synthesized by General Biosystems (Chuzhou, Anhui, China) according to published sequences. (Five Fab fragments were arranged as a bicistronic unit with the light chain and heavy chain in different reading frames, each under a phoA promoter and an STII signal peptide (Fig. 1). The fragments encoding the whole units were amplified by PCR and digested with restriction enzymes *Nde* I/*Bgl* II (NEB; Ipswich, MA, USA). The backbone vector was derived from pRSFDuet with phoA promoter instead of T7 and pre-treated with the same enzymes. Then the fragments and vectors were ligated by T4 DNA ligase (Takara, Tokyo, Japan) and the sequences were further confirmed by sequencing (Sangon Biotech; Shanghai, China). The PCR Purification kit, Plasmid Mini-Prep Kit, and DNA Gel Extraction Kit were purchased from Axygen (Hangzhou, China).

Shake flask cultivations

For batch culture experiments, 5 mL of each overnight culture was inoculated into 800 mL of LB medium supplemented with kanamycin (100 μg/mL). These cultures were grown at 37 °C at a shaking speed of 220 rpm. During the exponential phase at OD_{600} of 0.8~1.0, cells were pelleted at room temperature by centrifugation for 10 min at 4000 × g. The pellet was resuspended in 800 mL of PLM medium (1 g $(NH_4)_2SO_4$, 49 mg $MgSO_4$, 2 g glucose, and 33.6 g PIPES for each liter and adjusted to pH 7.0) and then incubated at 20 °C overnight with a shaking speed of 200 rpm for expression induction.

It was reported that heating can suppress synthesis of proteins involved in membrane functions, thus possibly damage

Table 1 Fabs used in this study

Fab name	αIGF1R	αHer	αVEGF	αRANKL	αPD
Antigen	IGF1R	Her2	VEGF-A	RANKL	PD-1
Type	Human IgG1κ	Humanized IgG1κ	Humanized IgG1κ	Human IgG2κ	Human IgG4κ
Accession No.	GenBank: AB981338& BC073766	GenBank: KY199430	GenBank: AOZ48529& AOZ48530	GenBank: AUF05858, AUF05857	DrugBank: DB09035

the integrity of outer membrane and in turn benefit the leakage of periplasmic proteins (Tian et al. 2018). Therefore, after induction, the incubation temperature was quickly raised to 59 °C in water bath and held for 1 h to improve Fab yields. The culture was then quickly cooled down to room temperature and centrifuged to collect media and cells separately. B-PER Reagent (MilliporeSigma, St. Louis, MO, USA) was used to separate the intracellular soluble and insoluble (IBs) fractions. All samples media, soluble fractions, and IBs were analyzed by Western blot to see the distribution of Fabs in different components, using goat anti-human kappa light chains antibody (MilliporeSigma, St. Louis, MO, USA) as the capture antibody, and rabbit anti-goat IgG antibody conjugated with HRP (Jackson ImmunoResearch; West Grove, PA, USA) as the second antibody.

Purification of Fabs

In this study, we aimed to facilitate the extracellular expression and purification of Fabs by phoA-STII system; therefore, we purified the products directly from the culture media which were collected immediately after fermentation by centrifugation at 12,000 × g for 10 min. To evaluate the secretion efficiency, the fermented cells were harvested and resuspended in phosphate- buffered saline (PBS) at 10% weight/volume ratio and disrupted by homogenization for six runs at 900 bar. After centrifugation at 12,000 × g for 30 min at 4 °C, the supernatant was collected as the cytoplasmic soluble fractions for purification.

Capto L has a strong affinity to the variable region of kappa light chain of immunoglobulins and is therefore capable of purifying a wide range of antibody fragments including Fabs. According to IgG types as listed in Table 1, Capto L was used for the purification of αHer, αVEGF, αRANKL, and αPDF. With PBS of pH 7.4 as the binding buffer (designated Buffer A1), the collected media or soluble cytoplasmic proteins were loaded onto the Capto L column (CV = 1 mL) at a flow rate of 0.5 mL/min. Then the column was washed with Buffer A2 (0.025 M sodium citrate + 0.025 M sodium phosphate, pH 7.4) until the absorbance of UV 280 nm reached baseline. The target protein was finally eluted by buffer B1 (0.025 M sodium citrate + 0.025 M sodium phosphate, pH 2.3) with a flow rate of 1.0 mL/min.

It's well known that Capto L binds to kappa light chains of type I, III, and IV of human IgG but not type II (Enokizono et al. 1997). Therefore, we used KappaSelect resin to purify αIGF1R which with a type II IgG kappa light chain. The purification process was similar to that of Capto L. Abovementioned Buffer A1 was used as the equilibration, loading, as well as wash buffer. The elution buffer was 0.1 M glycine buffer of pH 2.7. The elutes from Capto L and KappaSelect chromatography were analyzed by 12% SDS-PAGE and Western blot to evaluate the purification performances, using the same antibodies mentioned above. Then,

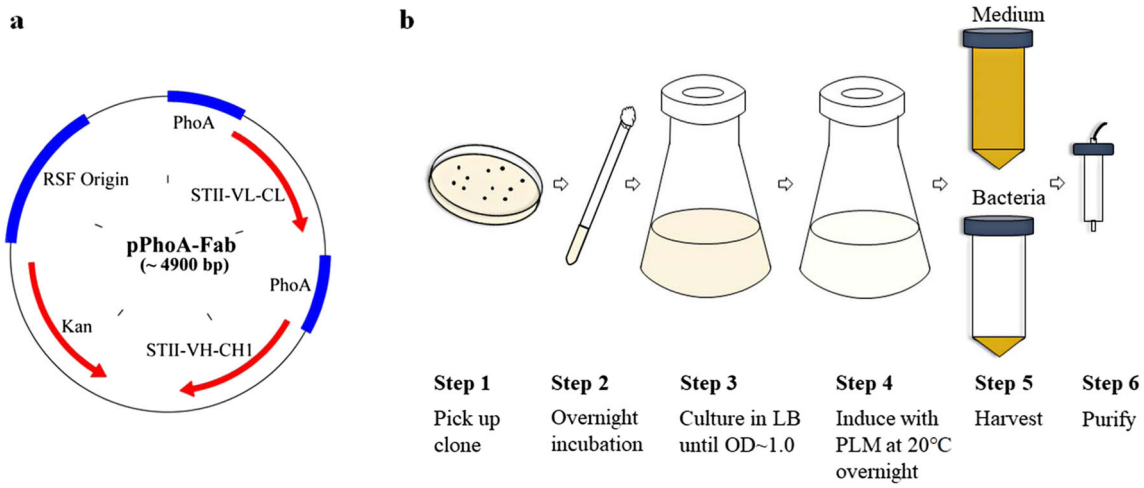


Fig. 1 Scheme of the extracellular expression and purification process for Fab production. **a** Construction of recombinant bicistronic plasmids for extracellular expression of Fabs in *E. coli*. **b** Flow chart of the secretory expression and purification process

the final products were further analyzed for characteristics including purity, integrity, affinity, and bioactivity.

Characterization of Fabs by SEC-HPLC and LC-MS

Size-exclusion high-performance liquid chromatography (SEC-HPLC) was used to determine the purity of the products. In detail, Agilent 1260 HPLC system (Agilent, Santa Clara, CA) was fitted with TSK G2000SW$_{XL}$ columns (TSK-GEL, G2000SW$_{XL}$, 5 μm, 0.78 × 300 mm; Tosoh Biosciences, King of Prussia, PA) at 30 °C. Both mobile and elution phases were PBS in HPLC-grade water, pH 7.4, flowed at 0.75 mL/min and monitored at 280 nm. Peak areas were integrated using the Agilent Chem Station software. The purity of each Fab was calculated as a percentage of the total peak area detected.

To confirm Fab products identities, a Waters' time-of-flight (Q-Tof) premier mass spectrometer interfaced with a Water's Acquity I-class ultra-high-performance liquid chromatography (LC-MS) was used for exact mass measurement. A gradient of 9.5% B/min (A, 0.1% aqueous formic acid; B, 0.1% formic acid in acetonitrile) was used with an Acquity UPLC BEH C18 column (1 × 100 mm, 1.7 μm, 300 Å). The column was heated to 45 °C. Mass spectrometer conditions are as follows: capillary voltage was 2 kV, cone voltage was 40 V, source temperature was 115 °C, desolvation temperature was 450 °C, and desolation gas flow was 900 L/min.

Binding affinity measurement of Fabs

We employed ForteBio Octet RED96e system (Pall ForteBio, Fremont, CA, USA), a special biolayer interferometry-based (BLI) technology, to perform a rapid antibody-antigen binding affinity quantitation as well as kinetic profiling of Fabs. Firstly, the sensors were immobilized with antigens at concentrations between 5 and 10 μg/mL: IGF1R for αIGF1R, Her2 for αHer, VEGF-A165 for αVEGF, RANKL for αRANKL, and PD-1 for αPD. Most of antigen proteins were purchased from Sino Biological Inc. (Beijing, China), except for VEGF-A165 which was purchased from PeproTech (Rocky Hill, NJ, USA). Then each Fab has diluted serially by 1:1 to 6 concentrations between 1.56 and 250 nM for measurement. The affinity was detected by procedures consisting of three steps: loading, regeneration, and equilibration. PBS of pH 7.4 containing 0.02% Tween 20 was used for loading and samples dilution buffer, and 10 mM Glycine of pH 1.5 was used for regeneration. Finally, the experimental data were processed by the data analysis software in ForteBio Octet RED96e system.

Biological function assays of Fabs

The biological activities of αIGF1R, αHer, αRANKL, and αPD were evaluated by their binding affinity to cell lines which express their antigens IGF1R, Her2, RANKL, and PD-1 respectively. HepG2 cells are positive for the surface expression of IGF1R. SKBR3 cell line is well-known for its Her2-overexpression (Oude Munnink et al. 2012). HUVEC cells were reported to produce RANKL (Naumnik 2017). Jurkat is a leukemic T cell line and expresses PD-1 when stimulated by phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Shanghai, China) (Skalniak et al. 2017). The cells were collected and suspended in 100 μL PBS at a density of 2.5 × 10^6 cells/mL, and Fabs were added to a final concentration of 10 μg/mL for binding. After incubation at 4 °C for 1 h, the cells were washed with PBS twice to remove unconjugated antibodies, suspended in 100 μL PBS containing 10 μg/mL FITC-labeled recombinant protein L (ACRO Biosystems, Shanghai, China), and further incubated at 4 °C for 45 min before detection by Flow cytometry LSRFortessa (BD Biosciences; Franklin Lakes, NJ, USA). For αIGF1R, we used FITC-conjugated goat anti-human IgG (Fab')$_2$ antibody (Abcam; Cambridge, MA, USA) as the second antibody. For αRANKL, cells were treated with Cytofix/Cytoperm reagents BD Biosciences (Franklin Lakes, NJ, USA) according to manufacturer's manuals, to allow the binding of αRANKL with the intracellular RANKL.

The VEGF neutralizing Fab is capable of binding to recombinant human VEGF165 and induce inhibition of VEGF-A-induced HUVEC proliferation in a concentration-dependent manner (Lowe et al. 2007). Therefore, we used a cell proliferation inhibitory assay to determine the bioactivity of αVEGF as described previously (Ji et al. 2017), using ranibizumab as control. Briefly, the purified αVEGF and the reference antibody were diluted to desired concentrations with cell culture medium containing 10 ng/mL of recombinant human VEGF-A165 protein (PeproTech; Rocky Hill, NJ, USA) and then added to 96-well plate which was seeded with HUVEC cells at 3000 per well. After cultivation for 3 days, cell proliferation was measured using CCK-8 reagent (Dojindo, Kumamoto, Japan). The absorbance was read at 450 nm, with a reference at 600 nm. The data were analyzed with GraphPad Prism software, and the IC_{50} values were calculated using the four-parameter non-linear logistic regression model as interpreted previously (Chen et al. 2017).

We performed above assays using purchased reference antibodies as controls. Trastuzumab (Roach/Genentech) was purchased from a pharmaceutical market, ranibizumab, denosumab, and nivolumab were biosimilar obtained as gifts from pharmaceutical companies. They were used as controls for αHer, αVEGF, αRANKL, and αPD, respectively. Since there are no approved anti-IGF1R antibodies available in the market, we used FITC-conjugated rabbit anti-human IGF1R mAb (Sino Biological; Beijing, China) as the control for αIGF1R.

Viability assessed by bacteria density and colony forming unit assays

The fermentation and heating procedures could be accompanied by cell lysis or loss of viability of bacteria. Therefore, we measured OD_{600} of the culture at each stage of fermentation to indicate bacteria density which is related to cell lysis. To evaluate the cell viability, the culture was collected at each stage (before induction, after induction, and after heating) of the fermentation process for colonies growth on LB agar supplemented with 100 μg/mL kanamycin in Petri dishes. The culture would be diluted by 10^{-1}–10^{-8} with sterile water before plating to guarantee 30–300 colonies per dish after incubation at 37 °C for 16 h. The colonies would be counted to represent the viable cells, which were further normalized to OD_{600}.

Mechanism studies for impact of host strain, promoter type, and signal peptide on Fabs extracellular expression

To reveal the mechanism of the phoA-STII approach to secret Fabs into media, we studied the roles of three important parameters including host strain, promoters, and signal peptides, with αVEGF as a representative protein. For the study of host strains, we transformed the plasmid pPhaA-Fab into 3 prevalent K-12 strains: Rosetta-gami 2, SHuffle T7, and MG1655, and their expression and secretion of αVEGF was compared with that of BL21(DE3). For the study of promoter and signal peptide, we constructed two new plasmids to replace phoA with T7 promoter (T7-STII-Fab) or replace STII with pelB signal peptide (phoA-pelB-Fab), respectively. Each plasmid was transformed into BL21(DE3) to evaluate their expression and secretion of αVEGF to be compared with that resulted from pPhoA-Fab (phoA-STII-Fab).

Results

Extracellular expression of Fabs mediated by phoA-STII system

Using phoA promoter and STII signal peptide, we successfully constructed recombinant plasmids to produce the selected Fabs as shown in Fig. 1. Fabs were induced by phosphate starvation, and the expression solubility and extracellular secretion were evaluated by Western blot. As shown in Fig. 2a, all five Fabs were expressed with high solubility and efficient secretion. There were apparently higher proportions of products in extracellular media than in the intracellular soluble fractions, while only minor products were expressed in the form of IBs. Western blot results of three batches of fermentation were statistically quantified by photodensity scanning. As shown in Fig. 2b, there were generally more product in media than intracellular soluble fractions or IBs, suggesting that phoA-STII expression system can efficiently express and transport target proteins out of the bacteria cells.

Purification of Fabs

To better investigate the secretion efficiency, we performed purification from the extracellular media and intracellular supernatant separately, with Capto L or KappaSelect resins as described in "Materials and methods". The ratio of extracellular/intracellular purification yield was calculated to evaluate the secretion efficiency. SDS-PAGE analysis showed that all Fabs have been purified with high purity, and there was only a single band of the target protein in the lane of elution (Fig. S1). As shown in Table 2, the purification yields of extracellular products were generally higher than those of the intracellular soluble products, and the average yield of Fab purified from fermentation media was up to 6.3 mg per liter. The result was consistent with the Western blot data as we observed in Fig. 2, demonstrating that phoA-STII system can profoundly express and secrete Fab fragments into extracellular media.

Characterization of Fabs with SEC-HPLC and LC-MS

The quality of purified Fab products was characterized using SEC-HPLC for purity and LC-MS for intact molecular weight (Mw). According to the HPLC results, the purified Fabs had similar retention time which may contribute to their similar Mw and tertiary structures (Fig. 3a). The purity of all Fab fragments was above 94%, as listed in Fig. 3b. The data confirmed that with phoA-STII system, Fabs were successfully produced with high purity.

We used LC-MS to detect the accurate Mw of the purified Fabs to explore whether the signal peptides have been removed during secretion. As shown in Fig. 4, the measured Mw of Fabs were very close to the calculated Mw, with a minor gap of about 1 Da. The results confirmed that the signal peptides have been precisely cleaved during the secretion, and no extra amino acids were left in the target protein in the medium.

Binding affinity of Fabs to their specific antigens

In order to verify the preserved functionality of Fabs, the five Fab molecules were subjected to kinetic binding analysis to their specific antigens, using real-time biosensor analysis on a ForteBio instrument. The proteins were injected at different concentrations over surfaces containing immobilized antigens respectively. Sensorgrams obtained from the biosensor analysis showed that both binding arms had preserved functionality and could bind to specific antigens, respectively. Furthermore, for the kinetic parameters, the association rate constant (k_a),

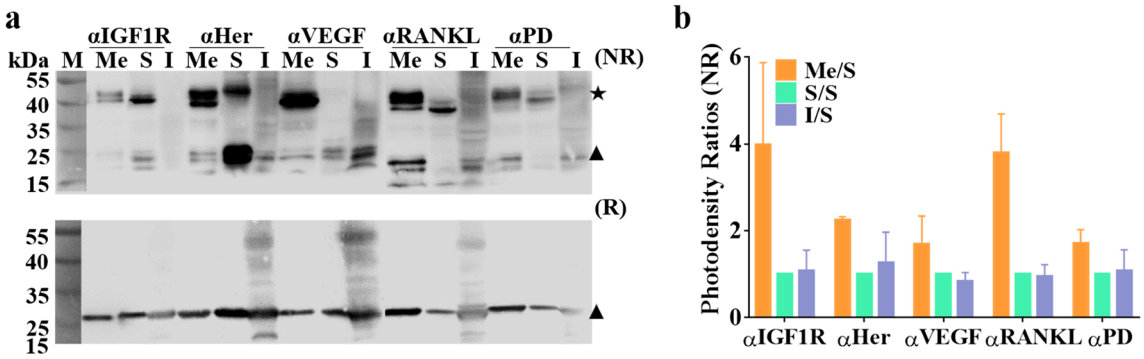


Fig. 2 Evaluation of extracellular secretion of Fabs. **a** Western blot analysis of Fabs expressed in *E. coli*. (Black star) Non-reducing (NR) Fab; (Black triangle) reduced (R) LC bands. Lane M, protein molecular weight standard; Me, medium; S, cytoplasmic soluble fraction; IB, inclusion body. **b** Fab expression levels in different space of "Me", "S", and "I" relative to "S" estimated by densitometry scanning of non-reducing samples (NR). The values were statistics of three independent fermentation and Western blot experiments

dissociation rate constant (k_d) and the dissociation equilibrium constant (K_D) of the Fabs, we obtained desired values in comparison with references as presented in Table 3 and Fig. S2. Except that αVEGF had a slightly higher K_D value than those reported in references, all other four Fabs showed comparative affinity with their references. Since most of the references used full-length IgG, but not Fab, the detected K_D values indicated that the absence of Fc fragment did not reduce the affinity of Fabs we purified.

Biological function assays of Fabs

As described in "Materials and methods", we used flow cytometry to detect the affinity of αIGF1R, αHer, αRANKL, and αPD with corresponding cell lines and used VEGF-stimulated HUVEC proliferation assay to evaluate the bioactivity of αVEGF. The IC_{50} of αVEGF to inhibit VEGF-dependent proliferation of HUVEC was 34.88 ng/mL, very similar to that of ranibizumab (47.26 ng/mL), suggesting that our purified product was of full bioactivity (Fig. 5a and b).

The antigen binding activities of αIGF1R, αHer, αRANKL, and αPD were analyzed by their binding affinity to the surface antigens of cell lines MCF-7, SKBR3, HUVEC, and Jurkat respectively, as described in "Materials and methods". The results were shown in Fig. 5c–f. In comparison with the bioactivities of their controls shown in Fig. 5g–j, all Fabs showed satisfying binding affinities to the surface antigens of the corresponding cell lines. The data supported that the Fab produced by the current strategy were biologically active.

Cell lysis and viability during process

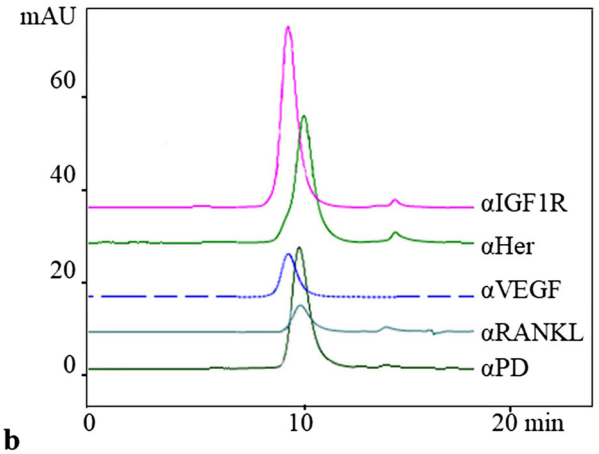
Since phosphorus compounds play important roles in building organism structures and sustain metabolic activities, phosphate starvation during fermentation of *E. coli* can significantly decrease cell concentrations (Marzan and Shimizu 2011). The heating procedure can also cause cell lysis. But we found that there were no significant difference of cell density between each stage of pre-induction (Pre), induction (Ind), and heat treatment (Heat), as indicated by OD_{600} (Fig. 6a). The result suggested that there was no obvious cell lysis during the entire fermentation process. Meanwhile, we observed comparable or higher amounts of Fab products in media of "Heat" than "Ind" (Fig. S3), indicating that the heating procedure may be beneficial for purification yields as we predicted.

We further evaluated cell viability by CFU counting at different stages of the process: pre-induction by phosphate starvation (Pre), immediately before heating (Ind), and right after heat treatment (Heat). The CFU counts were normalized to OD_{600}, and there was a logarithmic decrease of cell viability during induction ($8.06 \pm 3.14 \times 10^9$ vs. $3.28 \pm 0.08 \times 10^7$ CFU per unit of OD_{600} for αVEGF representatively), which continued going down sharply after heating ($1.89 \pm 1.24 \times 10^4$ CFU per unit of OD_{600} for αVEGF) as shown in Fig.

Table 2 Extracellular secretion efficiency of Fabs from two batches of purification

		αIGF1R	αHer	αVEGF	αRANKL	αPD
Batch 1	Extracellular (mg/L)	10.1	6.25	2.65	2.4	4.5
	Intracellular (mg/L)	10.5	3.3	1.5	0.55	3
Batch 2	Extracellular (mg/L)	2.5	6.3	1.65	0.6	1.2
	Intracellular (mg/L)	2.5	3.65	0.35	0.75	1.2
Average ratios (extracellular/intracellular)		0.98	1.81	3.24	2.58	1.25

a



b

	αIGF1R	αHer	αVEGF	αRANKL	αPD
Purity (%)	99.9	94.3	98.3	99.6	99.1
Mw (kDa)	47.9	47.8	48.4	47.6	47.1

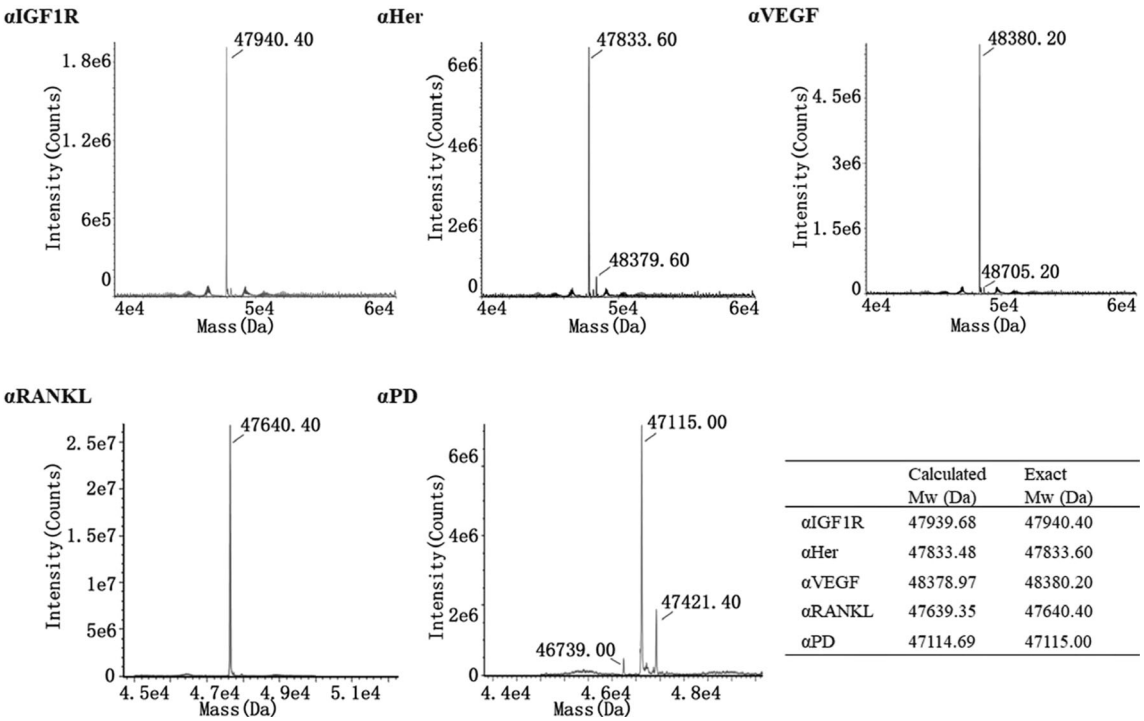
Fig. 3 SEC-HPLC analysis for Fab products purity. **a** SEC-HPLC spectrum of Fabs. **b** Purity of Fab analyzed by HPLC

6b, illustrating that most of the cells entered into death during the fermentation process. The contradiction between stable cell density and reduced viability suggested that the phosphate starvation induction, and heating processes did not destroy the outer membrane or cell wall of *E. coli* but corrupted the intracellular biochemical activities which could ultimately cause the death of cells.

Mechanism studies of Fabs extracellular expression

There have been many studies about the extracellular production of proteins in *E. coli*, which demonstrated that the producing efficiency was related to multiple parameters, especially host strains, promoters, and signal peptides. Firstly, we studied the impact of host strains using several widely used K-12 strain bacteria: Rosetta-gami 2, SHuffle T7, and MG1655. To our surprise, we found that the expression in K-12 strain bacteria was much lower than that of BL21(DE3). The target protein could be hardly detected after induction using Rosetta-gami 2 or SHuffle T7. In MG1655, only a little expression was detected, with very limited extracellular secretion (Fig. 7b). According to the photodensity analysis of Western blot of Fig. 7a and b, the ratios of medium/cell fractions were about 202% for BL21(DE3) and only about 37.5% for MG1655. Therefore, BL21(DE3) was the cell strain with the best expression and secretion performances for this platform.

Then, we replaced phoA promoter with T7 promoter to investigate its function and responsibility in the process. As shown in Fig. 7a and c, T7 promoter can also promote the soluble expression of αVEGF and the secretion into extracellular medium. However, in comparison with phoA promoter, the proportion secreted into the medium was lower when promoted by T7. It may contribute to that T7 is a strong promoter which accumulates target proteins rapidly to form IBs, therefore, impedes their entering into periplasm and secretion into medium. Moreover, we observed leakage expression before



	Calculated Mw (Da)	Exact Mw (Da)
αIGF1R	47939.68	47940.40
αHer	47833.48	47833.60
αVEGF	48378.97	48380.20
αRANKL	47639.35	47640.40
αPD	47114.69	47115.00

Fig. 4 LC-MS analysis of exact molecular weight of Fab products. The detected Mw were tightly close to the predicted values as listed in that table. The gap between the measured and predicted Mw were less than 1 Da for most of the Fabs

Table 3 The binding affinity of Fab products detected by BLI technology

Fabs	Target	Affinity (K_D)	Literature reference (K_D)
αIGF1R	IGF1R	21 nM	4–945 nM (Calzone et al. 2013; Moody et al. 2014)
αHer	Her2	29 pM	22 pM–0.11 nM (Ekerljung et al. 2012)
αVEGF	VEGF	2.4 nM	192 pM–1.33 nM (Khalili et al. 2012; Khalili et al. 2015; Lowe et al. 2007; Papadopoulos et al. 2012; Yu et al. 2008)
αRANKL	RANKL	0.23 nM	0.15–680 nM (Liu et al. 2015; Willard et al. 2000)
αPD	PD-1	8.6 nM	0.7–98.9 nM (Akiyama et al. 2016)

IPTG induction when using T7 promoter, while no expression before induction was observed when using phoA promoter.

Signal peptides play critical roles for secretion of target proteins into media. STII is a SecB signal peptide which belongs to type II secretion system and employs two steps to secrete proteins: first, export proteins to the periplasm, subsequently, the target protein traverses the outer membrane to the extracellular medium. To explore the function of STII, we substituted it with PelB, one of the most widely used SecB pathway signal peptides for secretion expression in *E. coli*. Using representative αVEGF, we found that pelB had poorer extracellular secretion efficiency than STII by comparing the Western blot results shown in Fig. 7a and d. There are several strategies to excrete the periplasmic proteins into the culture

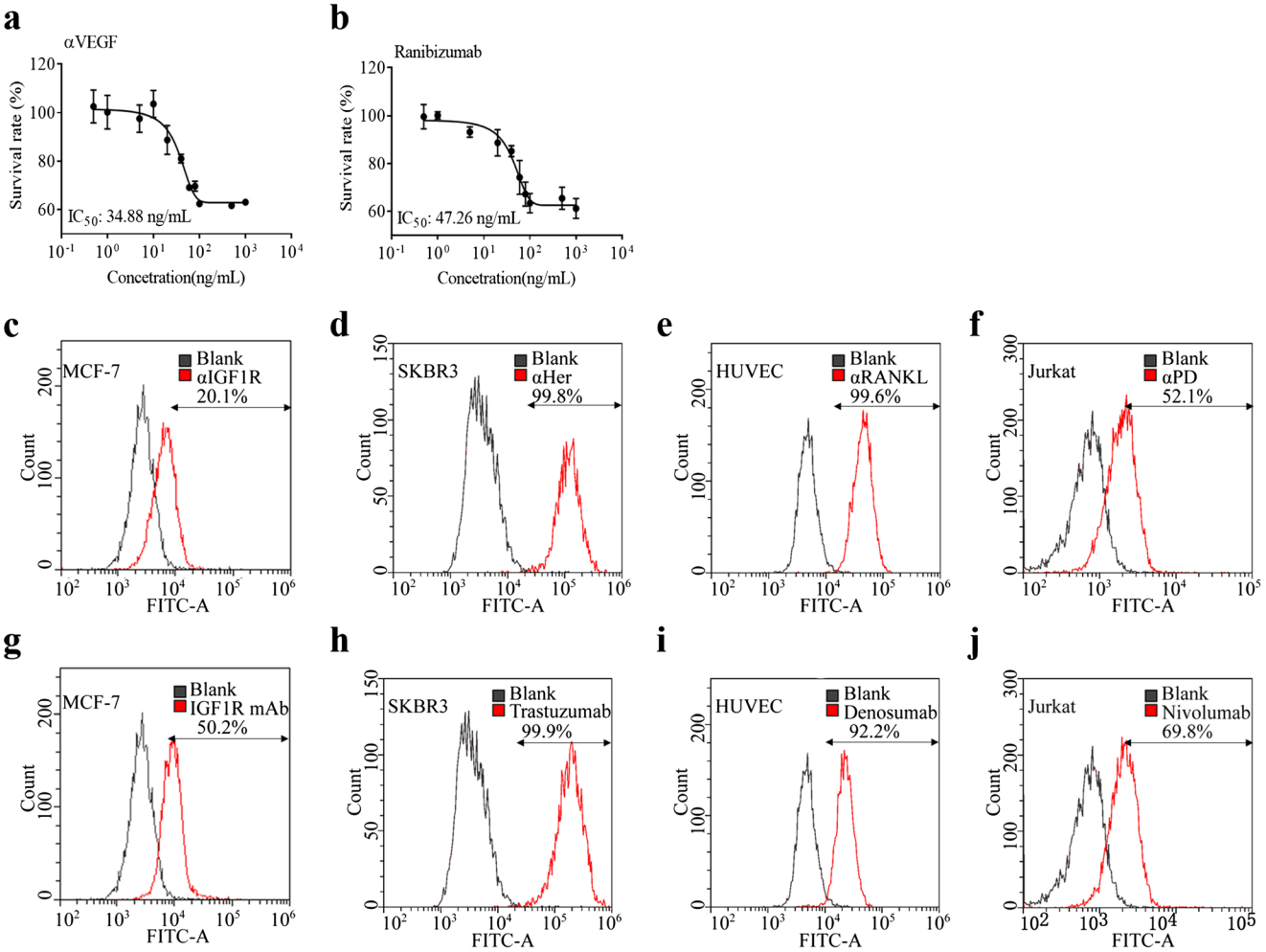


Fig. 5 In vitro bioactivity of Fab products. **a** HUVEC cell proliferation inhibition by αVEGF was detected using CCK8 assay. **b** Ranibizumab was evaluated as control of αVEGF; **c** FACS analysis of αIGF1R binding to the surface IGF1R on MCF7 cells. **d** FACS analysis of αHer binding to the surface Her2 on SKBR3 cells. **e** FACS analysis of αRANKL binding to the intracellular RANKL in HUVEC cell. **f** FACS analysis of αPD binding to the surface PD-1 on Jurkat cell. **g**–**j** Control assays using rabbit anti-human IGF1R mAb, Trastuzumab, Denosumab, and Nivolumab as controls for αIGF1R, αHer, αRANKL, and αPD

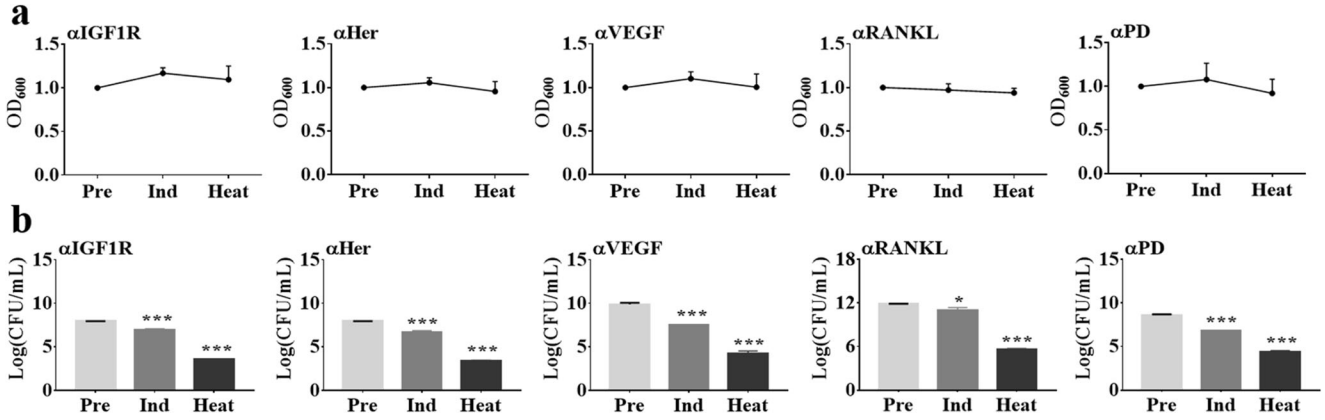


Fig. 6 The influence of expression process on bacterial cells. **a** Bacteria density were evaluated by OD600 values during stages of fermentation. **b** Viability of bacteria cells in different stages of induction were evaluated by colony counting units (CFU) relative to OD600 values ($n > 3$). The columns stand for the Log (CFU) values in different production stages. Pre, pre-induction; Ind, after induction and immediately before heat treatment; Heat, after heat treatment. The data represent three independent experiments

medium, such as the out-membrane leakage, co-expression with outer membrane proteins, or transmembrane proteins (Choi and Lee 2004). The higher secretion efficiency of STII may contribute to the helping of TolC system as previously reported (Yamanaka et al. 2001; Yamanaka et al. 2008).

Upon all the above investigated parameters that influence expression and secretion of Fabs, we demonstrated that the extracellular expression efficiency was the most superior when using BL21(DE3) strain, phoA promoter and STII signal peptide, which was exactly we used to develop the current platform (summarized in Fig. 7e).

Discussion

Fabs account for the majority of antibody fragments in ongoing clinical or pre-clinical development. Due to their small size and lack of complicated post-translational modifications, Fabs can be efficiently expressed in non-mammalian expression systems such as yeast and *E. coli*. Until now, *E. coli* has been used in the production of approximately 40% of the biopharmaceutical products launched in the market (Gupta and Shukla 2017) and is the host that is mostly used for the production of Fabs because of various advantages including low cost, easy manipulation, and scalable manufacturing.

However, difficulties in expressing Fabs in *E. coli* have limited its use as an expression system. Like most biopharmaceuticals, Fabs contain pairs of intrachain and interchain disulfide-bonds which determine their tertiary structure and biological functions. The lack of an optimal redox environment and assisting elements such as chaperones leads to the accumulation of unfolded proteins in IBs. Once IBs have formed, Fabs must be recovered using complicated denaturation-refolding procedures as reported previously (Fujii et al. 2007). Therefore, soluble expression systems yielding correctly folded, biologically functional products have attracted considerable attention. Many strategies for improving the soluble expression of Fabs have been studied so far, typically using low temperature induction, ventilation adjustment, fusion with solubilizing partners, host bacteria mutants, or secretion of the product into the periplasmic space or extracellular medium (Kim et al. 2018; Nesmeyanova et al. 1991; Rezaie et al. 2017). Among these strategies, extracellular secretion is of particular interest since the product can be extracted directly from the medium, avoiding the need for cell lysis procedures to release the Fabs from the cells.

The system established in this study enabled the majority of the Fab proteins to be secreted directly into the medium and folded correctly to form active proteins rather than IBs. The system was based on the phoA promoter and the STII signal peptide and employed the prevalent bacteria BL21(DE3) as the host strain. All five Fabs were expressed successfully, and the majority of the products were secreted into the media (Fig. 2). Products were obtained with a purity of up to 99% using a single affinity chromatography step, as determined by SEC-HPLC, demonstrating the high efficiency of this strategy (Fig. 3). Even though the fermentation conditions were very harsh because of phosphate starvation and heat treatment, no obvious cell lysis was observed. This avoided the release of intracellular proteins with misfolded structures or uncleaved signal peptides and resulted in all the products having the predicted molecular weights, as determined by LC-MS.

It was particularly important to explore the parameters contributing to the success of our extracellular expression platform. *E. coli* possesses six secretion pathways, each involving different molecules and outer membrane proteins (Yamanaka et al. 2008). One approach used to accelerate extracellular secretion has been to increase periplasmic leakage by targeting essential membrane proteins (Shin and Chen 2008). Therefore, the secretion process is highly dependent on parameters such as the bacterial strain, promoter, and signal peptide (Jalalirad 2013; Zhao et al. 2018). To investigate the

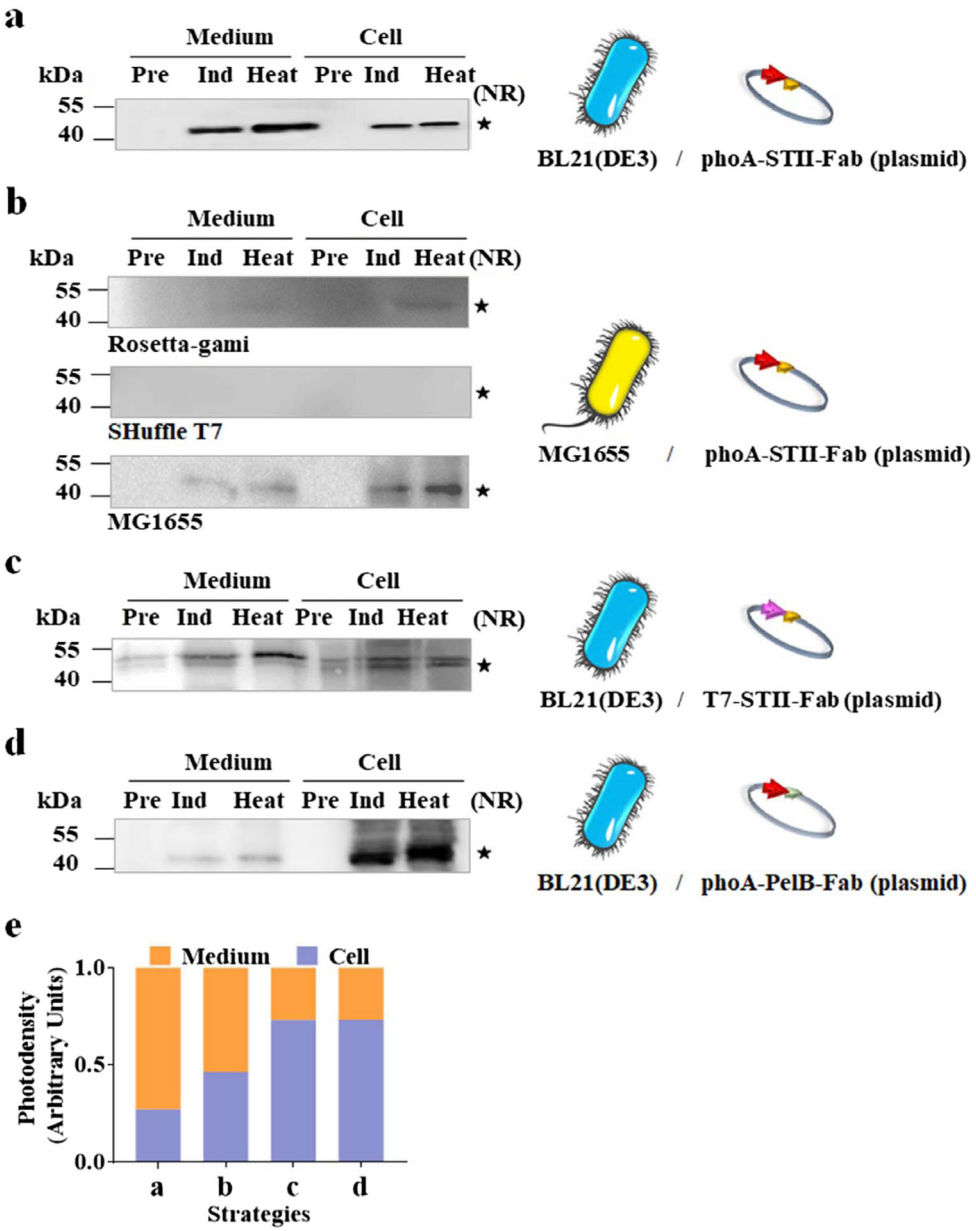


Fig. 7 Parameters influence Fab expression and secretion in *E. coli*. The expression and secretion situations of a representative Fab fragment, αVEGF, was observed by Western blot. Parameters were investigated using different host bacteria strains, promoters, and signal peptides as shown by cartoons. **a** expression with phoA promoter and STII leader in BL21(DE3). **c** expression with phoA promoter and STII leader in K-12 strains Rosetta-gami 2, Shuffle T7, and MG1655.**c**: expression with T7 promoter and STII leader in BL21(DE3). **d** expression with phoA promoter and pelB leader in BL21(DE3). Lane M, protein molecular weight marker; Pre, before induction; Ind, induction; Heat, after heating. (black star) indicated Fab bands about 45 kDa. Extracellular secretion efficiencies of strategies **a**~**d** were compared by photodensity scanning of Western blot bands of conditions **a**–**d**, using MG1655 strain to represent the data of K-12 strains for condition (**c**). The data represent three independent experiments

influence of host strains on secretion, the majority of *E. coli* strains used were derivatives of either the non-pathogenic K-12 or B strains. Recently, the metabolic and physiological differences between the two strains have been shown to facilitate potential biotechnological applications (Han et al. 2014). Here, we used αVEGF as a model protein to investigate protein expression and secretion in several K-12 strains, including Rosetta-gami 2, Shuffle T7, and MG1655, compared with BL21(DE3). Rosetta-gami 2 has thioredoxin reductase (trxB) and glutathione reductase (gor) gene mutations, Shuffle T7 has trxB and gor gene mutations as well as constitutive DsbC expression, and MG1655 has generally high growth rate and productivity (Sun et al. 2016; Zhou et al. 2018). All three K-12 strains showed lower expression and secretion than BL21(DE3) (Fig. 7b), demonstrating that BL21(DE3) is an ideal host strain for the extracellular expression of Fabs, possibly due to its genotypic properties as well as its metabolic and physiological characteristics (Han et al. 2014).

We then investigated the effect of promoters on the system by constructing plasmids with the widely used T7 promoter instead of the phoA promoter. As one of the strongest bacterial promoters, T7 is able to achieve high target protein yields, whereas, the phoA promoter is generally described as being a weaker promoter. The T7 promoter did induce higher cytoplasmic expression; however, it did not produce a higher level of extracellular products. In other words, the secretion of protein products into the growth medium appears to be controlled by mechanisms other than transcription or translation.

Signal peptides play a dominant role in the secretion and translocation of proteins after translation. The type II system is the most widely used secretion system of all those found in *E. coli*. Of the three pathways in the type II system, the SecB pathway was reported to be optimal for alpha helix-free molecules (Thie et al. 2008). Since Fabs do not have alpha-helices, we selected the STII signal sequence from the SecB family to help refold and transport the target proteins. We compared its secretion efficiency with pelB, one of the most prevalent SecB signal sequences, however, pelB showed poor secretion of Fabs into the medium (Fig. 7d). The higher secretion ability of STII may be due to the fact that the heat-stable enterotoxin (ST) produced by enterotoxigenic *E. coli* is an extracellular peptide toxin. In addition, it has been reported that ST is translocated across the outer membrane via a channel made by TolC and that the MacAB system can cooperate with TolC to form a unique pathway for the STII enterotoxin (Yamanaka et al. 2008). The overexpression of TolC has been shown to dramatically increase the extracellular secretion of the STII enterotoxin (Yamanaka et al. 2001). Therefore, we hypothesize that the secretion efficiency of the STII signal peptide could be further improved by TolC coexpression.

Generally, secretory expression in *E. coli* is the most suitable process for producing disulfide bond-containing antibody fragments such as Fab, Fab', and $(Fab')_2$ (Ellis et al. 2017). A similar phoA-STII system was used for the periplasmic expression and purification of the anti-VEGF Fab (Baca et al. 1997). However, our work on extracellular expression is superior to this previous study due to the use of different expression cassette designs, host strains, and fermentation conditions. As shown in Table 2, our approach yielded up to 10 mg/L of purified Fab with a final cell density of OD_{600} 1.0 for a 16 h induction in shake flasks. Considering that the intracellular Fab fractions were discarded during purification, a much higher expression titer was actually achieved by our system. In comparison with the periplasmic preparation strategy reported previously, which achieved a final periplasmic expression level of over 2.4 g/L (final cell density of OD_{600} 105) Fab' with a 40 h induction (Ellis et al. 2017), our approach has a similar expression efficiency (per OD_{600}) but with a considerably reduced time and cost, as well as simplified purification process.

For the first time, our work has provided a general platform for the efficient extracellular expression and purification of Fab fragments and other recombinant proteins in *E. coli*. Using the preparation of five pharmaceutical Fabs, we demonstrated that the products were of high purity, integrity, and bioactivity, thus illustrating the reliability of this strategy. Moreover, due to the ease of scaling up the processes of extracellular expression and purification, the strategy has been successfully scaled up to 200 L in our cooperation biopharma to produce anti-VEGF Fabs for clinical trials (data not shown). We believe that this extracellular expression technique could be further improved to meet the requirements of laboratory and industrial manufacturing in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animal experiments.

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