Production and Purification of A Cell-Penetrating Peptide-Erythropoietin Fusion Protein and Optimization of an in Vitro Blood-Brain Barrier Model for Central Nervous System Drug Delivery

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Research

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Abstract

Drug delivery into the central nervous system (CNS) is a brilliant research field, and the development of protein production and purification procedures for novel therapeutic proteins is crucial. Erythropoietin (EPO) is a glycoprotein with tremendous neuroprotective potential, but its bulky size prevents easy penetration across the blood-brain barrier (BBB). EPO-HBHAc is a promising cell-penetrating peptide modified protein for CNS diseases, necessitating an appropriate in vitro BBB model for further evaluation. The plasmid of EPO-HBHAc was constructed by DNA recombinant technology, and the Chinese Hamster Ovary (CHO-K1) cell expression system was selected to generate target proteins. His-tag and size exclusion purification were used to purify the target protein from the cell-conditioned medium; target proteins were further evaluated by western blotting and Coomassie blue staining. Moreover, the endothelial cells (bEnd.3) and astrocytes (CTX TNA2) were used to generate the in vitro BBB model, and transepithelial electrical resistance (TEER) and paracellular diffusion were measured to evaluate barrier integrity. The EPO-HBHAc plasmid was successfully constructed, and a stable cell line expressing EPO-HBHAc was generated. A higher protein expression level was observed in serum-containing medium than in serum-free medium. His-tag purification is not sufficient to remove impurities from target proteins, and thus size exclusion purification was performed to increase the purity of the protein of interest. In contrast, a higher TEER value and lower paracellular diffusion were observed in the co-culture model than in the mono-culture model. Furthermore, the higher TEER value was observed in inserts with a larger growth area (4.67 cm\(^2\)) than in those with a smaller area (0.33 cm\(^2\)). In conclusion, we demonstrated that some critical points might impact protein production and the in vitro BBB model construction in this study. Importantly, our research will provide valuable information in the field of CNS drug delivery.

1. Introduction

Neurological disorders are reportedly leading causes of disability-adjusted life-years (DALYs), with the prevalence of these diseases steeply increasing owing to an aging population [1]. Globally, neurological disorders have revealed a significant impact on both the economy and society. Thus, the development of therapeutics for central nervous system (CNS) disorders is urgent. Macromolecular drugs such as proteins and peptides demonstrate higher efficacy when compared with small molecular drugs, as well as present good efficiency and low toxicity owing to their target specificity [2]. Furthermore, owing to advancements in biotechnology, large-scale production of biologics is now increasingly cost-effective. Additionally, according to a report by the U.S. Food and Drug Administration (FDA), the number of approved biologics has been growing in recent years. In 2018, 17 biologics were approved, representing a record that exceeds the number of approved biologics in previous years. Moreover, these biologics account for more than 25% of all approved drugs in the last five years [3]. Hence, the growth potential of the therapeutic protein market is tremendous.

Erythropoietin (EPO) is a glycoprotein that has been used for the treatment of different types of anemia for decades. Apart from the erythropoiesis activity of EPO, several studies have reported the neuroprotective functions of this protein, eliciting protective effects in neurodegenerative and ischemic...
brain diseases [4–7]. Owing to its bulky size, EPO does not easily penetrate the blood-brain barrier (BBB), and an extremely high EPO dose is needed to attain a therapeutic concentration in the brain tissue to demonstrate a neuroprotective effect. In our previous publication, we reported that the BBB permeability of EPO was enhanced using heparin-binding hemagglutinin adhesion c (HBHAc), a cell-penetration peptide (CPP), modification [8].

Chinese hamster ovary (CHO-K1) cells are the primary expression system for therapeutic proteins [9] and were selected to produce EPO-HBHAc. To obtain high-quality proteins, the choice of the culture medium is a critical point in protein production. Although serum-free medium is widely used for FDA-approved products [10], the impact of serum-starvation on cell viability and protein expression remains unpredictable and depends on the cell type [11]. Moreover, the optimized purification process is an essential step. For purification of this novel CPP-modified protein, poly-histidine tags (His-tags) were used, coupled to a Nickle-NTA matrix. The protein can be eluted under non-denaturing conditions by adding an adequate concentration of imidazole to the elution buffer. The selected His-tag location is based on the protein of interest and is related to the post-translational modification of proteins, including removal of the N-terminal signal peptide [12]. Moreover, a specific protease site added between the His-tag motif and protein of interest allows the tag removal from the protein.

Conversely, the construction of the in vitro BBB model is important to develop an appropriate CNS drug delivery system. Currently, brain endothelial cells are cultured alone, co-cultured with astrocytes, or triple co-cultured with astrocytes and pericytes [13]. The advantages of transwell systems include the relative ease of setup and control. Transepithelial electrical resistance (TEER) and permeability measurements of these models provide reliable quantitative evaluations of barrier integrity [14].

The development of novel therapeutic proteins, including EPO-HBHAc, remains a complex task, and the production process requires step by step evaluation. Thus, in the present study, we demonstrated the procedure of EPO-HBHAc construction and generated an optimized in vitro BBB model.

2. Materials And Methods

2.1 Materials

All restriction enzymes were purchased from New England Biolabs (MA, USA). pUC57 EpoAB-HBSP-HBHAc and pUC57 EPO plasmids were purchased from Genomics (Taipei, Taiwan). The Plasmid Miniprep Purification Kit and Micro-Elute DNA Clean/Extraction Kit were procured from GMbiolab (Taichung, Taiwan). Bacto-tryptone, yeast extract, and agar were purchased from BD Bioscience (CA, USA). G418 (Geneticin) was purchased from Invivogen (CA, USA). Opti-MEM I Reduced Serum Media, HisPur Ni-NTA Resin, and Lipofectamine® 3000 reagent were all purchased from Thermo Fisher Scientific (MA, USA). FITC-4K-dextran and FITC-70K-dextran were purchased from Sigma-Aldrich (MO, USA). EPO antibody and anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (TX, USA).

2.2 Cell cultures
CHO-K1 cells were maintained in Ham's F12 medium, and bEnd.3 cells (mouse brain endothelial cells) and CTX TNA2 cells (rat astrocytes) were maintained in DMEM (Dulbecco's Modified Eagle's medium). All media were supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin. Cells were cultured at 37 °C and in 5% CO₂, and the subculturing procedure was performed according to American Type Culture Collection (ATCC).

2.3 Plasmid construction and stable clone selection of EPO-HBHAc

The process of plasmid construction was described in our previous study [8]. The precise plasmid encoding EPO or EPO-HBHAc was transfected into CHO-K1 cells using Lipofectamine 3000 reagent. After overnight incubation, the transfected cells were divided between 10-cm dishes and incubated for an additional 24 h. G418 was used for stable clone cell line selection, and cells with the neomycin resistance gene survived and formed colonies. Then, cells were cultured with a medium containing 1 mg/mL G418, and the medium was replaced every 2 days for 2 to 4 weeks. Until cell colony formation, the colonies were transferred into a 6-well plate (one colony/well). On reaching approximately 80% confluency, the cells were expanded. The target proteins expressed in both the cell culture supernatant and cell lysate were analyzed by western blotting with an anti-EPO antibody.

2.4 Optimization of protein expression and purification

To evaluate the protein expression profile, CHO-K1 cells with EPO or EPO-HBHAc gene were seeded in a 6-well plate at a density of $5 \times 10^5$ cells/well and incubated overnight. Next, the cells were incubated in Opti-MEM I reduced serum medium, and a medium, with 2% FBS or without FBS, was used to evaluate the effect of FBS on target protein expression. To determine the protein expression level at different incubation time intervals, the cell culture supernatant was collected after 2, 3, and 4 days incubation. Protein expression levels in different culture intervals were evaluated by western blotting using the EPO antibody.

For the large-scale production of fusion proteins, CHO-K1 cells were divided into 15-cm dishes, with the cell culture supernatant collected every 4 days. The aggregates and cell pellets were removed using a 0.22 µm filter cup and concentrated using a Tangential Flow Filtration (TFF) system (Millipore, USA). The concentrated supernatant was mixed with HisPur Ni-NTA Resin, and then the mixture was transferred to a column. The column packed with the protein-resin mixture was equilibrated with an equilibration buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4). To determine the appropriate concentration of imidazole in the wash and elution steps, the column was washed with a gradient concentration of imidazole from 25 to 100 mM, and the collected fractions were analyzed by western blotting. Finally, the target proteins were purified under the optimized Ni-NTA purification process. During the elution step, fractions were collected in 1 mL/tube and analyzed by western blotting. To increase the purity of target proteins after Ni-NTA purification, the fractions containing target proteins were collected and further purified using the size exclusion column. The purified recombinant fusion proteins were confirmed by Coomassie blue staining and western blotting.
2.5 *In vitro* BBB model construct

For the brain endothelial cell mono-culture BBB model, bEnd.3 cells were seeded on the upper surface of the insert (cell growth area: 4.67 cm$^2$) and cultured for several days. In contrast, for the brain endothelial cell and astrocyte co-culture BBB model, CTX TNA2 cells were seeded on the lower surface of the insert and allowed to adhere overnight. The insert was flipped back and CTX TNA2 cells were cultured for an additional two days. Then, bEnd.3 cells were seeded on the upper surface of the insert and co-cultured with CTX TNA2 cells. The TEER value of each well was measured daily using the Millicell ERS Voltohmmeter (Millipore, MA, USA). Furthermore, to evaluate paracellular diffusion for both the monoculture and co-culture BBB models, FITC-4K-dextran and FITC-70K-dextran were used as tracers. FITC-4K-dextran or FITC-70K-dextran was added to the BBB models when the TEER value reached the plateau, and the basolateral medium was collected 3, 6, 12, and 24 h after dosing. The fluorescence intensity in the collected medium was detected using a fluorescence microplate reader. To compare the effect of the growth area of the insert on the BBB model, bEnd.3 and CTX TNA2 cells were additionally co-cultured on the insert with smaller growth areas (0.33 cm$^2$), using the previously described cell culture process. Fluorescence-labeled-70K-dextran was added to the model as the TEER value reached the plateau, and the fluorescence intensity in the basolateral chamber was detected 2 h after dosing.

2.6 Statistics

All values are presented as mean ± SD. A significant difference was evaluated by ANOVA, followed by the Bonferroni modified t-test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1 Expression vector construction

The digested pcDNA3.1(+) His-tag vector was separated using a 1% agarose gel and the predicted size was 5424 bp (Fig. 1 Lane 1). The digested EPO gene was 596 bp, demonstrating a clear band on the gel between 500 to 600 bp (Fig. 1 Lane 2). The predicted size of the digested pcDNA3.1(+) HBHAc His-tag vector was 5501 bp (Fig. 1 Lane 3). The digested EPO DNA fragment was inserted into the digested pcDNA3.1(+) His-tag vector and digested pcDNA3.1(+) HBHAc His-tag vector to generate pcDNA3.1(+) EPO-HBHAc and pcDNA3.1(+) EPO, respectively. pcDNA3.1(+) EPO-HBHAc and pcDNA3.1(+) EPO were confirmed by DNA gel electrophoresis with *Hind*III digestion or *Hind*III/*Age*I double-digestion. The predicted size of the DNA fragment for *Hind*III digested pcDNA3.1(+) EPO and pcDNA3.1(+) EPO-HBHAc were 6020 bp and 6097 bp, respectively (Fig. 1B Lane 1 and Fig. 1C Lane 1). The predicted size of DNA fragments of *Hind*III/*Age*I double-digested pcDNA3.1(+) EPO and pcDNA3.1(+) EPO-HBHAc were 614 bp/5406 bp and 614 bp/5483 bp, respectively (Fig. 1B Lane 2 and Fig. 1C Lane 2). The correctness of the sequences was further confirmed by DNA sequencing and both plasmids were correct [8].

3.2 Stable clone selection
After confirming the correctness of constructed plasmids, the plasmids containing the gene of EPO and EPO-HBHAc were transfected into CHO-K1 cells, respectively. The colonies were formed and selected from the cells transfected with specific plasmids after G418 selection, and the target protein expression in cell culture supernatant and cell lysate were detected by western blotting. Proteins with the precise molecular weight were observed in the cell culture supernatant of both the EPO (Fig. 2 Lane 2) and EPO-HBHAc groups (Fig. 2 Lane 4). Low molecular products were detected in the cell lysate of EPO (Fig. 2 Lane 1) and EPO-HBHAc groups (Fig. 2 Lane 3). This result indicated that the plasmids with the target gene were successfully transfected into CHO-K1 cells, and the target proteins were mainly released into the cell culture medium.

### 3.3 Large-scale protein expression and purification

As shown in Fig. 3A, the intensity of the target protein increased with increasing incubation intervals in both 2% FBS and FBS-free groups. Furthermore, the highest EPO expression level was observed in cells treated with serum-free medium over a 4-day incubation (Fig. 3A Lane 7). However, in the FBS-free cultured group, the protein expression level decreased after the replacement of fresh medium for an additional 4 days of incubation (Fig. 3A Lane 8), demonstrating weak cell adherence. Consistent protein expression was observed in the 2% FBS cultured group after replacement with fresh medium for an additional 4 days incubation (Fig. 3A Lane 4). Conversely, the highest level of EPO-HBHAc fusion protein expression was detected in the cell group incubated in medium supplied with 2% FBS for 4 days (Fig. 3B Lane 4), and the proteins were consistently expressed even after replacement with fresh medium for an additional 4 days of incubation (Fig. 3B Lane 8).

For large-scale of production of the target protein, the transfected CHO-K1 cells were divided between 15-cm cell culture dishes and incubated with the medium containing 2% FBS. According to the evaluated protein expression profile, the cell culture supernatant containing target proteins was collected every 4 days. The collected supernatant was filtered and the volume of filtered supernatant was reduced using the TFF system before purification using the Ni-NTA column. To determine the suitable concentration of imidazole for the wash process, gradient concentrations from 25 to 100 mM imidazole were used. The chromatogram is shown in Fig. 4A and selected fractions were further evaluated by western blotting. Only small amounts of protein were detected in the equilibration step and at the beginning of wash step (Fig. 4B Lane 1 to Lane 3); however, a significant amount of EPO fusion proteins was detected in fraction No. 45 (40 mM imidazole) and fraction No. 81 (76 mM imidazole) (Fig. 4B Lane 4 and Lane 5). To reduce the loss of target proteins during the wash step, the imidazole concentration should not exceed 40 mM imidazole. Therefore, the buffer containing 30 mM of imidazole was used for the wash step during purification. The Ni-NTA chromatogram is shown in Fig. 5A and collected fractions were evaluated by Coomassie blue staining. Although several impurities could be removed by washing with 30 mM of imidazole, the purity of fusion proteins remained low after Ni-NTA purification (Fig. 5B). EPO fusion proteins were further purified using the size exclusion column, and major impurities were separated from our target protein (Fig. 6). The purification process for EPO-HBHAc fusion proteins followed the same procedure of EPO fusion proteins, and the chromatography of Ni-NTA and size exclusion purification are
shown in Fig. 7A and 7B, respectively. Unfortunately, the major impurities could not be precisely separated from EPO-HBHAc owing to the small molecular weight difference between them (Fig. 7C Lane 4 to Lane 6). However, the purity of EPO-HBHAc improved after purification by size exclusion column. The purity of the purified proteins was analyzed by Coomassie blue staining. EPO and EPO-HBHAc demonstrated a purity of > 80% and > 60%, respectively.

3.4 *In vitro* BBB model construct

To establish a suitable *in vitro* BBB model, the endothelial cell mono-culture and endothelial cell and astrocyte co-culture models were evaluated. The TEER values 7 days after bEnd.3 cells were seeded are shown in Fig. 8A. The co-culture model demonstrated higher TEER values than the mono-culture model ($208 \pm 4.6 \, \Omega \cdot \text{cm}^2$ vs. $150 \pm 9.1 \, \Omega \cdot \text{cm}^2$). Additionally, the paracellular diffusion assay was performed on day 7 after bEnd.3 cells were seeded, and FITC-4K-dextran and FITC-70K-dextran were used as tracers. As shown in Fig. 8B, $51.1 \pm 0.5\%$ and $37.5 \pm 1.7\%$ of FITC-4K-dextran were transported from the upper chamber to the basolateral chamber in the mono-culture model and co-culture model after 24 h of incubation, respectively. In contrast, only $7.3 \pm 1.1\%$ and $2.8 \pm 0.1\%$ of FITC-70K-dextran was transported from the upper chamber to the basolateral chamber in the mono-culture model and co-culture model after 24 h of incubation, respectively. In the co-culture model, lower paracellular diffusion was observed for both 4K and 70K dextran, with higher TEER values. These results indicated that the CTX TNA2 and bEnd.3 co-culture model would be a superior BBB model to evaluate EPO-HBHAc transcytosis *in vitro*. Furthermore, bEnd.3 cells and CTX TNA2 cells were co-cultured on the smaller inserts (growth area: $0.33 \, \text{cm}^2$), and the TEER value is shown in Fig. 9. The TEER value reached the plateau 4 days after bEnd.3 cells were seeded. The fluorescence intensity of 70K-dextran in the basolateral chamber was detected 2 h after dosing, and $14.9 \pm 2.2\%$ of 70K-dextran was transported from the upper chamber to the basolateral chamber in the co-culture model ($0.33 \, \text{cm}^2$).

4. Discussion

CHO-K1 cells were chosen to generate EPO and EPO-HBHAc fusion proteins, and stable clones expressing EPO and EPO-HBHAc were selected and cryopreserved. Furthermore, the target proteins were purified under a two-step purification process. Chemical conjugation is another approach to generate EPO-HBHAc; however, it is challenging to control the specific site of conjugation between EPO and HBHAc. A heterogeneous mixture would be generated, and EPO bioactivity could be affected if critical sites on EPO were occupied by HBHAc. The production of proteins in mammalian cells is an important tool not only for basic research but also in the biotech industry. Mammalian proteins, including tissue plasminogen activator and EPO, require a mammalian cell production system for better bioactivity, and this could be attributed to the ability of mammalian cells to generate proteins with appropriate molecular structures and biochemical properties [15]. Furthermore, compared with transient transfection, stable expression of the transgene is generally more desirable for large-scale production, demonstrating higher protein quality and homogeneity [16].
The adherent expression system is a traditional method for protein expression and was selected to generate EPO-HBHAc on a bench-scale in this study. However, the surface area of the flask might limit the yield of target proteins [17]. This is a proof-of-concept study, evaluating the protein production and purification process for a novel CPP-modified protein, and bench-scale production was sufficient for this purpose. However, a scale-up protein expression system for target proteins is necessary for further in vivo evaluations, with the suspension-adapted cell culture system providing an alternative to increasing the yield of this novel CPP-modified protein. In previous study investigating EPO production, the conditioned medium was collected every 2 days [18]; however, in the present study, the highest protein expression was observed after 4 days of incubation. Protein expression levels can vary among the different groups, and thus, we suggest that evaluating the protein expression profile is crucial when setting up a new protein production platform.

In our in vitro co-culture BBB model, the TEER value was significantly higher than that observed in the mono-culture model, and the penetration of 70K-dextran was significantly blocked from the upper chamber to the basolateral chamber. It remains a challenge to design an optimized in vitro experimental model to mimic the physiological and functional characteristics of the BBB. High junctional tightness measured as TEER is an important feature for an appropriate model mimicking the BBB in vitro. However, the optimal value of TEER for experiments could vary when obtained from different studies. This could be attributed to differences in measuring equipment and temperature, as well as the handling of the cells [14]. Hence, the tightness of in vitro BBB models was validated using permeability studies with hydrophilic tracers [19].

Additionally, our mono-culture model showed lower TEER values and higher paracellular diffusion when compared with the co-culture model, which is consistent with previous studies. Owing to inadequate tight junctions in endothelial cell mono-culture models, several research groups have attempted to reduce paracellular diffusion in these mono-culture models. One approach is to co-culture the endothelial cells with astrocytes [20, 21]. Previous studies have reported that cultured astrocytes implanted into areas with normally leaky vessels were able to induce the tightening of the endothelium, demonstrating that astrocytes play a major role in inducing barrier properties [22]. To better mimic the physiological structure of the BBB, endothelial cells are co-cultured with astrocytes, and the interaction between endothelial cells and astrocytes increases the expression of transporters, as well as that of tight junctions in endothelial cells. Furthermore, this interaction induces the formation of cell polarity in endothelial cells. Collectively, these advantages reveal that the endothelium-astrocyte co-culture model is more representative of the BBB [23–27].

A large amount of 4K-dextran was transported from the upper chamber to the basolateral chamber in both the monoculture and co-culture BBB models. This demonstrates that our models failed to afford sufficient barrier properties to small peptides/proteins with a molecular weight of approximately 4,000 Dalton. In contrast, only a limited amount of 70K-dextran was transported from the upper chamber to the basolateral chamber in both the monoculture and co-culture BBB models. Based on our results, the extents of EPO and EPO-HBHAc transportation differed in the co-culture model, suggesting that our co-
culture model could be applied in BBB penetrating investigations for substances with a molecular weight larger than that of EPO.

**Conclusion**

The optimized protein production and purification for EPO-HBHAc were successfully established, and the protein expression level was affected by the composition of the culture medium and incubation period. Conversely, higher barrier integrity was observed in the co-culture BBB model than in the mono-culture model, and the integrity was affected by the growth area of the insert. This comprehensive study revealed some critical factors for CPP-modified protein generation and BBB model construction, providing valuable information in the field of CNS drug delivery.

**List Of Abbreviations**

EPO, Erythropoietin; BBB, Blood-brain barrier; CNS, Central nervous system; CPP, Cell-penetrating peptide; DALYs, disability-adjusted life-years; TEER, Transepithelial electrical resistance; TFF, *Tangential flow filtration*.

**Declarations**

*Ethics approval and consent to participate*

Not applicable

*Consent for publication*

Not applicable

*Availability of data and material*

Please contact author for data requests.

*Competing interests*

The authors declare that they have no competing interests.

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*Authors' contributions*
PCC carried out all the experiments and drafted the manuscript. LJS conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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