International Journal of Pharmaceutics 386 (2010) 156–164

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Efficient preparation and PEGylation of recombinant human non-glycosylated erythropoietin expressed as inclusion body in *E. coli*

Yin-Jue Wang[a,b], Yong-Dong Liu[a], Jing Chen[a,b], Su-Juan Hao[c], Tao Hu[a], Guang-Hui Ma[a], Zhi-Guo Su[a,*]

[a] National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China
[b] Graduate School, Chinese Academy of Sciences, Beijing 100190, China
[c] Beijing University of Chemical Technology, Beijing 100029, China

ARTICLE INFO

Article history:
Received 31 August 2009
Received in revised form
11 November 2009
Accepted 15 November 2009
Available online 20 November 2009

Keywords:
Recombinant erythropoietin
Non-glycosylated
Refolding
Purification
PEGylation
Half-life

ABSTRACT

Recombinant human erythropoietin produced by mammalian cells contains about 40% carbohydrates which maintain its stability and long residence in body. However, mammalian derived Epo has low yields and high costs of production. In this article, a cost-effective strategy of producing non-glycosylated Epo from *Escherichia coli* and then PEGylating it to replace the role of sugar chains was investigated. Recombinant human non-glycosylated erythropoietin (rh-ngEpo) was overexpressed as inclusion body in *E. coli*. As the routine inclusion body washing step resulted in poor protein recovery and purity, a new process scheme of using strong ion-exchange chromatography to purify denatured rh-ngEpo from inclusion body before refolding was developed. The purity of the denatured rh-ngEpo was increased from 59% to over 90%. Rh-ngEpo was then refolded and subsequently purified by one step of weak cation-exchange chromatography to 98% pure. Final protein yield was 129 mg/l, a significant improvement from 49 mg/l obtained via the conventional practice. The *in vitro* bioactivity of purified rh-ngEpo was comparable with the CHO-expressed Epo and the formation of native secondary structure was also confirmed by CD spectra. Rh-ngEpo was then modified by a 20 kDa methoxy polyethylene glycol (PEG) succinimidyl carbonate. The monoPEGylated protein, which retained 68% bioactivity, had enhanced thermal stability and a remarkably prolonged circulating half-life in rats as compared with that of the unmodified protein. These studies demonstrated the feasibility of PEGylating rh-ngEpo as a promising way for the development of new Epo drugs.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Erythropoietin (Epo) is a key factor regulating erythrocytes production in human body (Krantz, 1991). It stimulates division and differentiation of erythroid progenitor cells in the bone marrow into mature erythrocytes. Recombinant human Epo has been clinically used in the treatment of anemia resulting from chronic kidney disease, chemotherapy, and complications from AIDS therapies (Markham and Bryson, 1995). Besides these well-established applications, Epo also has therapeutic potentials in the treatment of acute brain, heart and kidney injury, currently under evaluation (Sharples et al., 2006).

Natural Epo contains three sites with N-glycosylation (N24, N38, and N83) and one site with O-glycosylation (S126). Due to the importance of glycosylation in maintaining its stability and *in vivo* hematopoietic activity (Dordal et al., 1985; Narhi et al., 1991; Yamaguchi et al., 1991; Higuchi et al., 1992) and the complexity of the glycosylation patterns, Epo or Epo analogues were mostly produced in mammalian cell cultures (Park et al., 2000; Irani et al., 2002; Wang et al., 2002; Egrie et al., 2003; Zanette et al., 2003; Schriebl et al., 2006). However, mammalian derived Epo also has several drawbacks, such as low yields and high costs of production. Furthermore, there are difficulties in characterization of Epo due to its complex sugar chains and also inter-batch variation occurs.

In contrast, *Escherichia coli* expression system allows economic and fast production of large amounts of homogeneous protein. It was reported that Epo deprived of carbohydrate chains had almost no *in vivo* bioactivity because deglycosylation greatly increased its clearance rate in body (Wasley et al., 1991). Although *E. coli* derived Epo lacks sugar chains, this deficiency may be overcome by covalent PEG conjugation as PEG modified proteins have several well-established advantages over their unmodified counterparts, including prolonged residence in body, and decreased degradation by metabolic enzymes (Veronese and Gianfranco, 2005). Therefore, a strategy of PEGylating recombinant human non-glycosylated Epo (rh-ngEpo) expressed in *E. coli* to enhance its stability and extend its *in vivo* circulating life may be a feasible and cost-effective way for the development of new Epo drugs.

* Corresponding author. Tel.: +86 10 62561817; fax: +86 10 62561813.
E-mail address: zgsu@home.ipe.ac.cn (Z.-G. Su).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.11.016

Several attempts have been made to express Epo in *E. coli* for structural or functional studies (Narhi et al., 1991, 2001; Boissel et al., 1993), but little details about the refolding and purification procedures were revealed. In this paper, the process of refolding, purification and PEGylation of non-glycosylated Epo from inclusion body was investigated. It was found that the routine inclusion body washing step resulted in poor protein recovery and purity, and the following refolding and purification efficiencies were thus compromised. In light of this, a new process scheme of chromatographically purifying crude inclusion body before refolding was developed. Compared with the conventional practice, this new scheme greatly enhanced protein yield and process efficiency. Moreover, results of *in vitro* bioactivity, thermal stability and *in vivo* half-life studies suggested that PEGylated rh-ngEpo was a promising candidate for the development of new Epo drugs.

2. Materials and methods

2.1. Construction of the plasmid

The plasmid pMD18T/Epo encoding a full length cDNA of human Epo was kindly donated by Professor Qingsheng Qi (Shandong University, China). This plasmid was used as a template to amplify Epo with two primers (forward 5′-ATTAATCCATGGCACCGCCGCGTCTGATTTGTGATAGC-3′ and reverse 5′-ACTTGCCTCGAGTTAACGGTCGCCGGTACGGC-3′) by polymerase chain reaction (PCR). The underline indicates NcoI site and XhoI site, respectively. The cloned DNA was digested with NcoI and XhoI and subcloned into the same sites of pET-15b (Novagen, Germany) to yield pET-15b/Epo. This plasmid was constructed for the overexpression of Epo and introduced into *E. coli* BL21 (DE3).

2.2. Preparation and solubilization of inclusion body

E. coli carrying Epo plasmid was first grown at 37 °C in five shake-flasks each containing 100 ml LB medium supplemented with 100 μg/ml ampicillin and then inoculated in a 7 l bioreactor (K&T, Korea) when cell density reached OD_{600} of 0.8–1.0. Cells were continuously grown in 5 l fermentation medium (yeast extract 10 g/l, tryptone 10 g/l, glucose 10 g/l, K_2HPO_4 5.2 g/l, Na_2HPO_4 2.8 g/l, $MgSO_4$ 0.3 g/l, and $(NH_4)_2SO_4$ 1.4 g/l) supplemented with 100 μg/ml ampicillin. Epo expression was induced when cell growth reached mid-exponential phase with 1 mM isopropyl-D-thiogalactopyranoside (IPTG). After 4 h induction, cells were harvested by centrifugation at 8000 × *g* for 15 min at 4 °C. The cell pellets were then resuspended in 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and were lysed by sonication at 150 kHz, using VC-600-2 sonicator (Sonics & Materials Inc.) with a 13 mm probe. This cycle was repeated 5 times for a total sonication time of 20 min with an interval of 2 min for cooling. Cell debris and soluble materials were removed by centrifugation at 16,000 × *g* for 20 min at 4 °C. The pellets containing inclusion body were washed with 20 mM Tris–HCl (pH 8.0) containing 1% Triton X-100, 2 M urea, 1 mM EDTA, and recovered through centrifugation. The pellets were resuspended and washed another time with the same buffer. The rh-ngEpo inclusion body was then solubilized in denaturing buffer (20 mM Tris–HCl, pH 8.5) containing 6 M guanidine chloride and 100 mM 2-mercaptoethanol and left for 7 h at room temperature with continuous stirring. The final protein concentration in the denaturing solution was kept at 20 mg/ml.

2.3. Screening of refolding conditions by HPSEC

Refolding was initiated by rapid 200-fold dilution of the denatured protein into refolding buffers of 20 mM Tris–HCl (pH 8.5) with or without various refolding additives at a predetermined concentration, including arginine (0.5 M), guanidine chloride (1 M), urea (2 M), sucrose (0.5 M), β-CD (24 mM), glycerol (10%), Tween 80 (0.2%), PEG 400 (5%), and PEG 6000 (0.5%). The refolded protein samples were analyzed by high performance size-exclusion chromatography (HPSEC) using a Superdex 75 HR (300 mm × 10 mm ID, GE Healthcare) on an AKTA Purifier system (GE Healthcare). The column was equilibrated with 20 mM PB buffer containing 0.15 M NaCl (pH 7.0), and 200 μl of protein sample was injected, followed by isocratic elution with the equilibration buffer at 0.5 ml/min. Absorbance was recorded at 280 nm. Refolding yield was the ratio of the refolded rh-ngEpo monomer mass to the denatured Epo mass (Epo accounted for 59% of total protein mass in inclusion body) and was calculated with the following equation:

$$\text{Refolding yield} = \frac{\text{Peak area of refolded rh} - \text{ngEpo monomer}}{\text{Total HPSEC peak area} \times 59\%}$$

2.4. Purification of refolded rh-ngEpo

The refolded mixture was dialyzed against 10 volumes of 20 mM Tris–HCl (pH 8.0) containing 1 M urea at 4 °C overnight. After clarification by centrifugation, the dialysate was adjusted to pH 7.5 and then loaded onto a cation-exchange column (XK 200 mm × 16 mm ID, GE Healthcare) containing 40 ml CM Sepharose Fast Flow (GE Healthcare) equilibrated with buffer A (20 mM Tris–HCl, pH 7.5) and connected to AKTA Purifier system. The column was then washed with buffer A until UV baseline was reached. The bound proteins were then eluted by buffer B (20 mM Tris–HCl, 0.5 M NaCl, pH 7.5). The ion-exchange chromatography fraction was further purified by a prepacked gel filtration column Hiload Superdex 75 (600 mm × 26 mm ID, GE Healthcare) equilibrated with 20 mM Tris–HCl (pH 7.5) containing 0.15 M NaCl. The major protein peak was collected and subjected to SDS-PAGE analysis.

2.5. New process scheme of rh-ngEpo production

Crude inclusion body was solubilized in 20 mM Tris–HCl (pH 7.0) containing 8 M urea and 50 mM 2-mercaptoethanol. After incubation at room temperature for 7 h, the denatured protein was loaded onto a cation-exchange column (XK 200 mm × 16 mm ID, GE Healthcare) containing 25 ml SP Sepharose Fast Flow (GE Healthcare) equilibrated with buffer C (20 mM Tris–HCl, pH 7.0) containing 8 M urea and 20 mM 2-mercaptoethanol. The column was washed with the same buffer and rh-ngEpo was then eluted by buffer D (20 mM Tris–HCl, pH 7.0) containing 8 M urea, 20 mM 2-mercaptoethanol and 0.7 M NaCl. The eluted and still denatured rh-ngEpo was diluted into 20 mM Tris–HCl (pH 8.5) containing 0.5 M arginine at 4 °C and the final protein concentration was kept at 100 μg/ml. After 24 h incubation, the refolded rh-ngEpo was then dialyzed and further purified by CM Sepharose as indicated above.

2.6. SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970). GE Healthcare BioKit low molecular weight standards were used. Samples were loaded on 15% polyacrylamide gels. Protein bands were developed by Coomassie blue staining.

2.7. RP-HPLC analysis

For the analysis of refolded rh-ngEpo, 100 μl of rh-ngEpo in different refolding additives were applied to a reversed-phase HPLC column (Vydac C4, 250 mm × 4.6 mm ID) connected to a HPLC system (Agilent 1100, USA). HPLC solvent A was H_2O (0.1% TFA). Solvent B was acetonitrile (0.1% TFA). A linear gradient from 39%

to 49% of solvent B in 30 min was used with a flow rate of 1 ml/min. Absorbance was recorded at 280 nm.

For the purity analysis of purified rh-ngEpo, 100 μl protein sample (1 mg/ml) was applied. A linear gradient from 5% to 95% of solvent B in 30 min was used.

2.8. HPLC–MS analysis of disulfide linkages

Purified rh-ngEpo was buffer-exchanged into 50 mM NH_4HCO_3 (pH 8.0) containing 2 M urea, using a 5 ml Hitrap desalting column (GE Healthcare). Trypsin (sequence grade) was added with an enzyme to substrate ratio of 1:50. Reaction was carried out at 37 °C overnight. One portion of the digest was then reduced by treatment with DTT.

The non-reduced and reduced peptide fragments of rh-ngEpo were then analyzed by HPLC–MS. The online chromatographic separation was performed by reversed-phased chromatography on an Agilent Zorbax SB C18 column (150 mm × 2.1 mm ID) using Agilent 1100 system. Solvent A was H_2O (0.1% TFA). Solvent B was acetonitrile (0.1% TFA). A linear gradient of 20 min was used with a flow rate of 0.2 ml/min. The outlet of the column was introduced into the ion source of electrospray ionization mass spectrometer (LCQ DecaXP, Thermo Electron, USA). A spray voltage of 4.5 kV was employed and the heat capillary was kept at 275 °C. The mass spectrometer was scanned from m/z = 300 to 2000 in positive ion mode. The mass spectra before and after reduction were analyzed. The fragments that were lost upon reduction were assumed to be involved in disulfide bridges.

2.9. Far-UV circular dichroism analysis

Measurements of far-UV circular dichroism (CD) spectra were taken on a JASCO J-810 spectropolarimeter using a 1 mm pathlength cuvette. The scanning was performed between 250 nm and 200 nm at 500 nm/min with a bandpass of 1 nm. Each spectrum was obtained after an average of three scans. Spectra were obtained at room temperature and blank buffer subtraction was used for baseline correction.

2.10. Cell proliferation assays

The human UT7/Epo cell line (Komatsu et al., 1991) (Peking Union Medical College, China) was maintained in RPMI1640 media supplemented with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM glutamine, and 1 U/ml CHO cell expressed recombinant Epo (Sunshine Pharmaceutical Company, Shenyang, China) at 37 °C in a humidified atmosphere containing 5% CO_2. For bioassays, the cells were washed and resuspended at a concentration of 2×10^5 cells/ml in RPMI1640 media containing 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM glutamine. Each well of the 96-well plates was distributed 100 μl of cells and serial 2-fold dilutions of the samples were, respectively, added in duplicate to the wells. After incubation at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h, cellular growth was determined by colorimetric MTT assay (Mire-sluis and Thorpe, 1998).

2.11. Protein concentration determination

For cell proliferation assay and pharmacokinetics study, protein concentrations were determined using an A_{280} value of a 1 mg/ml solution as 1.24 (Narhi et al., 1991) for both unmodified and PEGylated rh-ngEpo. When referring to the mass of the PEGylated protein, the contribution of the PEG moiety is not included.

Rh-ngEpo concentrations during refolding and purification were determined by Bradford method using bovine serum albumin as standard protein (Bradford et al., 1976).

2.12. Preparation of monoPEGylated rh-ngEpo

Purified rh-ngEpo (1 mg/ml in 20 mM PB, pH 7.0) was treated with 8-fold molar excess of 20 kDa succinimidyl carbonyl PEG (SC-PEG, synthesized in our laboratory) for 2 h at 4 °C. The reaction mixture was then directly loaded onto a 5 ml Source 30 S (GE Healthcare) column that had been equilibrated in 20 mM PB (pH 7.0). After washing the column, bound protein was eluted with a linear gradient of 0–0.5 M NaCl for 8 column volumes. Protein peaks were collected and analyzed by SDS-PAGE.

2.13. Thermal stability

Rh-ngEpo, monoPEGylated rh-ngEpo (1 mg/ml in PB buffer, pH 6.5) were incubated at 37 °C. After 48 h incubation, samples were centrifuged at 18,000 × g for 10 min. The absorbance of supernatant was measured at 280 nm to determine the remaining protein quantity. CD spectra of the remaining soluble protein were also measured.

2.14. Pharmacokinetics studies

Three groups of three male Sprague–Dawley rats, weighing 270–290 g each, were used for this study. Animals received a single subcutaneous injection of rh-ngEpo, monoPEGylated rh-ngEpo or the CHO-expressed Epo (Sunshine Pharmaceutical Company) at a dose of 100 μg protein/kg body weight. Blood samples (0.4 ml) were drawn at 0.5 h, 2 h, 5 h, 10 h, 24 h, 48 h, and 72 h after injection into tubes containing EDTA-2K, and then centrifuged and the plasma samples were stored at −70 °C. Plasma samples were diluted appropriately and the protein concentrations were quantitated using human Epo ELISA kits (R&D).

3. Results

3.1. Preparation of rh-ngEpo inclusion body

Rh-ngEpo expression was induced 2.5 h after inoculation by adding 1 mM IPTG into the bioreactor (Fig. 1A). Fig. 1B revealed that in comparison with uninduced bacteria, induced cell expressed a polypeptide that corresponded to the predicted size of the rh-ngEpo (18,396 Da). Expression of rh-ngEpo showed no significant increase in proportion to the whole cell protein between 1 h and 4 h after induction. A total of 99 g wet weight bacteria was obtained from the 5 l culture. After sonication of the bacteria, inclusion body (15 g) containing the target protein was isolated by centrifugation and then washed by a buffer consisted of 1% Triton X-100 and 2 M urea to remove contaminants. After two rounds of washing, only 8.8 g wet pellet was left, and the purity of rh-ngEpo was only increased slightly from 54% to 59% (Fig. 2).

3.2. Optimization of refolding conditions

To date, different refolding strategies have been developed, and dilution with additives is considered to be an efficient and easy scale-up method (Tsumoto et al., 2003). As the refolding conditions optimal for a particular protein were not readily predictable, several typical additives were chosen to check their effectiveness on rh-ngEpo refolding. The results were shown in Fig. 3. As compared with only 27% mass recovery from no additive dilution, arginine (0.5 M), guanidine chloride (1 M), and urea (2 M) were all effective in suppressing the formation of insoluble aggregates during protein refolding with a mass recovery over 90%, while other additives have no significant or even adverse effects (Fig. 3A). It has been reported that there may be more than one refolded structures present in the refolding supernatant when refolding process

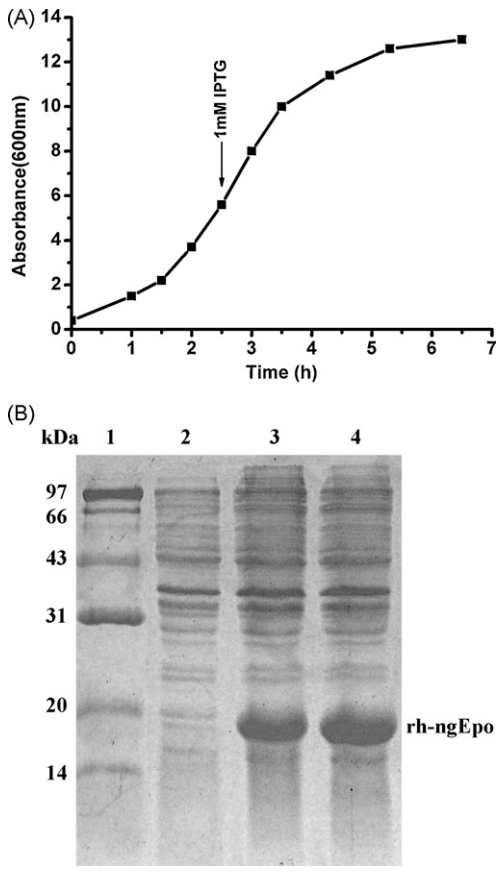

Fig. 1. Cell growth and expression during fermentation. (A) Cell growth curve. The curve is generated by measuring OD_{600} following inoculation of 500 ml LB media into 7 l bioreactor with 4.5 l starter media. Cells were induced 2.5 h after inoculation by addition of IPTG (1 mM final concentration). (B) SDS-PAGE analysis of protein expression. Lane 1: molecular weight standards; lane 2: cell lysate before induction; lane 3: cell lysate 1 h after induction; lane 4: cell lysate 4 h after induction.

reaches equilibrium (De Bernardez Clark et al., 1998). Therefore refolded samples were subjected to HPSEC and RP-HPLC analysis to investigate whether all the soluble protein formed the productive structure (Fig. 3B and C). It could be seen that although the three additives were all effective inhibitors of insoluble aggregates, they differed greatly in promoting the formation of active monomer.

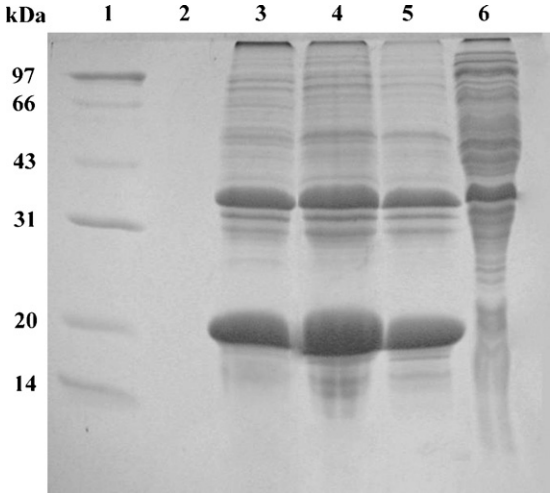

Fig. 2. Inclusion body washing. Lane 1: molecular weight standards; lane 2: blank; lane 3: crude inclusion body; lane 4: inclusion body washed one time; lane 5: inclusion body washed 2 times; lane 6: supernatant after sonication.

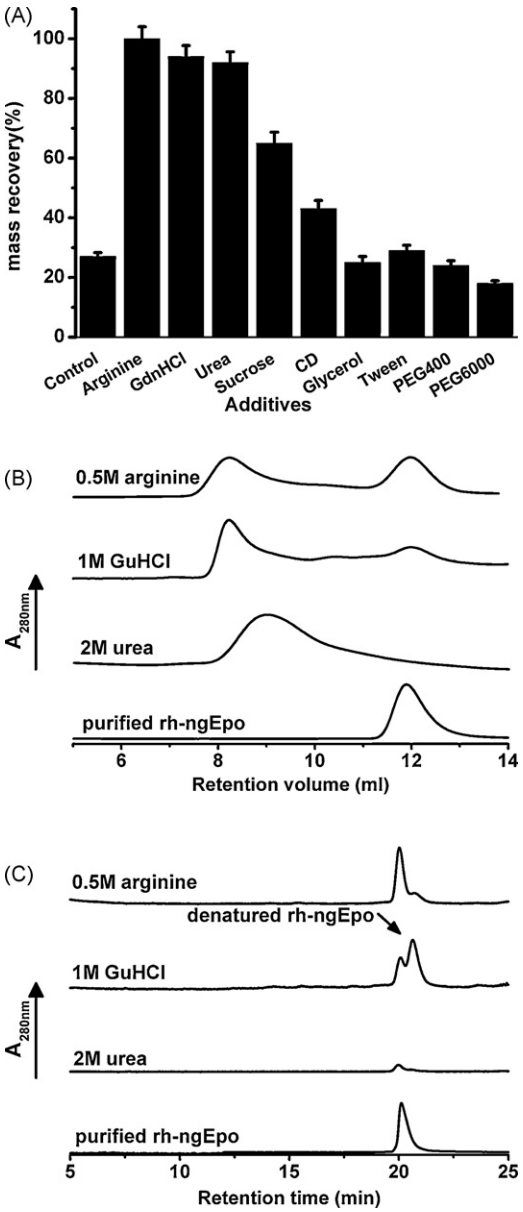

Fig. 3. Effects of additives on rh-ngEpo refolding. Denatured rh-ngEpo was diluted into refolding buffers with various additives at 4 °C. Final protein concentration was 100 μg/ml. (A) Effects of additives on protein mass recovery. Mass recovery is the ratio of the soluble proteins mass to the sample mass from denatured protein. (B) HPSEC analyses of refolded rh-ngEpo with additives of 0.5 M arginine, 1 M guanidine chloride or 2 M urea. A Superdex 75 HR (300 mm × 10 mm ID) was used. (C) RP-HPLC analyses of refolded rh-ngEpo with additives of 0.5 M arginine, 1 M guanidine chloride or 2 M urea. A RP-HPLC column (Vydac C4, 250 mm × 4.6 mm ID) was used. The analyses were carried out 24 h after protein refolding was initiated.

In the presence of 0.5 M arginine, 73% of the denatured rh-ngEpo refolded into the monomeric form. In contrast, a smaller monomer peak was detected in the presence of 1 M guanidine chloride and almost no such peak appeared in 2 M urea. Combined with RP-HPLC analyses, it can be concluded that in the presence of 2 M urea, the denatured protein refolded in the form of soluble aggregates and eluted together with the unwashed contaminants in the HPSEC analysis, while the low refolding yield obtained in 1 M guanidine chloride was due to the accumulation of unfolded structures. These results suggested that arginine acted as a more efficient suppressor of soluble aggregates than urea, and also has a much less denaturing effect than guanidine chloride, hence facilitating the correct on-pathway refolding of rh-ngEpo.

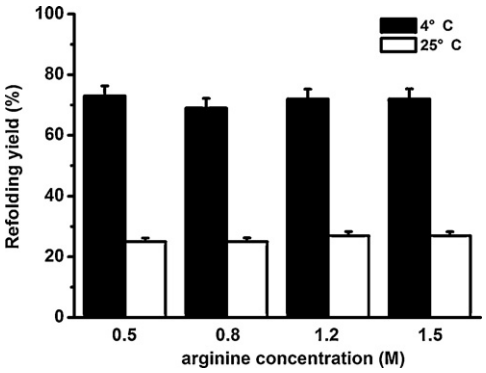

Fig. 4. Effects of arginine concentration and temperature on the refolding yield of rh-ngEpo. Denatured rh-ngEpo was diluted into refolding buffers with different concentrations of arginine at 4 °C or 25 °C. Final protein concentration was 100 μg/ml. Refolding yields were calculated from HPSEC analysis results.

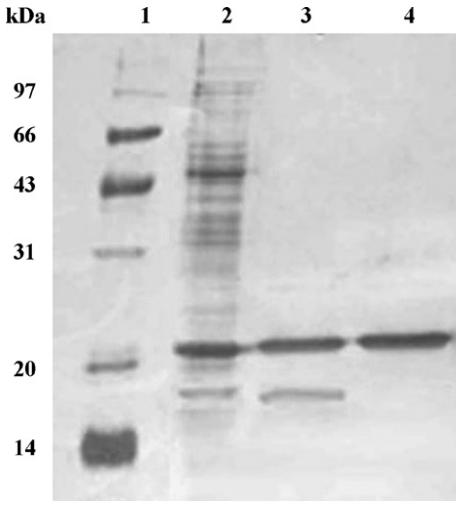

Fig. 5. SDS-PAGE analysis of rh-ngEpo during purification. Lane 1: molecular weight standards; lane 2: dialysate mixture; lane 3: protein peak eluted from IEC; lane 4: major protein peak eluted from GF.

The effects of temperature and arginine concentration on the refolding yield of rh-ngEpo were also investigated. As shown in Fig. 4, refolding yields were much higher at 4 °C than at 25 °C when arginine concentration increased from 0.5 M to 1.5 M, and arginine concentration had little effect on the refolding yield at the same temperature. The great gap in refolding yields between 4 °C and 25 °C were attributed to the different amount of soluble rh-ngEpo aggregates formation, as indicated by HPSEC analyses (data not shown). It was reported that hydrophobic interaction has a strong dependence on temperature in the refolding of bovine carbonic anhydrase II (Xie and Wetlaufer, 1996). Raising the temperature may enhance the hydrophobic interaction among refolding rh-ngEpo intermediates, resulting in more aggregated protein at 25 °C than at 4 °C. Finally, the optimal refolding condition adopted was 20 mM Tris–HCl (pH 8.5) containing 0.5 M arginine at 4 °C with a protein concentration of 100 μg/ml.

3.3. Purification of refolded rh-ngEpo

The refolded rh-ngEpo was dialyzed to remove arginine and the dialysate was then clarified by centrifugation. After pH was adjusted to 7.5, the dialysate was loaded onto a CM Sepharose column equilibrated with 20 mM Tris–HCl (pH 7.5) since non-glycosylated Epo has an isoelectric point of 9.2 (Davis et al., 1987). After washing, the bound protein was eluted directly by 0.5 M NaCl in a major protein peak. SDS-PAGE analysis showed that most high molecular weight impurities were removed from the dialysate mixture, but a relatively small amount of low molecular weight contaminant was coeluted with rh-ngEpo (Fig. 5). Although a linear salt gradient (0–0.5 M NaCl) across 10 column volumes was previously performed, little improvement of resolution was obtained. Therefore, a polishing step of size-exclusion chromatography (Superdex 75 prep grade) was adopted to finally separate rh-ngEpo to homogeneity, as suggested by SDS-PAGE analysis (Fig. 5).

3.4. Productivity enhanced by new process scheme

It was reported that contaminants, especially with high molecular weights, would greatly decrease protein renaturation efficiency and overall yield (Ouellette et al., 2003). Our results showed that the purity of inclusion body only increased a little after the two washing steps. To avoid the negative impact of remaining contaminants on the refolding and purification process, a new scheme was designed that adopted a chromatographic method to purify the protein from inclusion body in its denatured state. SP Sepharose Fast Flow resin (GE Healthcare) was selected for the purification under denaturing conditions. 2-Mercaptoethanol was included in the mobile phase to prevent possible undesirable oxidization during purification. As seen in Fig. 6A and B, most of the contaminants flowed through the column, and rh-ngEpo was then eluted in denaturing buffer with a much higher purity (>90%) than that of the washed inclusion body (59%). The eluted and denatured rh-ngEpo was then refolded at the optimal condition determined before. Fig. 6C shows that as high as 87% of the refolded rh-ngEpo was in monomeric state, thanks to the effective removal of contaminants. After dialysis and one step of weak cation-exchange purification, a protein purity of 98% was reached as determined by HPSEC and RP-HPLC analyses (Fig. 7). Final protein yield was greatly increased from 17% to 44%. Step recoveries of rh-ngEpo in these two schemes were summarized in Fig. 8.

3.5. Characterization of the purified rh-ngEpo

Fig. 9 was the far-UV circular dichroism spectra of purified rh-ngEpo and CHO-expressed Epo (Sunshine Pharmaceutical Company). It could be seen that they have almost the same profile with the characteristic minima observed at 208 nm and 222 nm, indicating that rh-ngEpo had the same second structure as the fully glycosylated one. Epo has two internal disulfide bonds: Cys7–Cys161 and Cys29–Cys33. Cys7–Cys161 is crucial for the stability of the molecular structure of Epo (Boissel et al., 1993). We unambiguously located the two disulfide bonds from proteolytic digestion followed by ESI-MS analysis (data not shown). The *in vitro* bioactivity of the purified rh-ngEpo was also assessed by measuring its ability in inducing proliferation of the Epo-dependent UT-7/Epo cells using MTT assay. In comparison with the CHO-expressed Epo (Sunshine Pharmaceutical Company), rh-ngEpo showed a minor decrease of activity (Table 1). The observed minor reduction of *in vitro* activity is likely to be due to the physical instability of the *E. coli* expressed Epo during long hours (48 h) of incubation

Table 1
In vitro bioactivities of rh-ngEpo, monoPEGylated rh-ngEpo and CHO-expressed Epo in a cell proliferation assay.

Sample	EC_{50} (ng/ml)
rh-ngEpo	0.40
monoPEGylated rh-ngEpo	0.58
CHO-expressed Epo	0.36

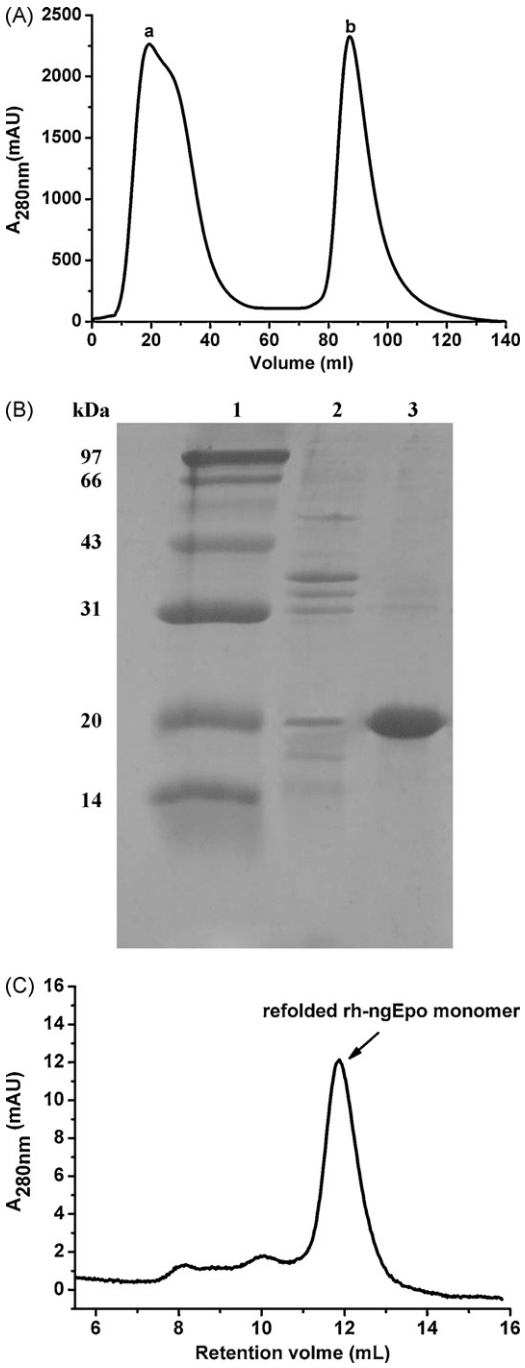

Fig. 6. Purification of denatured rh-ngEpo from solubilized inclusion body and its effect on refolding. (A) Ion-exchange chromatogram of solubilized inclusion body. 25 ml SP Sepharose Fast Flow was packed in an XK column (200 mm × 16 mm ID). Sample: inclusion body solubilized in 20 mM Tris–HCl, 8 M urea, 50 mM 2-mercaptoethanol, pH 7.0. Protein concentration was 10 mg/ml; 15 ml sample was loaded onto the column equilibrated with buffer C: 20 mM Tris–HCl (pH 7.0) containing 8 M urea and 20 mM 2-mercaptoethanol. Peak a flowed through the column, and peak b was then eluted by buffer D: 20 mM Tris–HCl (pH 7.0) containing 8 M urea, 20 mM 2-mercaptoethanol and 0.7 M NaCl. (B) SDS-PAGE analysis. Lane 1: molecular weight standards; lane 2: peak a; lane 3: peak b. (C) HPSEC analysis of refolded rh-ngEpo after denaturing purification. A Superdex 75 HR column (300 mm × 10 mm ID) was used.

at 37 °C. However, the immunoreactivity of rh-ngEpo assayed by ELISA was 2.13×10^5 IU/mg, even higher than that of the glycosylated form (1.81×10^5 IU/mg). The sugar chains on the surface of the CHO-expressed Epo may spatially hinder the antigen–antibody interaction, leading to the minor reduction of immunoreactivity.

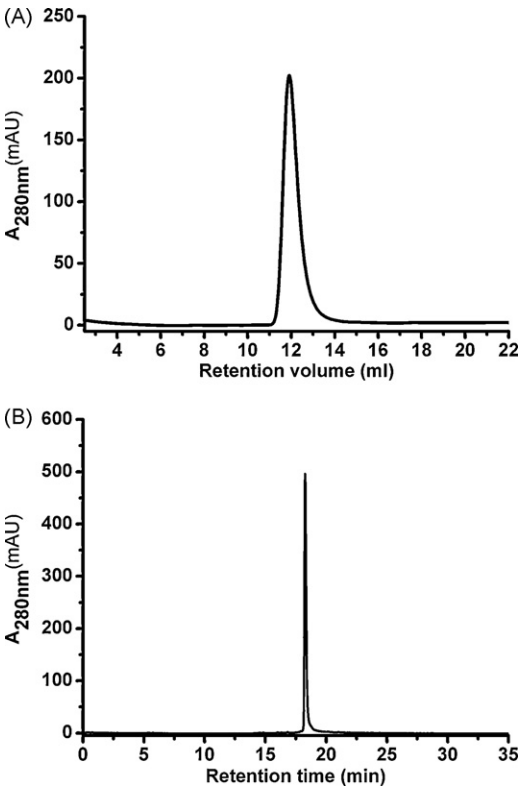

Fig. 7. Purity analyses of purified rh-ngEpo after the new process scheme. (A) HPSEC analysis of the purified rh-ngEpo. 120 μg of purified rh-ngEpo was analyzed on a Superdex 75 HR column (300 mm × 10 mm ID). (B) RP-HPLC analysis of the purified rh-ngEpo. 100 μg of purified rh-ngEpo was loaded on a Vydac C4 column (250 mm × 4.6 mm ID).

3.6. Preparation of monoPEGylated rh-ngEpo

With the aim of preparing a PEGylated form of rh-ngEpo with improved pharmacokinetic properties, rh-ngEpo was modified with 20 kDa mPEG-succinimidyl carbonate. The N-hydroxysuccinimide active ester derivative was chosen because of the mild reaction conditions required for coupling under which stability of rh-ngEpo could be maintained at high concentrations of 1–2 mg/ml. The unmodified, monoPEGylated and diPEGylated forms were separated by a cation-exchanger Source 30 S. Fig. 10 shows that the targeted singly modified protein was purified to homogeneity. Cell proliferation assays revealed that the monoPEGylated rh-ngEpo retained approximately 68% bioactivity of that of the non-glycosylated Epo (Table 1).

3.7. Thermal stability

The ability of PEGylation to protect rh-ngEpo against thermal aggregation was tested at 37 °C. After 48 h incubation, rh-ngEpo precipitated and only less than 20% remained in the supernatant. In contrast, the monoPEGylated rh-ngEpo solution remained clear and above 90% of protein mass was recovered through centrifugation. The CD spectra showed that the helices structure of rh-ngEpo was greatly destroyed, while the monoPEGylated rh-ngEpo still maintained structural integrity (Fig. 11).

3.8. Pharmacokinetics evaluations

Fig. 12 illustrates the mean serum concentration–time curves obtained after subcutaneous administration of the unmodified, monoPEGylated rh-ngEpo or CHO-expressed Epo. The pharmacokinetic parameters were summarized in Table 2. Plasma

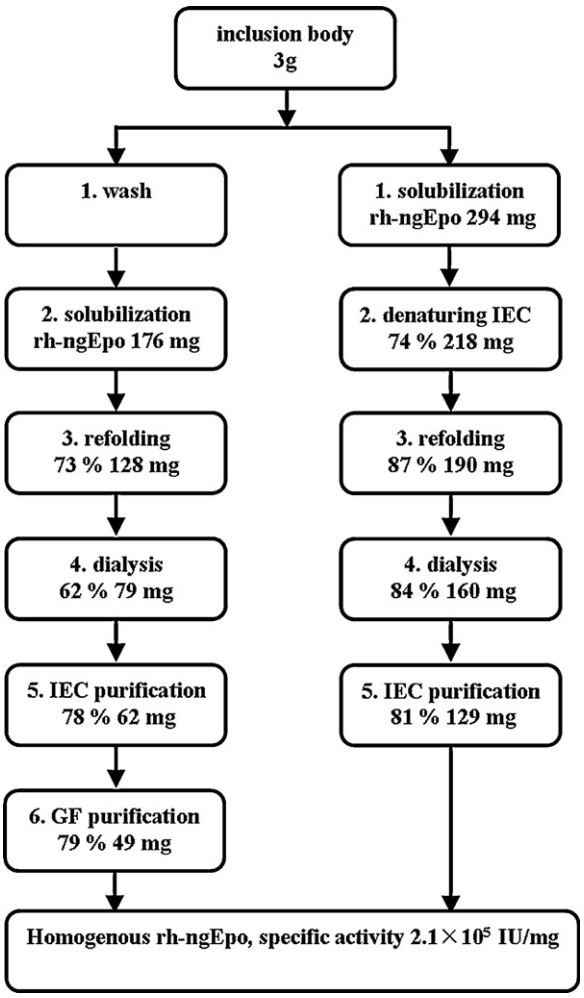

Fig. 8. Efficiency comparison of the two process schemes. Rh-ngEpo yield of each step was indicated. Contents of rh-ngEpo was assessed by densitometry of SDS-PAGE stained by Coomassie blue.

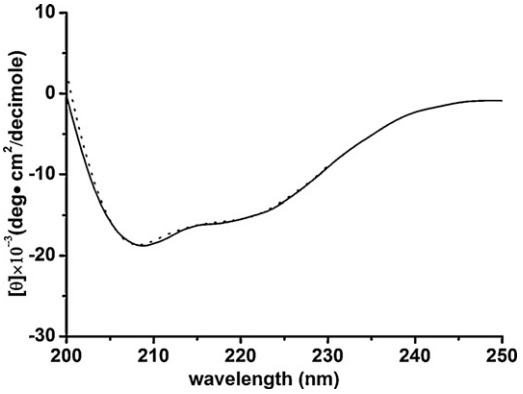

Fig. 9. CD spectra of purified rh-ngEpo (solid line) and CHO-expressed Epo (dotted line). Measurements were taken on a JASCO J-810 spectropolarimeter. The sample concentration was 0.2 mg/ml.

Table 2
Summary of pharmacokinetic parameters of rh-ngEpo, monoPEGylated rh-ngEpo and CHO-expressed Epo.

Sample	AUC (ng·h/ml)	CL (ml/h/kg)	T_{max} (h)	$T_{1/2}$ (h)
rh-ngEpo	148	676	0.5	1.6
monoPEGylated rh-ngEpo	2165	46	11	18.2
CHO-expressed Epo	3308	30	15	20.6

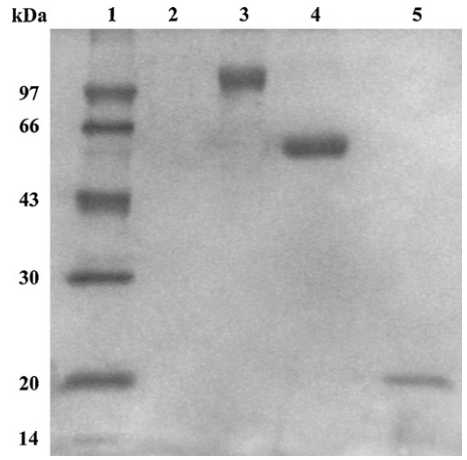

Fig. 10. SDS-PAGE of purified PEGylated and unmodified rh-ngEpo. Lane 1: molecular weight standards; lane 2: blank; lane 3: purified diPEGylated rh-ngEpo; lane 4: purified monoPEGylated rh-ngEpo; lane 5: purified unmodified rh-ngEpo.

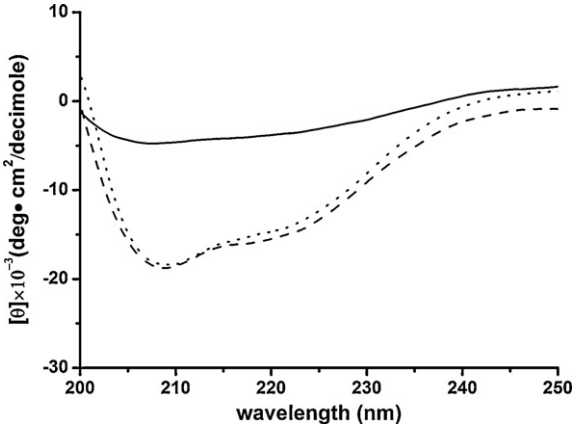

Fig. 11. CD spectra of remaining soluble rh-ngEpo (solid line), monoPEGylated rh-ngEpo (dotted line) after incubation at 37 °C for 48 h, and control purified rh-ngEpo (dashed line). Measurements were taken on a JASCO J-810 spectropolarimeter.

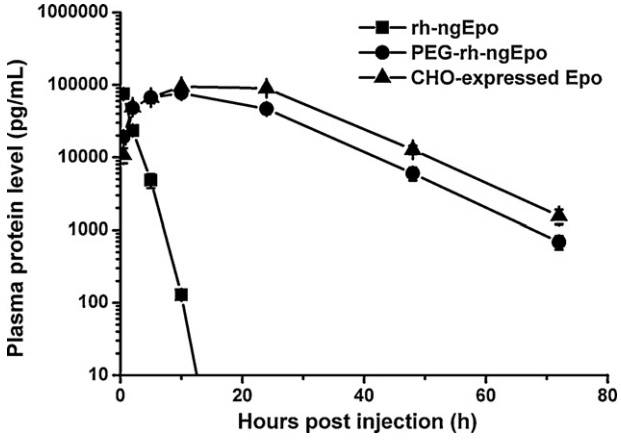

Fig. 12. Mean serum activity versus time of rh-ngEpo, monoPEGylated rh-ngEpo and CHO-expressed Epo after subcutaneous injection in rats. Data are means ± SD for three rats per group. Each rat received a 100 μg protein/kg dose. Plasma levels of the proteins were measured by ELISA.

concentrations of rh-ngEpo peaked at 0.5 h postinjection, then decreased rapidly, and were undetectable about 15 h postinjection. In contrast, maximum plasma levels of the monoPEGylated and CHO-expressed Epo were not attained until at least 10 h postinjection. The level of systemic exposure AUC for the PEGylated protein group was also significantly increased compared with the rh-ngEpo-treated group. Terminal half-life for the PEGylated protein increased 11-fold compared with that of the unmodified one, but still shorter than that of the CHO-expressed Epo.

4. Discussion

Generally, recombinant protein expressed as inclusion body in *E. coli* is recovered as active protein by four conventional procedures in sequence: washing, denaturation, refolding and purification. However, the purity of rh-ngEpo inclusion body cannot be effectively improved by washing. The deleterious effect of impurities on the ensuing refolding and purification procedures, combined with the great loss of targeted protein during washing, resulted in a low final protein yield of 17%. A new procedure of chromatographic purification was therefore developed which greatly reduced rh-ngEpo loss and increased protein purity in comparison with the washing procedure (Fig. 8). On the other hand, the dialysate mixture in the previous process often turned cloudy when dialysis was completed and a centrifugation procedure was always needed to remove the protein precipitates. In contrast, the final dialysate in the new scheme remained much clearer and therefore can be loaded right onto the column. In addition to the aforementioned benefits, the laborious efforts put into the inclusion body washing step was also saved. Although an extra denaturing chromatography step was added in the new scheme, removal of the low-capacity and time-consuming gel filtration unit actually enhanced the overall efficiency. Our simple and cost-effective production of rh-ngEpo offers a helpful alternative for the production of *E. coli* expressed recombinant protein when the conventional inclusion body washing procedure was inefficient or even counterproductive.

The refolded and purified rh-ngEpo displayed full immunoreactivity and most of the *in vitro* bioactivity compared with the CHO-expressed fully glycosylated Epo. The two disulfide bonds and the secondary structure are also consistent with those of native human Epo. These data further confirmed that glycosylation is not necessary for the preservation of native conformation and *in vitro* bioactivity of erythropoietin.

An early research (Narhi et al., 1991) found that the non-glycosylated form of Epo is much less stable to thermal denaturation than the glycosylated molecule. Our investigations demonstrated that incorporation of a PEG molecule also enhanced the thermal stability and retarded the *in vivo* elimination of the unmodified protein. The PEGylated molecule also retained the hematopoietic ability of the fully glycosylated Epo in our initial *in vivo* activity assay in mice (data not shown). These results suggested that PEG have a similar protective function compared with carbohydrate chains on protein surface.

The monoPEGylated rh-ngEpo was assayed to suffer a moderate 32% loss of *in vitro* bioactivity compared with the parent Epo molecule. It was reported that rh-interferon-α2a was modified primarily at a histidine residue by SC-PEG at mildly acid pH and this PEG-His-interferon possessed higher specific bioactivity than those modified at amino groups (Wylie et al., 2001). It is highly possible that the two histidine residues (His32, His94) in rh-ngEpo also served as the sites of PEGylation, since the pH value adopted in our PEGylation reaction is similar to that of rh-interferon-α2a. Considering His32 and His94 are not critical in the ligand–receptor interaction (Cheetham et al., 1998; Syed et al., 1998), PEGylation at these sites may compensate for the possible great loss of bioactivity caused by random modifications on the functionally important lysine residues (Elliott et al., 1997; Cheetham et al., 1998; Syed et al., 1998). As a result, considerable bioactivity was retained in the PEG–rh-ngEpo conjugate.

As the capability of PEG replacing carbohydrate chains to enhance the structural stability and extend *in vivo* residence of rh-ngEpo have not been fully exploited, and there are a wide variety of PEG reagents commercially available with different linking chemistries, molecular weights, and structures, etc., future work should be directed towards optimization of PEG coupling strategies in order to further improve the pharmacokinetic properties of rh-ngEpo and better preserve its *in vitro* bioactivity. These efforts would be useful for maximizing the pharmacological efficacy of rh-ngEpo. The above "PEGylation replacing glycosylation" strategy provides opportunities for the development of new glycoprotein therapeutics.

Acknowledgments

The authors are thankful for the financial support from the National Nature Science Foundation of China (Contract Nos. 20976178, 20636010 and 20820102036) and The National 863 High-Tech Project (Contract No. 2007AA021604).

References

Boissel, J.P., Lee, W.R., Presnell, S.R., Cohen, F.E., Bunn, H.F., 1993. Erythropoietin structure–function relationships. Mutant proteins that test a model of tertiary structure. J. Biol. Chem. 268, 15983–15993.

Bradford, M.M., Mcrorie, R.A., Williams, W.L., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cheetham, J.C., Smith, D.M., Aoki, K.H., Stevenson, J.L., Hoeffel, T.J., Syed, R.S., Egrie, J., Harvey, T.S., 1998. NMR structure of human erythropoietin and a comparison to its receptor bound form. Nat. Struct. Biol. 5, 861–866.

Davis, J.M., Arakawa, T., Strickland, T.W., Yphantis, D.A., 1987. Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. Biochemistry 26, 2633–2638.

De Bernardez Clark, E., Hevehan, D., Szela, S., Reddy, J.M., 1998. Oxidative renaturation of hen egg-white lysozyme: folding vs aggregation. Biotechnol. Prog. 14, 47–54.

Dordal, M.S., Wang, F.F., Goldwasse, E., 1985. The role of carbohydrate in erythropoietin action. Endocrinology 116, 2293–2299.

Egrie, J.C., Dwyer, E., Browne, J.K., Hitz, A., Lykos, M.A., 2003. Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. Exp. Hematol. 31, 290–299.

Elliott, S., Lorenzini, T., Chang, D., Barzilay, J., Delorme, E., 1997. Mapping of the active site of recombinant human erythropoietin. Blood 89, 493–502.

Higuchi, M., Oh-eda, M., Kuboniwa, H., Tomonoh, K., Shimonaka, Y., Ochi, N., 1992. Role of sugar chains in the expression of the biological activity of human erythropoietin. J. Biol. Chem. 267, 7703–7709.

Irani, N., Beccaria, A.J., Wagner, R., 2002. Expression of recombinant cytoplasmic yeast pyruvate carboxylase for the improvement of the production of human erythropoietin by recombinant BHK-21 cells. J. Biotechnol. 93, 269–282.

Komatsu, N., Nakauchi, H., Miwa, A., Ishihara, T., Eguchi, M., Moroi, M., Okada, M., Sato, Y., Wada, H., Yawata, Y., Suda, T., Miura, Y., 1991. Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. Cancer Res. 51, 341–348.

Krantz, S.B., 1991. Erythropoietin. Blood 77, 419–434.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Markham, A., Bryson, H.M., 1995. Epoetin alpha. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use in nonrenal applications. Drugs 49, 232–254.

Mire-sluis, A.R., Thorpe, R., 1998. Laboratory protocols for the quantitation of cytokines by bioassay using cytokine responsive cell lines. J. Immunol. Methods 211, 199–210.

Narhi, L.O., Arakawa, T., Aoki, K.H., Elmore, R., Rohde, M.F., Boone, T., Strickland, T.W., 1991. The effect of carbohydrate on the structure and stability of erythropoietin. J. Biol. Chem. 266, 23022–23026.

Narhi, L.O., Arakawa, T., Aoki, K., Wen, J., Elliott, S., Boone, T., Cheetham, J., 2001. Asn to Lys mutations at three sites which are N-glycosylated in the mammalian protein decrease the aggregation of *Escherichia coli*-derived erythropoietin. Protein Eng. 14, 135–140.

Ouellette, T., Destrau, S., Ouellette, T., Zhu, J., Roach, J.M., Coffman, J.D., Hecht, T., Lynch, J.E., Giardina, S.L., 2003. Production and purification of refolded recombinant human IL-7 from inclusion bodies. Protein Expr. Purif. 30, 156–166.

Park, J.H., Kim, C., Kim, W.B., Kim, Y.K., Lee, S.Y., Yang, J.M., 2000. Efficiency of promoter and cell line in high-level expression of erythropoietin. Biotechnol. Appl. Biochem. 32, 167–172.

Schriebl, K., Trummer, E., Lattenmayer, C., Weik, R., Kunert, R., Müller, D., Katinger, H., Vorauer-Uhl, K., 2006. Biochemical characterization of rh-ngEpo-Fc fusion protein expressed in CHO cells. Protein Expr. Purif. 49, 265–275.

Sharples, E.J., Thiemermann, C., Yaqoob, M.M., 2006. Novel applications of recombinant erythropoietin. Curr. Opin. Pharmacol. 6, 184–189.

Syed, R.S., Reid, S.W., Li, C., Cheetham, J.C., Aoki, K.H., Liu, B., Zhan, H., Osslund, T.D., Chirino, A.J., Zhang, J., Finer-Moore, J., Elliot, S., Sitney, K., Katz, B.A., Matthews, D.J., Wendoloski, J.J., Egrie, J., Stroud, R.M., 1998. Efficiency of signaling through cytokine receptors depends critically on receptor orientation. Nature 395, 511–516.

Tsumoto, K., Ejima, D., Kumagai, I., Arakawa, T., 2003. Practical considerations in refolding proteins from inclusion bodies. Protein Expr. Purif. 28, 1–8.

Veronese, F.M., Gianfranco, P., 2005. PEGylation, successful approach to drug delivery. Drug Discov. Today 10, 1451–1458.

Wang, M.D., Yang, M., Huzel, N., Butler, M., 2002. Erythropoietin production from CHO cells grown by continuous culture in a fluidized-bed bioreactor. Biotechnol. Bioeng. 77, 194–203.

Wasley, L.C., Timony, G., Murtha, P., Stoudemire, J., Dorner, A.J., Caro, J., Krieger, M., Kaufman, R.J., 1991. The importance of N- and O-linked oligosaccharides for the biosynthesis and *in vitro* and *in vivo* biologic activities of erythropoietin. Blood 77, 2624–2632.

Wylie, D.C., Voloch, M., Lee, S., Liu, Y.H., Cannon-Carlson, S., Cutler, C., Pramanik, B., 2001. Carboxyalkylated histidine is a pH-dependent product of PEGylation with SC-PEG. Pharm. Res. 18, 1354–1360.

Xie, Y., Wetlaufer, D.B., 1996. Control of aggregation in protein refolding: the temperature-leap tactic. Protein Sci. 5, 517–523.

Yamaguchi, K., Akai, K., Kawanishi, G., Ueda, M., Masida, S., Sasaki, R., 1991. Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties. J. Biol. Chem. 266, 20434–20439.

Zanette, D., Soffientini, A., Sottani, C., Sarubbi, E., 2003. Evaluation of phenylboronate agarose for industrial-scale purification of erythropoietin from mammalian cell cultures. J. Biotechnol. 101, 275–287.