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BIOTECHNOLOGY METHODS

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Functional expression of a single-chain antibody fragment against human epidermal growth factor receptor 2 (HER2) in *Escherichia coli*

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Abstract The human epidermal growth factor receptor (HER) family plays an important role in cell growth and signaling and alteration of its function has been demonstrated in many different kinds of cancer. Receptor dimerization is necessary for the HER signal transduction pathway and tyrosine kinase activity. Recently, several monoclonal antibodies have been developed to directly interfere with ligand–HER receptor binding and receptor dimerization. A single chain variable fragment (ScFv) is a valuable alternative to an intact antibody. This report describes the production and purification of an ScFv specific for domain II of the HER2 receptor in *Escherichia coli* BL21 (DE3) cytoplasm. The majority of expressed of anti-her2his-ScFv protein was produced as inclusion bodies. A Ni-NTA affinity column was used to purify the anti-her2his-ScFv protein. The molecular weight of anti-her2his-ScFv protein was estimated to be approximately 27 kDa, as confirmed by SDS-PAGE and Western blotting assay. The anti-her2his-ScFv showed near 95 % purity and reached a yield of approximately 29 mg/l in flask fermentation. The purified anti-her2his-ScFv showed its biological activity by binding to HER2 receptor on the surface of BT-474 cells. This ScFv may be a potential pharmaceutical candidate for targeting tumour cells overexpressing HER2 receptor.

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Introduction

The human epidermal growth factor receptor (HER) family plays an important role in cell growth and signaling, and its expression is altered in many kinds of cancer cells. This family consists of four members that are similar in structure, i.e. HER1 (also known as EGFR), HER2 (also known as ErbB2, c-erbB2 or HER2/neu), HER3 and HER4. Receptor dimerization or receptor pairing is necessary for signal transduction via HER and HER family members are able to form homodimers or heterodimers among themselves. Dimerization leads to activation of the intracellular tyrosine kinase domain of HER family members, resulting in phosphorylation of specific tyrosine residues and initiation of signaling cascades including mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathways. These downstream signaling pathways potentially increase cell proliferation and decrease cell apoptosis [3].

HER2 is a 185-kDa transmembrane protein (p^{185}) and, unlike other HER family members, it has no identified ligand. Being a preferred partner for heterodimerization, this receptor is in an open conformation to form heterodimers with other HER members. Previous studies have shown that heterodimers containing HER2 have a high cell transformation ability [8, 36] and overexpression of HER2 can potentially lead to poor prognosis of cancers [5]. As a result of the important biological role associated with cell growth and differentiation, HER2 has been identified as an effective target for cancer therapy [38].

Monoclonal antibodies have been developed to inhibit the biological activity of HER2 through (1) interference of ligand–receptor binding and receptor dimerization [23, 41], (2) reduction of receptor on cell surface and (3) binding to the Fcγ receptors on effector cells to cause tumour cell killing [4, 22]. In particular, a novel humanized monoclonal antibody, pertuzumab (Perjeta®) developed by Genentech with the approval from the US Food and Drug Administration (FDA), was used in combination with trastuzumab and docetaxel for treating metastatic HER2-overexpressing breast cancer [39]. Mechanistically, pertuzumab can bind to domain II of HER2 and sterically block its dimerization with other HER members [17]. On the other hand, the pertuzumab Fab fragment lacking the Fc region blocked phosphorylation of HER2/HER3 heterodimer. Since both intact pertuzumab and its Fab fragment inhibited tumour growth, it has been suggested that the presence of the Fc region is not necessary for growth inhibition of tumour cells [2].

Antibody fragments can be used in many therapeutic and diagnostic applications as a substitute for large intact antibodies, especially when the presence of the crystallizable region (Fc region) is not critical for biological activities, such as inactivation of cytokines, blocking of receptors and neutralization of viruses [21, 44]. Single chain variable fragments (ScFv), representing one of the most common types of antibody fragments, consist of the variable regions of the heavy and light chains of antibodies [6]. Because of their small size (26–28 kDa), ScFv molecules often have effective biodistribution and better tumour penetration in comparison with intact antibodies or other types of antibody fragments [51, 52].

Recently, a number of ScFv molecules have been successfully developed and produced using various gene expression systems [18, 32]. *Escherichia coli* is a bacterial host suitable for expressing small non-glycosylated recombinant antibody fragments, including ScFv [19, 43]. Using this expression system, high antibody titres up to 2 g/l can be achieved [10]. Owing to these advantages, the production of ScFv in *E. coli* can represent an effective bioprocess for large-scale manufacturing of antibody fragments.

In addition to monoclonal antibodies, the therapeutic potential of antibody fragments for treating HER2-related cancers has also been reported. Several versions of ScFv with different epitopes have been developed to target HER2 receptor. For example, tumour targeting for an engineered anti-p185$^{HER\text{-}2}$ (ScFv-CH3)$_2$ antibody fragment in athymic mice bearing MCF7/HER2 xenografts has been evaluated [33]. However, up to now, no ScFv targeting HER2 has been approved by the US FDA, although a few them are under clinical trials, such as bispecific ScFv (HER2 and HER3, phase II) [13] and HER2-targeted doxorubicin liposome (phase I) [46].

The aim of this study was to derive the ScFv version of pertuzumab and to express it using the bacterial expression system of *E. coli*. The biological activity of the expressed and purified pertuzumab ScFv was evaluated and its potential as an anticancer therapeutic candidate for targeting tumour cells overexpressing the HER2 receptor was demonstrated.

Materials and methods

Bacterial strains, plasmid, and reagents

Escherichia coli XL1-Blue (Stratagene, La Jolla, CA, USA) was used for molecular cloning and BL21 (DE3) (Novagen, Madison, WI, USA) was used as a host for ScFv expression. The pET22b(+) vector (Novagen, Madison, WI, USA) was used to clone the ScFv gene whose expression was under the control of the T7 promoter. Chemicals and reagents used in this study were of analytical grade and obtained from Merck (Darmstadt, Germany).

Cell culture

The human breast carcinoma cell lines BT-474 and MDA-MB-231 were purchased from the Pasture Institute of Iran, Tehran. They were grown in Roswell Park Memorial Institute (RPMI) 1,640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Cloning of the *anti-her2his-ScFv* gene

The *anti-her2his-ScFv* gene was designed based on the amino acid sequences of the variable heavy (VH) and light (VL) chains of pertuzumab using the optimum codons for expression in *E. coli*. The vector containing the synthesized *anti-her2his-ScFv* gene (provided by Integrated DNA Technologies, Coralville, USA) was used as the template for polymerase chain reaction (PCR) to append the *Nde*I and *Xho*I restriction sites for cloning using the primer pair of 5′ CATATGGAAGTGCAGCTGGTCGAATC 3′ (forward) and 5′ CTCGAGGGTACGTTTGATTTCCACC 3′ (reverse). PCR was performed in a thermal cycler (Bio-Rad, Hercules, CA, USA) with the following temperature profile: (1) initial DNA denaturation at 94 °C for 5 min, (2) 30 PCR cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min, (3) extension period at 72 °C for 15 min. The PCR product containing the *anti-her2his-ScFv* gene (741 bp) was restrictionally digested with *Nde*I and *Xho*I, gel-extracted using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), and then cloned into the

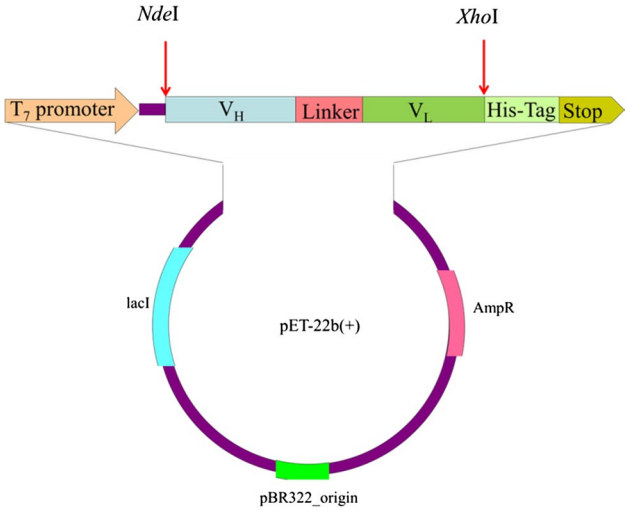

Fig. 1 Schematic diagram of the pET22b (*anti-her2his-ScFv*) expression vector. The position of the enzymatic cleavage is indicated by the *red arrow*. The gene encoding anti-her2his-ScFv protein was inserted into the pET22b vector under the control of the T7lac promoter, in frame with a histidine (6× His) tag. (Colour figure online)

pET22b vector using the corresponding restriction sites to form the expression plasmid pET22b(*anti-her2his-ScFv*) (Fig. 1). *E. coli* XL1-Blue was transformed with the ligation mixture using ampicillin for selection. The cloned *anti-her2his-ScFv* gene was sequenced to ensure no mutations and to confirm the correct frame for expression. For protein detection and purification purposes, a hexa-histidine tag was fused with the C terminus of the expressed anti-her2his-ScFv.

Expression of the anti-her2his-ScFv

E. coli BL21 (DE3) was transformed with the expression plasmid pET22b (*anti-her2his-ScFv*). Cultivation of BL21 (DE3) harbouring pET22b (*anti-her2his-ScFv*) started with a single colony, which was inoculated in 150 ml Luria–Bertani (LB) broth containing 100 μg/ml ampicillin, and the culture was shaken at 37 °C and 180 rpm until the cell density (OD_{600}) reached 0.4–0.6. Then, the expression of anti-her2his-ScFv was induced by adding 1 mM IPTG and the induced culture was shaken for another 2 h under the same conditions. For optimization of anti-her2his-ScFv expression, cultivations were performed under different IPTG concentrations (0.25, 0.5, 1 or 2) and temperatures (25, 30 or 37 °C). At the final expression time, cell density of each sample (OD_{600}) was measured. The cells were harvested by centrifugation at 7,500*g* for 10 min and the pellet was resuspended in a phosphate buffer (50 mM NaH_2PO_4 and 0.5 M NaCl, pH 8.0). Then, cells were treated with three freeze/thaw cycles. For cell lysis, cells were incubated in the presence of 1 mg/ml lysozyme (Cinagene, Tehran, Iran) on ice for 1 h. This lysate was further incubated in the presence of 10 U/ml benzonase (Sigma, St Louis, MO, USA) for 15 min at 25 °C to eliminate DNA and RNA. The soluble and insoluble fractions of the cell lysate were separated by centrifugation at 7,500*g* for 10 min and were stored at −20 °C for future analysis. The total protein assay was conducted according to a published protocol [7].

Purification and refolding of anti-her2his-ScFv

The expressed anti-her2his-ScFv protein was purified with the Ni-NTA affinity chromatography (Invitrogen, Carlsbad, CA, USA) under the following three conditions as described by the manufacturer with some modifications.

Purification under native conditions

The soluble fraction of the lysate was applied to a chromatographic column containing Ni-NTA agarose resin for incubation at 25 °C for 1 h with gentle agitation. The column was washed four times with the native wash buffer (50 mM NaH_2PO_4, 0.5 M NaCl and 20 mM imidazole, pH 8.0), and anti-her2his-ScFv was then eluted using the native elution buffer (50 mM NaH_2PO_4, 0.5 M NaCl and 150 mM imidazole, pH 8.0).

Purification under denaturing conditions

The insoluble fraction containing misfolded anti-her2his-ScFv was dissolved in a denaturing buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8) for incubation for 30 min at 37 °C and 180 rpm. Cell debris was removed by centrifugation at 7,500*g* for 10 min. The supernatant was injected into a chromatographic column containing Ni-NTA agarose resin for incubation at 25 °C for 1 h with gentle agitation. The column was washed twice with the denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8), twice with the denaturing wash buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.0) and finally twice with the denaturing wash buffer (pH 5.3), anti-her2his-ScFv was eluted from the column using the denaturing elution buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4). The solubilized anti-her2his-ScFv was refolded through dialysis using a dialysis membrane with 12,000 molecular weight cut-off (MWCO) against a dialysis buffer (10 mM Tris, 0.1 % Triton X-100, pH 5.5) at 4 °C to remove urea. After dialysis, misfolded protein aggregates were eliminated by centrifugation. The refolding efficiency was defined as the ratio of biological activity (based on the ELISA described below) of the refolded insoluble fraction to that of the soluble fraction.

Purification under hybrid conditions

To purify anti-her2his-ScFv from total cellular protein a combination of denaturing and native methods was used. Briefly, bacterial pellet was lysed in a denaturing buffer containing 6 M guanidine hydrochloride incubated for 1 h at 37 °C and 180 rpm. Cell debris was removed by centrifugation at 7,500*g* for 10 min. The supernatant was applied to a chromatographic column containing Ni-NTA agarose for incubation at 25 °C for 1 h with gentle agitation. The column was washed twice with the denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8), twice with the denaturing wash buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 6.0) and then twice with the denaturing wash buffer (pH 5.3). Finally, the resin was washed with the native wash buffer (50 mM NaH_2PO_4 and 0.5 M NaCl, pH 8.0). Anti-her2his-ScFv was eluted from the column using the native elution buffer (50 mM NaH_2PO_4, 0.5 M NaCl and 150 mM imidazole, pH 8.0).

Endotoxin detection and removal

Extraction with Triton X-114 was used to remove endotoxins from the purified anti-her2his-ScFv. Briefly, Triton X-114 was added to the protein solution to a final concentration of 1 %. The sample was shaken gently at 4 °C for 30 min and then was incubated at 37 °C for 10 min. Subsequently, the sample was centrifuged at 20,000*g* and 25 °C for 10 min to form two phases and the upper phase containing anti-her2his-ScFv was collected. This extract procedure was repeated three times. The limulus amebocyte lysate (LAL) assay was conducted to measure the endotoxin level of the purified anti-her2his-ScFv using a commercial kit (Lonza, Verviers, Belgium).

SDS-PAGE and immunoblotting analysis

The protein samples were prepared in a gel loading buffer (0.25 M Tris–HCl, pH 6.8, 5 % glycerol, 5 % 2-mercaptoethanol, 3 % SDS and 0.2 mg/ml bromophenol blue) and were heated to 95 °C for 5 min to denature proteins. Proteins were then separated by SDS-PAGE (80 V for 5 % gel and 150 V for 12 % gel). The expression level and purity of protein were determined by densitometry analysis of polyacrylamide gels using TL120 software (Nonlinear Inc, Durham NC, USA). Purity was calculated as a percentage of total protein [49]. The concentration of protein was measured according to the method of Bradford [7] using bovine albumin as the standard.

For immunoblotting analysis, the separated proteins were electrophoretically transferred from the polyacrylamide gel onto a nitrocellulose membrane (100 V for 1 h). The blotted membrane was incubated in a blocking buffer containing 3 % skim milk in phosphate buffered saline/Tween 20 (TPBS) overnight at 4 °C and then probed with anti-his(C-term)-HRP antibody (Invitrogen, Carlsbad, CA, USA, 1:5,000) at 25 °C for 1 h. Subsequently, the membrane was washed with TPBS three times, reacted with enhanced chemiluminescence (ECL) Western blotting reagents (Roche, Mannheim, Germany) for 2 min and exposed to X-ray film for 15–60 s. Dot blotting was performed for the soluble fraction, insoluble fraction and total fraction of protein samples. The samples were spotted onto a nitrocellulose membrane and the membrane was incubated with a blocking buffer (3 % skim milk in TPBS) for 1 h at 25 °C. The membrane was incubated in the presence of anti-His(C-term)-HRP antibody for 1 h at 25 °C. The membranes were washed with TPBS three times and the blots were then developed with *ortho*-phenylenediamine (OPD) solution.

Enzyme-linked immunosorbent assays (ELISA)

ELISA was conducted to evaluate the biological activity for binding of anti-her2his-ScFv to HER2-overexpressing and HER2-low-expressing cells. To do this, BT-474 and MDA-MB-231 cells were seeded (4×10^5 cells/ml) in 96-well plates for attachment for 24 h. Cell monolayers were incubated with serum-free medium for 4 h and subsequently purified anti-her2his-ScFv or mouse anti-HER2 antibody (Invitrogen) as positive control was added to wells for binding for 2 h at 4 °C. Cells were rinsed three times with PBS to remove unbound anti-her2his-ScFv or mouse anti-HER2 antibody and then fixed with 4 % formalin buffer. After fixation, they were further rinsed three times with TPBS and incubated with PBS containing 1 % BSA for 2 h. Then, cells were incubated in the presence of anti-his(C-term)-HRP antibody or anti-mouse IgG (Fc specific)-HRP (Sigma) for 1 h at 25 °C. The cells were washed three times with TPBS and then 50 µl of OPD solution was added to each well for incubation for 20 min. The colour-producing reaction was stopped by addition of 25 µl of 4.5 N sulfuric acid and the absorbance was measured at 490 nm.

Results and discussion

Expression of anti-her2his-ScFv

The *anti-her2his-ScFv* gene was synthesized for its heterologous expression in *E. coli*. The gene sequence was determined on the basis of codon optimization of the amino acid sequences of the variable heavy and light chains of pertuzumab [17]. Such codon optimization can potentially increase the anti-her2his-ScFv expression level through an

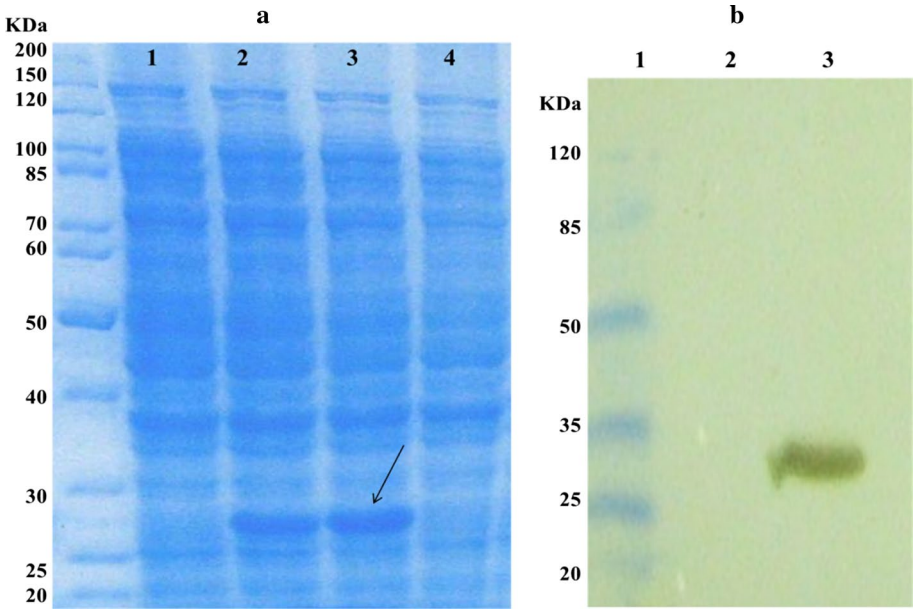

Fig. 2 **a** Proteins were separated on a 15 % SDS-PAGE gel and visualized by Coomassie brilliant blue R250 staining. *Lane 1* protein marker (Fermentase SM0661); total protein from *E. coli* BL21 (DE3) containing pET22b (*anti-her2his-ScFv*) plasmid before induction (*lane 2*) and after induction with 1 mM IPTG for 1 h at 37 °C (*lane 3*); total protein from *E. coli* BL21(DE3) containing pET22b (*anti-her2his-ScFv*) plasmid (*lane 4*) or pET22b (without insert) (*lane 5*) after induction with 1 mM IPTG for 2 h at 37 °C. The anti-her2his-ScFv (27 kDa) is denoted by an *arrow*. **b** Western blot analysis with anti-His(C-term) antibody: *lane 1* prestained protein marker (Thermo Scientific, 26612); total protein from *E. coli* BL21 (DE3) containing pET22b (*anti-her2his-ScFv*) plasmid before induction (negative control) *(lane 2*) and after induction with 1 mM IPTG for 2 h at 37 °C (*lane 3*)

improved translational efficiency [45]. The variable heavy and light chains of the antibody can be joined by a linker in either the VH-linker-VL or VL-linker-VH orientation. As more amino acids involved in the binding of pertuzumab to HER2 are located in variable heavy chains [17], the *anti-her2his-ScFv* gene was designed in such a way that VH was located in the N terminal (Fig. 1). On the other hand, the polyhistidine tag was located in the C terminal to preserve the binding ability of anti-her2his-ScFv. Other studies showed that domain arrangement can potentially influence expression [30], stability and antigen binding ability of the antibody gene product [14].

We used *E. coli* BL21(DE3) as a host cell and the two knockouts of the protease genes of *ompT* and *lon* can potentially minimize protein degradation during expression and purification stages. The expression of the *anti-her2his-ScFv* gene in *E. coli* BL21(DE3) was regulated by the strong T7 promoter system. Results of cultivation for anti-her2his-ScFv expression at 37 °C are summarized in Fig. 2. The envisioned molecular weight of anti-her2his-ScFv was approximately 27 kDa, which was confirmed by SDS-PAGE analysis (Fig. 2a). Western blotting analysis using the anti-his(C-terminal) antibody indicated that expressed anti-her2his-ScFv was a his-tagged fusion protein (Fig. 2b). After cell disruption, samples of total cell, soluble fraction and insoluble fraction were analysed by SDS-PAGE (Fig. 3a) and immune-dot blotting (Fig. 4). While the level of total expressed anti-her2his-ScFv could reach as high as 30 % of total cellular protein, most of it was detected in the insoluble fraction with a potentially minimum biological activity. Heterologous expression of recombinant proteins in a reducing environment of the cytoplasm of *E. coli* is often more effective than the expression in an oxidative environment of the periplasm though the overexpressed gene products tend to aggregate as inclusion bodies [12, 31]. Nevertheless, inclusion bodies are often more resistant to proteolysis by *E. coli* proteases and such an expression strategy can facilitate downstream processing while allowing high yields of protein production. Other studies also reported similar observations. For example, Zhang et al. [53] reported that the majority of anti-HA (hemagglutinin antigen of avian influenza virus) ScFv (constituting approximately 35 % of total cellular protein) was expressed in an insoluble form. A reason for misfolding of recombinant proteins is associated with the inability of forming disulfide bond(s) in the reducing cytoplasm of *E. coli*. Markiv et al. [29] reported soluble expression and isolation of antibody fragment–fluorophore conjugates using an *E. coli* expression host, i.e. Rosetta gami B(DE3), which carries the two gene knockouts of *gor/trxB* to transform the cytoplasm to an oxidative environment.

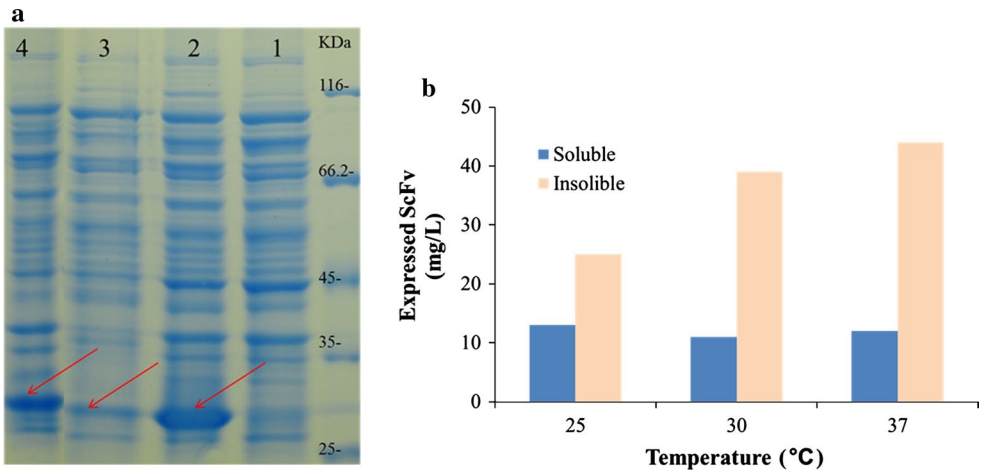

Fig. 3 **a** Proteins were separated on a 12 % SDS-PAGE gel and visualized by Coomassie brilliant blue R250 staining. Total protein from *E. coli* BL21 (DE3) containing pET22b (without insert) (*lane 1*) or pET22b (*anti-her2his-ScFv*) plasmid (*lane 2*) after induction with 1 mM IPTG for 2 h at 37 °C; soluble (*lane 3*) and insoluble (*lane 4*) fraction from *E. coli* BL21 (DE3) containing pET22b (*anti-her2his-ScFv*) plasmid after induction with 1 mM IPTG for 2 h at 37 °C. **b** Comparison between the amount of soluble and insoluble anti-her2his-ScFv expressed after induction with 1 mM IPTG for 2 h at the three temperatures. The protein quantification was performed by densitometry analysis using TL120 software. The band intensities of expressed ScFv were analysed and normalized using a standard of bovine serum albumin

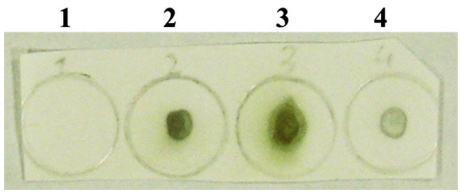

Fig. 4 Dot-blot assay by anti-His (C-term) antibody. *1* negative control (whole cell from *E. coli* BL21 (DE3) containing pET22b (without insert) after induction with 1 mM IPTG for 2 h at 37 °C); *2* insoluble fraction, *3* whole cell and *4* soluble fraction from *E. coli* BL21 (DE3) containing pET22b (*anti-her2his-ScFv*) after induction with 1 mM IPTG for 2 h at 37 °C

Similar to most other studies for recombinant protein production, two major factors affecting the expression performance of anti-her2his-ScFv are inducer concentration and temperature. Depending on various host/vector systems, the IPTG concentration used for induction and cultivation temperatures can range widely from 0.005 to 5 mM [50] and 24 to 37 °C [35], respectively. Larentis et al. [24] reported improved expression of mature rPsaA in recombinant *E. coli* with a low IPTG concentration for induction. Lim et al. [25] studied the induction conditions for anti-exotoxin ScFv expression and observed that higher IPTG concentrations (>1 mM) tended to inhibit ScFv expression, presumably because of an increased metabolic load and induction of bacterial proteases degrading heterologously expressed proteins. Volontè et al. [42] reported that optimal expression of recombinant protein occurred at a growth temperature of 37 °C with IPTG addition at the middle-exponential growth phase. On the other hand, Santala and Lamminmäki [37] observed that expression of anti-thyroid stimulating hormone (TSH) ScFv in *E. coli* Origami B was optimal at a low temperature of 24 °C. These studies suggest the significant variance of culture conditions for different host/vector systems. The present study showed that optimal production of anti-her2his-ScFv occurred when 1 mM IPTG was used with a cultivation temperature of 37 °C (Fig. 5). The effect of temperature on the expression of anti-her2his-ScFv is summarized in Fig. 3b. While the ratio of soluble to insoluble ScFv was highest at a low temperature of 25 °C, the expression of total ScFv (both soluble and insoluble fractions) increased with temperature. Since several *E. coli* chaperones are actively expressed at low temperatures [16], the stability and folding of recombinant ScFv were enhanced at 25 °C. However, cell growth appeared to be retarded, resulting in a decreased volumetric protein concentration.

In light of the high-level expression but significant formation of protein aggregates of the expressed anti-her2his-ScFv, we proceeded with various downstream processing strategies for purification and refolding for maximum recovery of biologically active antibody.

Purification, refolding, and endotoxin removal of anti-her2his-ScFv

Purification of expressed anti-her2his-ScFv was conducted using Ni-NTA affinity chromatography under the native, denaturing and hybrid conditions and the purification

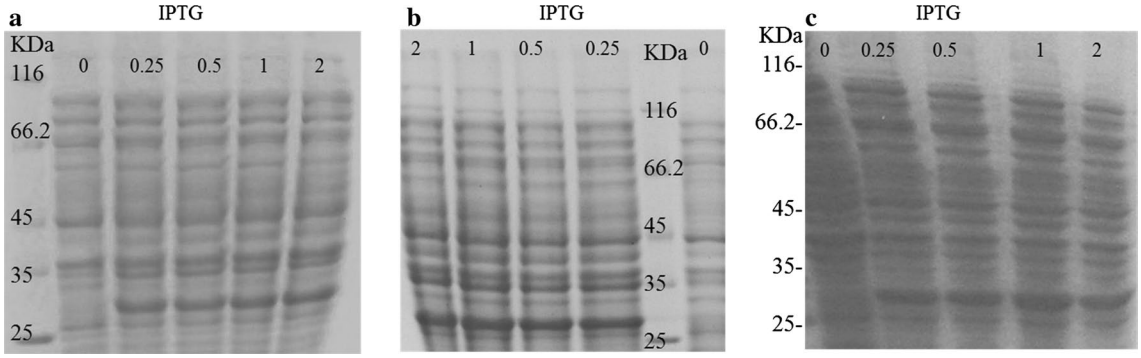

Fig. 5 The effects of incubation temperature and IPTG concentration (mM) on protein expression. Proteins were separated on a 12 % SDS-PAGE gel and visualized by Coomassie brilliant blue R250 staining. Protein expression was induced with different concentration of IPTG (mM) at **a** 25 °C, **b** 30 °C and **c** 37 °C for 2 h

Table 1 Summary of the yields of anti-her2his-ScFv protein during procedures of purification, refolding, and endotoxin removal

Procedure	ScFv (mg)	Purity (%)	Yield (%)	Relative activity[f]
Whole cell lysate[a]	2.8	34	100	–
Soluble protein	0.6	7	21.4	–
Insoluble protein	2.2	30	78.6	–
(1) Hybrid NIT purification[a]	1	97	35.7	77
(2) Native NIT purification[b]	0.25	95	41.7	100
(3a) Denaturing NIT purification[c]	1.2	95	54.5	–
(3b) Dialysis[d]	0.9	96	75.0	87
(3c) Endotoxin removal[e]	0.8	96	88.9	86

[a] From pellet obtained from 50 ml cell culture

[b] From soluble fraction

[c] From insoluble fraction

[d] After denaturing NIT purification

[e] Only the NIT-purified sample under denaturing conditions was processed for endotoxin removal

[f] Relative activity = binding activity of refolded protein/binding activity of native protein purified from soluble fraction × 100 %

performance is summarized in Table 1. SDS-PAGE and densitometry analyses show that all three approaches led to satisfactory purification performance with a high purity up to 97 % (Fig. 6). While most of the expressed anti-her2his-ScFv accumulated in the insoluble fraction, most of it can be recovered by Ni-NTA affinity chromatography since the yield of this purification step was approximately 54.5 % under the denaturing conditions. On the other hand, conducting Ni-NTA affinity chromatography under the hybrid conditions might be unfavourable as a result of its low yield of 35.7 %. The yield of Ni-NTA affinity chromatography under the native conditions at only 41.7 % was similar to but relatively lower than that under the denaturing conditions, implying that the binding affinity might be affected by the folding/unfolding state of anti-her2his-ScFv. Protein concentrations were measured using the Bradford assay (Table 1) and the data were in agreement with the band intensity analysis. As a result, significantly pure anti-her2his-ScFv can be obtained using one-step Ni-NTA affinity chromatography under both native and denaturing conditions and the overall yield of anti-her2his-ScFv from both soluble and insoluble fractions was approximately 29 mg based on processing of 1 l *E. coli* culture (Table 1). Bu et al. [9] reported expression of anti-brain natriuretic peptide ScFv using a similar approach to purify ScFv under denaturing conditions with a final yield of 10.2 mg/l of culture. Woestenenk et al. [48] reported that only 10 out of 20 his-tagged human proteins expressed in *E. coli* could be purified under native conditions and most proteins could be purified under denaturing conditions. These observations, along with ours, suggest that hexa-histidine epitope tag might not be accessible for all recombinant his-tagged protein molecules depending on their folding/unfolding state and denatured forms might have better accessibility.

Although the expression level of recombinant ScFv in *E. coli* can be potentially high, refolding of ScFv to

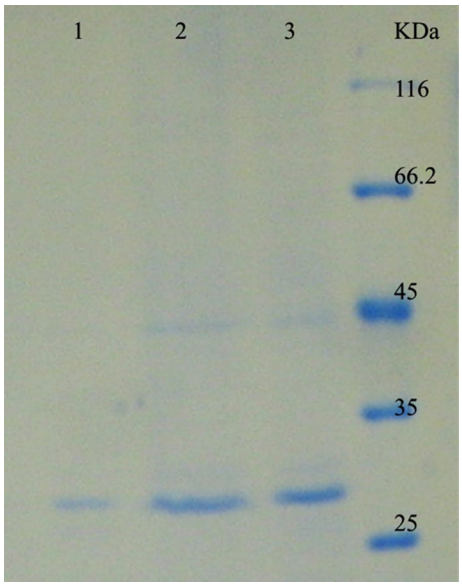

Fig. 6 SDS-PAGE analysis of the purified his-tagged ScFv using a Ni-NTA column. Proteins were separated on a 15 % SDS-PAGE gel and visualized by Coomassie brilliant blue R250 staining. *Lane 1* soluble protein purified under native conditions; *lane 2* total protein purified under hybrid conditions and *lane 3* insoluble protein purified under denaturing conditions

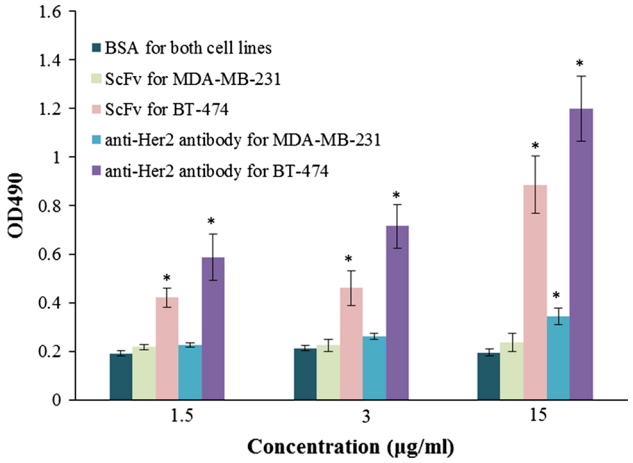

Fig. 7 Binding assay of anti-her2his-ScFv protein, mouse anti-HER2 antibody (Clone TAB 250, Invitrogen) as positive control and bovine serum albumin (BSA) as a negative control to BT-474 and MDA-MB-231 cells utilizing ELISA. The *vertical bars* indicate the standard deviations ($n = 6$). *Asterisks* indicate the means which were significantly different ($P < 0.05$) from the negative control

develop its biological activity can be a challenging and complex process [20]. Nevertheless, this ScFv production strategy can be rather effective since inclusion bodies are easily recovered and purified with a high yield. In the present study, two refolding approaches were explored. First, anti-her2his-ScFv was purified under the denaturing conditions and the denatured anti-her2his-ScFv was refolded via dialysis by slowly reducing the urea concentration of the protein solution. It was observed that the pH of dialysis buffer could affect the efficacy of refolding. Neutral pH 7.5 resulted in protein precipitation, whereas acidic pH 5.5 led to less precipitation with a 25 % product loss for this step (Table 1). Note that we performed dialysis without reducing agents (e.g. 2-mercaptoethanol, dithiothreitol or glutathione) though these reducing agents can potentially influence the refolding yield [11]. Alternatively, anti-her2his-ScFv was refolded while attaching on Ni-NTA agarose resin in an affinity chromatographic column under the hybrid conditions. A similar approach was previously conducted for purification and refolding of anti-tumour necrosis factor (TNF) ScFv with a high yield and purity [26]. Such an on-column refolding strategy can minimize protein aggregation associated with the exposure of hydrophobic surfaces by reducing the interaction between protein molecules during the refolding process.

Endotoxin is one of the most common contaminants in therapeutic proteins produced in gram-negative bacteria such as *E. coli*. These lipopolysaccharide (LPS) molecules are stable in a wide range of temperature and pH, making this cleaning step rather challenging. Several methods have been developed to eliminate endotoxin from therapeutic proteins, including two-phase separation, ultrafiltration, LPS affinity resins, membrane absorbers, hydrophobic interaction and ion exchange chromatography [28]. The effectiveness of these methods is related to the properties of therapeutic protein of interest [28]. In this study, extraction with Triton X-114 was applied to reduce endotoxin from purified anti-her2his-ScFv. Triton X-114 treatment successfully decreases the level of endotoxin by more than 10,000-fold (from 3,000 to 0.3 EU/mg) with approximately 90 % protein yield and normal biological activity (Table 1). In contrast to our approach, Abéngozar et al. [1] observed a slightly better removal efficiency using polymyxin B affinity chromatography with an endotoxin level of 0.05 EU/ml for the purified anti-ephrinB2 ScFv.

Biological activity of anti-her2his-ScFv

To evaluate the biological activity of purified anti-her2his-ScFv, the binding activity of anti-her2his-ScFv proteins to HER2-overexpressing cells BT-474 and HER2-low-expressing cells MDA-MB-231 were analysed by cell-based ELISA and the results are summarized in Fig. 7. The recognition and binding of purified anti-her2his-ScFv to BT-474 cells was clearly demonstrated, and the binding was dose-dependent. Anti-her2his-ScFv can bind to MDA-MB-231 at a lower level than BT-474 cells, demonstrating the specificity of anti-her2his-ScFv to HER2. Such a difference in antigen–antibody interaction was more observable when

a high concentration of anti-her2his-ScFv was used. Note that BT-474 and MDA-MB-231 cells express 7.6×10^5 and 2.8×10^4 copies of HER2, respectively, on the cell surface [34]. A similar observation was previously reported for anti-HER2 ScFv (ScFv800E6) produced in a yeast expression system with a high binding affinity to HER2-positive MCF7 breast cancer cells but no binding affinity to HER2-negative MDA cells [40]. In another study, in vitro specificity of pertuzumab to uterine serous papillary adenocarcinoma cells with high expression of HER2 was reported [15]. Our results suggest that anti-her2his-ScFv retains the affinity and specificity of its parental antibody, pertuzumab. ScFvs molecules often have two disulfide bonds for stabilizing the molecular structure. While these disulfide bonds seldom formed in the reducing cytoplasm of *E. coli*, certain ScFvs expressed in *E. coli* can have a stable folding structure without the aiding effect of the disulfide bonds depending on the primary sequence [47]. Functional expression of biologically active anti-HER2 ScFv (ScFv800E6) in a bacterial reducing environment has also been reported [27]. We also compared the biological activity of anti-her2his-ScFv purified under denaturation/refolding and native conditions (Table 1). The efficiencies of refolding, defined as relative bioactivities, for the dialysis and on-column approaches were 87 and 77 %, respectively. These results suggest that, even though anti-her2his-ScFv might not be solubly expressed in *E. coli*, the in vitro refolding is a feasible approach for the production and purification of biologically active anti-her2his-ScFv.

Conclusion

In this study, we reported an effective strategy for the expression of the ScFv version of pertuzumab specific to domain II of the HER2 receptor (i.e. anti-her2his-ScFv) in *E. coli* followed by one-step affinity chromatography for purification. While soluble expression was observed to certain extents, the overexpressed anti-her2his-ScFv primarily aggregated as inclusion bodies which can be recovered and refolded in vitro during the chromatographic processing. Most importantly, the purified anti-her2his-ScFv showed a promising bioactivity with a selective binding affinity towards HER2-overexpressing tumour cells. The molecular size of the anti-her2his-ScFv of 27 kDa is only one-sixth of the intact antibody of pertuzumab. For therapeutic and diagnostic applications, such a small size can potentially facilitate molecular delivery of anti-her2his-ScFv into the blood circulation systems surrounding tumour cells.

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References

1. Abéngozar MA, de Frutos S, Ferreiro S, Soriano J, Perez-Martinez M, Olmeda D, Marenchino M, Cañamero M, Ortega S, Megias D (2012) Blocking ephrinB2 with highly specific antibodies inhibits angiogenesis, lymphangiogenesis, and tumor growth. Blood 119(19):4565–4576
2. Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, Lofgren JA, Tindell C, Evans DP, Maiese K (2002) Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell 2(2):127–137
3. Bazley L, Gullick W (2005) The epidermal growth factor receptor family. Endocr Relat Cancer 12(Suppl 1):S17–S27
4. Ben-Kasus T, Schechter B, Lavi S, Yarden Y, Sela M (2009) Persistent elimination of ErbB-2/HER2-overexpressing tumors using combinations of monoclonal antibodies: relevance of receptor endocytosis. Proc Natl Acad Sci 106(9):3294–3299
5. Berchuck A, Kamel A, Whitaker R, Kerns B, Olt G, Kinney R, Soper J, Dodge R, Clarke-Pearson D, Marks P (1990) Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 50(13):4087–4091
6. Bird R, Hardman K, Jacobson J, Johnson S, Kaufman B, Lee S, Lee T, Pope S, Riordan G, Whitlow M (1988) Single-chain antigen-binding proteins. Science 242(4877):423
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1):248–254
8. Brandt BH, Roetger A, Dittmar T, Nikolai G, Seeling M, Merschjann A, Nofer JR, Dehmer-Möller G, Junker R, Assmann G (1999) c-erbB-2/EGFR as dominant heterodimerization partners determine a motogenic phenotype in human breast cancer cells. FASEB J 13(14):1939–1949
9. Bu D, Zhou Y, Tang J, Jing F, Zhang W (2013) Expression and purification of a novel therapeutic single-chain variable fragment antibody against BNP from inclusion bodies of *Escherichia coli*. Protein Expr Purif 92(2):203–207
10. Chen C, Snedecor B, Nishihara J, Joly J, McFarland N, Andersen D, Battersby J, Champion K (2004) High-level accumulation of a recombinant antibody fragment in the periplasm of *Escherichia coli* requires a triple-mutant (degP prc spr) host strain. Biotechnol Bioeng 85(5):463–474
11. Chen L-H, Huang Q, Wan L, Zeng L-Y, Li S-F, Li Y-P, Lu X-F, Cheng J-Q (2006) Expression, purification, and in vitro refolding of a humanized single-chain Fv antibody against human CTLA4 (CD152). Protein Expr Purif 46(2):495–502
12. Cornelis P (2000) Expressing genes in different *Escherichia coli* compartments. Curr Opin Biotechnol 11(5):450–454
13. Denlinger C, Beeram M, Tolcher A, Goldstein L, Slichenmyer W, Murray J, Mcdonagh C, Andreas K, Moyo V (2010) A phase I/II and pharmacologic study of MM-111 in patients with advanced, refractory HER2-positive (HER2+) cancers. J Clin Oncol 28:169
14. Desplancq D, King DJ, Lawson AD, Mountain A (1994) Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. Protein Eng 7(8):1027–1033
15. El-Sahwi K, Bellone S, Cocco E, Cargnelutti M, Casagrande F, Bellone M, Abu-Khalaf M, Buza N, Tavassoli F, Hui P (2009) In vitro activity of pertuzumab in combination with trastuzumab in uterine serous papillary adenocarcinoma. Br J Cancer 102(1):134–143
16. Ferrer M, Chernikova TN, Yakimov MM, Golyshin PN, Timmis KN (2003) Chaperonins govern growth of *Escherichia coli* at low temperatures. Nat Biotechnol 21(11):1266–1267
17. Franklin M, Carey K, Vajdos F, Leahy D, de Vos A, Sliwkowski M (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell 5(4):317–328

18. Furuta T, Ogawa T, Yamaji H (2012) Production of antibody fragments using the baculovirus-insect cell system. Method Mol Biol (Clifton, NJ) 907:371
19. Guo J, You S, Li L, Zhang Y, Huang J, Zhang C (2003) Construction and high-level expression of a single-chain Fv antibody fragment specific for acidic isoferritin in *Escherichia coli*. J Biotechnol 102(2):177–189
20. Hoyer W, Ramm K, Pluckthun A (2002) A kinetic trap is an intrinsic feature in the folding pathway of single-chain Fv fragments. Biophys Chem 96(2–3):273–284
21. Hudson P, Souriau C (2003) Engineered antibodies. Nat Med 9(1):129–134
22. Keler T, Graziano RF, Mandal A, Wallace PK, Fisher J, Guyre PM, Fanger MW, Deo YM (1997) Bispecific antibody-dependent cellular cytotoxicity of HER2/neu-overexpressing tumor cells by Fcγ receptor type I-expressing effector cells. Cancer Res 57(18):4008–4014
23. Kiewe P, Hasmüller S, Kahlert S, Heinrigs M, Rack B, Marmé A, Korfel A, Jäger M, Lindhofer H, Sommer H (2006) Phase I trial of the trifunctional anti-HER2× anti-CD3 antibody ertumaxomab in metastatic breast cancer. Clin Cancer Res 12(10):3085–3091
24. Larentis AL, Argondizzo APC, Esteves GdS, Jessouron E, Galler R, Medeiros MA (2011) Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expr Purif 78(1):38–47
25. Lim K-P, Li H, Nathan S (2004) Expression and purification of a recombinant scFv towards the exotoxin of the pathogen *Burkholderia pseudomallei*. J Microbiol 42(2):126–132
26. Liu M, Wang X, Yin C, Zhang Z, Lin Q, Zhen Y, Huang H (2006) One-step on-column purification and refolding of a single-chain variable fragment (scFv) antibody against tumour necrosis factor α. Biotechnol Appl Biochem 43(3):137–145
27. Lombardi A, Sperandei M, Cantale C, Giacomini P, Galeffi P (2005) Functional expression of a single-chain antibody specific for the HER2 human oncogene in a bacterial reducing environment. Protein Expr Purif 44(1):10–15
28. Magalhães PO, Lopes AM, Mazzola PG, Rangel-Yagui C, Penna T, Pessoa A Jr (2007) Methods of endotoxin removal from biological preparations: a review. J Pharm Pharm Sci 10(3):388–404
29. Markiv A, Beatson R, Burchell J, Durvasula RV, Kang AS (2011) Expression of recombinant multi-coloured fluorescent antibodies in gor-/trxB-*E. coli* cytoplasm. BMC Biotechnol 11(1):117
30. Merk H, Stiege W, Tsumoto K, Kumagai I, Erdmann VA (1999) Cell-free expression of two single-chain monoclonal antibodies against lysozyme: effect of domain arrangement on the expression. J Biochem 125(2):328–333
31. Moore JT, Uppal A, Maley F, Maley G (1993) Overcoming inclusion body formation in a high-level expression system. Protein Expr Purif 4(2):160–163
32. Nettleship JE, Flanagan A, Rahman-Huq N, Hamer R, Owens RJ (2012) Converting monoclonal antibodies into Fab fragments for transient expression in mammalian cells. Method Mol Biol (Clifton, NJ) 801:137
33. Olafsen T, Tan GJ, Cheung C-W, Yazaki PJ, Park JM, Shively JE, Williams LE, Raubitschek AA, Press MF, Wu AM (2004) Characterization of engineered anti-p185HER-2 (scFv-CH3) 2 antibody fragments (minibodies) for tumor targeting. Protein Eng Des Sel 17(4):315–323
34. Robinson MK, Hodge KM, Horak E, Sundberg ÅL, Russeva M, Shaller CC, von Mehren M, Shchaveleva I, Simmons HH, Marks JD (2008) Targeting ErbB2 and ErbB3 with a bispecific single-chain Fv enhances targeting selectivity and induces a therapeutic effect in vitro. Br J Cancer 99(9):1415–1425
35. Rodríguez-Carmona E, Cano-Garrido O, Dragosits M, Maurer M, Mader A, Kunert R, Mattanovich D, Villaverde A, Vázquez F (2012) Recombinant Fab expression and secretion in *Escherichia coli* continuous culture at medium cell densities: influence of temperature. Process Biochem 47(3):446–452
36. Rubin I, Yarden Y (2001) The basic biology of HER2. Ann Oncol 12(Suppl 1):S3–S8
37. Santala V, Lamminmäki U (2004) Production of a biotinylated single-chain antibody fragment in the cytoplasm of *Escherichia coli*. J Immunol Methods 284(1):165–175
38. Schmitz KR, Ferguson KM (2009) Interaction of antibodies with ErbB receptor extracellular regions. Exp Cell Res 315(4):659–670
39. Sheridan C (2012) FDA approves pertuzumab. Nat Biotechnol 30(7):570
40. Sommaruga S, Lombardi A, Salvadè A, Mazzucchelli S, Corsi F, Galeffi P, Tortora P, Prosperi D (2011) Highly efficient production of anti-HER2 scFv antibody variant for targeting breast cancer cells. Appl Microbiol Biotechnol 91(3):613–621
41. Vasconcellos FA, Aleixo PB, Stone SC, Conceição FR, Dellagostin OA, Aleixo JAG (2012) Generation and characterization of new HER2 monoclonal antibodies. Acta Histochem 115(3):240–244
42. Volontè F, Piubelli L, Pollegioni L (2011) Optimizing HIV-1 protease production in *Escherichia coli* as fusion protein. Microb Cell Fact 10:53
43. Wang H, Dai J, Li B, Fan K, Peng L, Zhang D, Cao Z, Qian W, Zhao J (2008) Expression, purification, and characterization of an immunotoxin containing a humanized anti-CD25 single-chain fragment variable antibody fused to a modified truncated Pseudomonas exotoxin A. Protein Expr Purif 58(1):140–147
44. Weisser NE, Hall JC (2009) Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. Biotechnol Adv 27(4):502–520
45. Welch M, Govindarajan S, Ness JE, Villalobos A, Gurney A, Minshull J, Gustafsson C (2009) Design parameters to control synthetic gene expression in *Escherichia coli*. PLoS One 4(9):e7002
46. Wickham T, Futch K (2012) A phase I study of MM-302, a HER2-targeted liposomal doxorubicin. Patients with advanced, HER2-positive breast cancer. Cancer Res 72:P5–P18
47. Woërn A, Pluëckthun A (1998) An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. FEBS Lett 427(3):357–361
48. Woestenenk EA, Hammarström M, van den Berg S, Härd T, Berglund H (2004) His tag effect on solubility of human proteins produced in *Escherichia coli*: a comparison between four expression vectors. J Struct Funct Genomics 5(3):217–229
49. Wu X, Zhang L, Zhang J, Zhang C, Zhu L, Shi Y (2008) Recombinant early secreted antigen target 6 protein as a skin test antigen for the specific detection of *Mycobacterium tuberculosis* infection. Clin Exp Immunol 152(1):81–88
50. Xie L, Hall D, Eiteman M, Altman E (2003) Optimization of recombinant aminolevulinate synthase production in *Escherichia coli* using factorial design. Appl Microbiol Biotechnol 63(3):267–273
51. Yazaki P, Kassa T, Cheung C, Crow D, Sherman M, Bading J, Anderson A, Colcher D, Raubitschek A (2008) Biodistribution and tumor imaging of an anti-CEA single-chain antibody–albumin fusion protein. Nucl Med Biol 35(2):151–158
52. Yokota T, Milenic D, Whitlow M, Schlom J (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res 52(12):3402
53. Zhang T, Wang C, Zhang W, Gao Y, Yang S, Wang T, Zhang R, Qin C, Xia X (2010) Generation and characterization of a fusion protein of single-chain fragment variable antibody against hemagglutinin antigen of avian influenza virus and truncated protamine. Vaccine 28(23):3949–3955