**Purification optimization for a recombinant single-chain variable
fragment against type 1 insulin-like growth factor receptor (IGF-1R)
by using design of experiment (DoE)**

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Abstract

Design of experiment (DoE) is a statistics-based technique for experimental design that could overcome the shortcomings of traditional one-factor-at-a-time (OFAT) approach for protein purification optimization. In this study, a DoE approach was applied for optimizing purification of a recombinant single-chain variable fragment (scFv) against type 1 insulin-like growth factor receptor (IGF-1R) expressed in *E. coli*. In first capture step using Capto L, a 2-level fractional factorial analysis and successively a central composite circumscribed (CCC) design were used to identify the optimal elution conditions. Two main effects, pH and trehalose, were identified, and high recovery (above 95%) and low aggregates ratio (below 10%) were achieved at the pH range from 2.9 to 3.0 with 32%-35% (w/v) trehalose added. In the second step using cation exchange chromatography, an initial screening of media and elution pH and a following CCC design were performed, whereby the optimal selectivity of the scFv was obtained on Capto S at pH near 6.0, and the optimal conditions for fulfilling high DBC and purity were identified as pH range of 5.9-6.1 and loading conductivity range of 5-12.5 mS/cm. Upon a further gel filtration, the final purified scFv with a purity of 98% was obtained. Finally, the optimized conditions were verified by a 20-fold scale-up experiment. The purities and yields of intermediate and final products all fell within the regions predicted by DoE approach, suggesting the robustness of the optimized conditions. We proposed that the DoE approach described here is also applicable in production of other recombinant antibody constructs.

Keywords: IGF-1R; ScFv; DoE; Design of experiment; Fractional factorial analysis; Central composite circumscribed design.

Introduction

Human insulin-like growth factor 1 receptor (IGF-1R) is a transmembrane receptor of insulin-like growth factor 1 (IGF-1) and IGF-2 [1]. It has been widely reported that IGF-1R is implicated in several cancers [2-3], suggesting a promising target for a wide range of cancers. The monoclonal antibodies against IGF-1R have been demonstrated to inhibit cancer cell growth [4].

Single-chain variable fragment (scFv) is a fusion protein of the variable domains of heavy chain and light chain of full-length antibody [5]. ScFv has better permeability to target site in tissues than full-length antibody because of its small size, and has been widely developed for therapeutic or diagnostic interest [5-6]. In our previous work, we have expressed a soluble bioactive scFv against IGF-1R in *E. coli* periplastic space [7]. In this study, we aimed to purify the scFv for further development.

Protein purification is a multifactorial exercise, as many factors such as sample composition, purification technique, chromatography media, binding, wash, and elution conditions all might play a role. In a traditional way for optimization of protein purification, one-factor-at-a-time (OFAT) approach is employed. Such approach involves varying one variable at a time, while keeping other variables constant. Thus it requires a number of experiments to be carried out, making this approach time-consuming and labor intensive. Moreover, it often fails to predict the genuine optimal conditions because the setup just covers a limited part of the experiment space and does not include the combined effects of all the variables

involved. These shortcomings can be overcome by design of experiment (DoE), which is a statistics-based technique for designing experiments and analyzing the information obtained [8]. The advantage of this technique is that a wide range of experimental parameters or variables could be varied systematically and simultaneously, enabling obtainment of sufficient information using minimum number of experiments. Furthermore, DoE allows detection of interactive effects of different factors, **thus ensuring the reliability and robustness of the optimal conditions obtained**. In recent years, a number of study cases have proved the high efficiency of DoE approach on protein purification optimization [8-10].

In this study, we described the optimization of purification process for a soluble scFv against IGF-IR using DoE approach to obtain the final product with high purity and recovery.

Materials and Methods

*Chemicals, chromatography **media** and equipment*

IPTG, arabinose, urea, PEG (MW 2,000), dextran, arginine, trehalose, sodium phosphate (monobasic and dibasic) and other commonly used chemicals were all purchased from Sigma–Aldrich (Shanghai, China). The chromatography **media** and prepacked columns for protein purification were all purchased from GE Healthcare (Shanghai, China). An ÄKTA Purifier 100 (GE Healthcare) was used for the purification process development.

Expression of the recombinant scFv against IGF-IR

The plasmid construction and expression of the recombinant scFv against IGF-IR

was conducted as described in our previous report [11] with an exception that the sequence encoding C-terminus 6×His tag was removed here. Briefly, the variable region of heavy chain (VH) and variable region of light chain (VL) derived from a monoclonal antibody (CP 751871) against IGF-1R [7] were connected into a scFv via a linker peptide (Gly₄Ser)₃. The cDNA encoding such scFv construct was codon optimized, synthesized, cloned into the *Nco* I-*Eco*R I site of pET-26b(+) (Novagen) and designated as pET_scFv. For soluble expression of the scFv, the pET_scFv and a vector pDsbA/C carrying two chaperone-encoding sequences (DsbA and DsbC) were co-transformed into *E. coli* BL21 (DE3) (more details can be seen in [11]). The positive transformant was inoculated into 2× YT media and grown at 30 °C until the OD₆₀₀ reached 0.5–0.8 and then induced with 0.2 % arabinose 1 h prior to the addition of 0.2 mM IPTG, following which the scFv production was typically allowed to continue for 4-10 h. Forty grams of wet weight cell pellets were suspended in 600 mL lysis buffer (20 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, pH 7.5), broken by a high pressure homogenizer (ATS, Canada) and then centrifuged. The supernatant of the cell lysate was collected and filtered by 0.22 µm membrane for further purification.

Purification optimization by using DoE in small scale

A three-step process consisting of a capture step by Capto LTM affinity chromatography and two polishing steps with cation exchange and gel filtration respectively was used to purify the target scFv. The DoE approach was applied in the first two steps. In general, an initial screening of wide range of parameters or factors,

including chromatography media, dynamic binding capacities (DBC), and wash and elution conditions, was carried out to identify the ones that have significant effects on the response, and to determine which factors need to be further optimized. Then an optimization was performed for determining optimal factor settings.

For the capture step, Capto LTM medium was chosen as it possesses specific affinity to antibody or antibody fragment with kappa domain. The DBC and elution pH range were firstly determined using a 1-mL preppacked column. The DBC at 10% breakthrough (QB10%) was determined by frontal analysis using the following parameters (column: 1-mL preppacked Capto LTM; residence time: 4 min; equilibration buffer: 20 mM sodium phosphate, 150 mM NaCl, pH 7.5; sample: purified scFv 2.5 mg/mL in equilibration buffer), and a 70% of the DBC at QB%10 was selected as loading condition in following experiments. Prior to a pH linear gradient elution from 6.0 to 2.5 performed to identify the appropriate elution pH, a washing step with five column-volumes (CV) of equilibration buffer was operated. For further optimizing the elution conditions, a 2-level fractional factorial analysis with resolution $\square$ was designed as shown in Table 1, seven factors were included as variables, and aggregates and recovery of the target protein were selected as responses. Two main factors, pH and trehalose, impacting the two responses were identified from the fractional factorial analysis, and then subjected to a central composite circumstances (CCC) analysis (see details in Table 2) to obtain the optimal experiment space of elution step. The Capto LTM elution pool was immediately diluted by 1 M Tris-Cl (pH 8.0) to neutralize the pH for temporary storage or further purification.

For the second purification step, a comparison of two cationic exchange **media**, Capto S and SP Sepharose[®] Fast Flow, mainly on the selectivity and recovery at three different **pHs**, was conducted in an initial screening phase with following loading parameters (column: 1 mL **HiTrap**; residence time: 2 min; equilibration buffer: 20 mM sodium **citrate** at pH 5.0 and 6.0, and 20 mM sodium phosphate at pH 7.0; sample: the Capto L eluent adjusted to 2 mg/mL in equilibration buffer). Accordingly, six runs, using two **media** and each at three **pHs**, were carried out. In each run, equal amount (10 mg) sample diluted in equilibration buffer was loaded, and a 5 CV washing with equilibration buffer and a 15 CV length of linear salt gradient elution from 0 to 1 M NaCl were followed. In addition, the pH values of all buffers used in each individual run were kept consistent. Based on the comparison, Capto S at pH 6.0 showed the best selectivity, thus it was selected in this step. Then, a central composite circumscribed (CCC) analysis (see details in Table 3), in which pH and conductivity were selected as variables and DBC as response, was used for loading conditions optimization by using a Tricorn[™] 5/100 column. Next, the purities of the target scFvs at all set points were also tested when 70% of the DBC was set as loading condition. The conditions where both criteria of purity and DBC were fulfilled were identified as operational space in this step.

For the final step, a gel filtration with **Superdex 75** 10/300 GL column (GE Healthcare) was used for further polishing. The running buffer is PBS and the loading volume is less than 5% CV of the column.

The experimental design and results analysis were performed by using the

software Minitab 16 (Minitab Inc., USA).

Verification of the optimized conditions in scale-up experiment

To verify the optimized conditions, the supernatant of cell lysates from 8 L cultures (250 mg target protein included) was loaded as starting sample. The column size in each purification step was accordingly scaled up to fit the change in loading amount, while other conditions remained the same as that optimized above of which the center point value of each optimal space was adopted. A Hiscale™ 16/40 column (16 mm diameter) (GE Healthcare) with Capto™ L volume of 20 mL and a Tricorn 10/100 column with Capto™ S volume of 4 mL were employed in the capture step and the cationic exchange step respectively. A Superdex 75 column of 800 mm in bed height and 16 mm in inner diameter was used in final step. Eluted peaks from all steps were collected and subjected to recovery and purity analysis.

Analytical methods

Protein quantification was performed by using Quant-iT Protein Assay Kit (Life **Technologies**). A Superdex 75™ 10/300 GL column (GE Healthcare) was used for determining aggregates and aggregate clearance. Briefly, 100 µl samples were loaded and run with PBS buffer at 0.5 mL/min, and UV absorbance at 280 nm was monitored. The percentage of aggregates was calculated as the aggregates peak area relative to the sum of the scFv peak area. Purity of the target protein was determined by using both SDS-PAGE analysis and SEC-HPLC analysis. The SEC-HPLC was performed on an Agilent 1200 HPLC system using BIOSep-SEC-S2000 column (300×7.8 mm, Phenomenex), and PBS buffer was used as mobile phase at 0.5 mL/min.

Results

Soluble expression of the scFv against IGF-IR in E. coli

In our previous work, we have achieved soluble expression of a bioactive scFv specific against IGF-IR in periplasmic space of *E. coli* [7]. In this study, the same recombinant scFv was expressed as described before except that the 6×His tag was removed. A comparable yield (2.5 mg/g of wet weight cells in shaking flask) of soluble target scFv to that in previous study was obtained.

Experimental design in the capture step using Capto L affinity chromatography

In the capture step, a HiTrapTM Capto LTM prepacked 1-ml column was chosen because of its specific affinity to kappa light chain which was included in the target scFv. Before the application of DoE, the DBC and elution pH range were firstly determined to define the initial loading and elution conditions. As the result, the DBC was 18 mg/mL with a residence time of 4 min, and the pH corresponding to the elution peak of the scFv was determined to be 3.25 based on a decreased pH linear gradient elution from 6.0 to 2.5. During this step, more than 30% of target protein was eluted as soluble aggregates, mainly in dimer form (see Fig.1, lane 2), although the purity of the target protein was above 90% if the aggregates were included. This suggested that the scFv might be aggregation-prone in acidic pH, thus preventing aggregation was a major task in this step. A following 2-level fractional factorial analysis to identify the main effects that inhibit aggregation during elution was conducted, where aggregates and recovery were defined as responses, and elution pH, salt concentration, and five commonly used additives for preventing aggregation were

defined as variables. The selection and the level values of five additives were set according to empirical data and literatures [12-14]. And, a 70% DBC, equal to 12.5 mg/mL, was adopted as loading quantities, and pH 3.25 was set as pH center point. The settings and values of responses were all shown in Table 1. According to the result of normal plot analysis, pH, dextran and trehalose were identified as main affects for aggregates ($p < 0.05$) (see Fig.2a). Meanwhile, pH was identified as main effect for recovery ($p < 0.05$). And, interactive effects mainly between pH and trehalose on both responses were also observed. Based on the significances of their effects on the aggregation, pH and trehalose were selected as variables for further optimization by using CCC analysis to obtain the optimal elution conditions for higher recovery and lower aggregates. The settings of the CCC were shown in Table 2. Fig. 3 showed the recovery and aggregates as a function of pH and trehalose concentration. The recovery was pH dependent and the optimal space was found at low pH. The low ratio of aggregates was achieved at high concentration of trehalose. An interactive effect was revealed between pH and trehalose. To fulfill both criteria of recovery (above 95%) and aggregates ratio (below 10%), it was decided to perform the elution at the pH range from 2.9 to 3.0 with 32%-35% (w/v) trehalose added.

Table 1 2-level fractional factorial design for screening of variables during elution and responses

Run order	Variables							Responses	
	A: pH	B: NaCl (mM)	C: Urea (M)	D: Arginine (M)	E: Trehalose (% w/v)	F: PEG (% w/v)	G: Dextran (% w/v)	Aggregates (%)	Recovery (%)
1	3.25 (0)	150 (0)	0.55(0)	0.3 (0)	17.5 (0)	5.5 (0)	5.5 (0)	18.5	93.0
2	3.0 (-1)	250 (+1)	1.0 (+1)	0.5 (+1)	5.0 (-1)	10 (+1)	1.0 (-1)	36.4	96.1
3	3.5 (+1)	250 (+1)	1.0 (+1)	0.5 (+1)	30 (+1)	10 (+1)	10 (+1)	8.8	91.3
4	3.0(-1)	50 (-1)	0.1 (-1)	0.1 (-1)	5.0 (-1)	1.0 (-1)	1.0 (-1)	35.2	97.0
5	3.5(+1)	250 (+1)	0.1 (-1)	0.5 (+1)	5.0 (-1)	1.0 (-1)	1.0 (-1)	21.1	89.5
6	3.5(+1)	50 (-1)	0.1 (-1)	0.1 (-1)	30 (+1)	1.0 (-1)	10 (+1)	8.3	92.0
7	3.0(-1)	250 (+1)	0.1 (-1)	0.5 (+1)	30 (+1)	1.0 (-1)	10 (+1)	12.5	95.2
8	3.5(+1)	50 (-1)	0.1 (-1)	0.5 (+1)	30 (+1)	10 (+1)	1.0 (-1)	9.4	90.0
9	3.0(-1)	250 (+1)	0.1 (-1)	0.1 (-1)	30 (+1)	10 (+1)	1.0 (-1)	13.0	96.0
10	3.25(0)	150 (0)	0.55 (0)	0.3 (0)	17.5 (0)	5.5 (0)	5.5 (0)	19.2	93.5
11	3.0(-1)	250 (+1)	1.0 (+1)	0.1 (-1)	5.0 (-1)	1.0 (-1)	10 (+1)	35.0	97.0
12	3.5(+1)	250 (+1)	1.0 (+1)	0.1 (-1)	30 (+1)	1.0 (-1)	1.0 (-1)	9.3	89.7
13	3.0(-1)	50(-1)	1.0 (+1)	0.1 (-1)	30 (+1)	10 (+1)	10 (+1)	12.5	95.4
14	3.0(-1)	50(-1)	0.1 (-1)	0.5 (+1)	5.0 (-1)	10 (+1)	10 (+1)	35.4	95.0
15	3.0(-1)	50(-1)	1.0 (+1)	0.5 (+1)	30 (+1)	1.0 (-1)	1.0 (-1)	13.3	96.0
16	3.25(0)	150(0)	0.55 (0)	0.3 (0)	17.5 (0)	5.5 (0)	5.5 (0)	18.9	92.5
17	3.5(+1)	50 (-1)	1.0 (+1)	0.1 (-1)	5.0 (-1)	10 (+1)	1.0 (-1)	20.0	88.9
18	3.5 (+1)	250 (+1)	0.1 (-1)	0.1 (-1)	5.0 (-1)	10 (+1)	10 (+1)	19.0	89.0
19	3.5 (+1)	50 (-1)	1.0 (+1)	0.5 (+1)	5.0 (-1)	1.0 (-1)	10 (+1)	18.6	91.0

Number +1, -1, and 0 in the bracket indicated the high level, low level, and center point of the variables, respectively.

Fig.1 Non-reducing SDS-PAGE analysis of the cell lysates (lane 1) and fraction eluted from Capto L (lane 2).

Fig. 2 Normal probability plot for the effects of pH, NaCl concentration and additives of the elution buffer on aggregates (a) and recovery (b) of the scFv during purification using Capto L chromatography. The main effects identified were indicated by red rectangle.

Table 2 CCC design and responses in first capture step

Run order	Variables		Responses	
	A:	B:	Aggregates	Recovery
	pH	Trehalose		
			(%)	(%)
1	0	0	17.2	92.0
2	1	-1	19.0	88.0
3	-1	-1	34.0	96.0
4	0	0	17.0	92.5
5	0	-1.414	35.0	92.0
6	-1.414	0	20.0	98.0
7	1	1	8.2	90.0
8	1.414	0	14.0	85.0
9	0	0	17.9	92.3
10	0	-1.414	7.6	93.0
11	0	0	17.5	92.8
12	0	0	17.8	92.5
13	-1	1	13.0	96.0

Note: Number 1.414, -1.414, 1, -1, and 0 represented pH 3.6, 2.9, 3.5, 3.0, and 3.25, and trehalose concentration of 35%, 0, 30%, 5%, and 17.5% (w/v), respectively.

Fig. 3 Contour plots for aggregates (a) and recovery (b) using Capto L. The ranges of the two responses represented by different colors were indicated on right. (color figure can be seen in the online version of this article).

Optimization of the cation exchange chromatography step

In the second purification step, the remaining dimer was the major impurity need to be separated. Considering the basic pI (8.7) of the target scFv, two cation exchange **media**, Capto S and SP Sepharose® Fast Flow, were initially chosen for the purification. The selectivity and recovery of each medium were tested at pH 5.0, 6.0 and 7.0, using a NaCl linear gradient elution from 0 to 1.0 M. Capto S presented better selectivity at pH 6.0 (data not shown), although there was little difference in recovery between all tested conditions, thus this medium was selected for further optimization. Next, a CCC design based on two variables (pH and loading conductivity) was set up to determine the salt concentration and the pH needed to obtain high DBC and purity. Accordingly, the DBC of the target protein as well as the purity of eluted product were set as responses. See Table 3 for setting details. Fig. 4a showed the contour plot for DBC. As expected, lower pH generated higher DBC, and this trend was slightly offset as the conductivity increased. Fig. 4b showed the contour plot for purity, the highest purity was found near pH 6.0, while the loading conductivity played a minor effect on this response. Based on these results, **pH 5.9-6.1** and loading conductivity 5-12.5 mS/cm were considered as optimal conditions for this step.

Table 3 CCC design and responses in the second step

Run order	Variables		Responses	
	A: pH	B: Conductivity	DBC	Purity
			(%)	(%)
1	0	0	130	93
2	1	-1	87	96
3	-1	-1	68	94
4	0	0	118	94
5	0	-1.414	88	96
6	-1.414	0	40	94
7	1	1	90	97
8	1.414	0	91	96
9	0	0	88	97
10	0	1.414	70	95
11	0	0	99	97
12	0	0	133	94
13	-1	1	39	93

Note: Number 1.414, -1.414, 1, -1, and 0 represented pH 7.4, 4.59, 7.0, 5.0, and 6.0, and conductivity of 17.1, 2.9, 15, 5, and 10 mS/cm, respectively.

Fig.4 Contour plots for DBC (a) and purity (b) using Capto S. $R^2=0.99$. (color figure

can be seen in the online version of this article)

Gel filtration and purity determination of final product

In final step of purification, the Superdex 75 medium was used to remove the trace aggregates and to desalt. The final products were stored in PBS. A following

HPLC detection was performed to determine the purity of the target protein. Accordingly, the final sample was 98% in purity (see Fig. 5). And the recovery was calculated as 94% in this step.

Fig. 5 Purity analysis of the purified scFv by using SEC-HPLC (a) and **non-reducing** SDS-PAGE (b).

Verification of the optimal purification conditions

Having optimized the three purification steps and having gained the optimal space for each step, a 20-fold scale-up process verification was performed. Accordingly, **88 grams wet cells (with 250 mg target protein included) from eight liters cultures were used as starting sample**. Purity and recovery of the target fraction in each purification step were calculated. The results were summarized in Table 4. All values fell within the predicted regions that were determined in small scale DoE design, implying the robustness of the optimal conditions. The total yield of final

product was 77% (equal to 1.9 mg purified scFv per g of wet weight cells), and the purity was up to 98.5% according to SEC-HPLC analysis.

Table 4 Summary of the verification experiment result

Fraction	Purity	% Yield
Lysate	ND	100
Capto L	86.4% (93% [*])	89 (95.6 [*])
Capto S	96%	82
Superdex 75	98.5%	77

ND, not determined.

^{*}if dimer aggregates were included.

Discussion

Design of experiments (DoE) is a structured and organized statistical method for planning experiments and analyzing the information obtained. Based on the information obtained, a mathematical model can be built to help understand the relationships between variables and responses, and help identify the main effects and predict their optimal space for certain objective. In this study, DoE was applied into purification optimization of a recombinant scFv against IGF-IR that is a promising target for a wide range of cancers, whereby the optimal purification conditions were efficiently established and the final product was obtained with high purity and yield. This laid a foundation for the bioprocess of the scFv in a further diagnostic or therapeutic application.

Affinity chromatography based on protein A or protein L has been commonly used for purifying antibodies or antibody fragments because of its high specificity. However, the acidic pH elution used in this method often leads to undesired aggregation or even precipitation of target proteins, which was the case here.

Generally, in this case, a wide range of additives such as amino acids, disaccharides, polyhydroxy alcohols, non-ionic detergents could be used to ameliorate the aggregation [12-14]. As the effects of these additives are case-by-case, there is no generic formulation available. Thus a wide screening for identifying the best ones or proper combinations needs to be performed, but this would be a tough task if using the traditional OFAT method for the screening. The fractional factorial analysis approach used here allowed analyzing many factors simultaneously and identifying the main effects rapidly, thus largely reducing the experiment number and saving time and labors. The CCC design employed then helped focus on the optimal conditions. In this study, trehalose was identified as the most effective additive for preventing aggregation of the target scFv upon acidic pH elution, while other additives showed minor effects. This again indicated that the effects of the additives are hard to be predicted, and the choice of the additives should be assessed on a case-by-case basis. Anyway, the DoE approach used in this step could be referenced when the aggregation issue happens in the protein A or protein L affinity chromatography of other antibodies or antibody fragments.

In conclusion, a three-step purification process for a scFv against human IGF-IR was optimized in a small scale using DoE approach, and then verified in a 20-fold scale-up experiment. The total yield reached 77%, and the purity of the final product was up to 98.5%. We proposed that the DoE design described in this study has wide applicability in the production of other recombinant scFvs with kappa light chain.

Acknowledgements

We thank Dr. Feng Zhang for his valuable and helpful suggestions on protein expression and DoE design.

Author's contribution

Yong-Hong Song and Xue-Wen Sun performed the experiments and wrote the manuscript. Xian-Hui Su designed and supervised the study. Bo Jiang assisted in performing the protein purification. Ji-En Liu provided consultation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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