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Molecular Design, Expression and Evaluation of PASylated Human Recombinant Erythropoietin with Enhanced Functional Properties

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Abstract   Erythropoietin (EPO) is the principal hormone which, has somewhat short half-life involved in the differentiation and regulation of circulating red blood cells. The present study was carried out to evaluate the capability of a polyethylene glycol mimetic technology as a biological alternative to improve pharmaceutical properties of human recombinant EPO. In silico models of EPO fused to 200 amino acids of proline, alanine, and serine (PAS) were initially generated and assessed by molecular dynamic (MD) simulation. The fluctuations of the modeled structure reached a plateau after 6000 ps of MD simulation. The Phi and psi analysis showed >99.2% of residues were located in the allowed regions. An expression vector consisting of EPO cDNA tagged to PAS coding sequences was synthesized and expressed in CHO-K1 Cells. The produced PASylated molecule was purified and characterized by standard analytical methods. The molecular weight of fusion protein was expanded to 70 kDa using sodium dodecyl sulfate polyacrylamide gel electrophoresis method. Analytical size exclusion chromatography revealed an approximately sevenfold increase in apparent size of produced protein. Although the in vitro potency of the fusion protein was significantly reduced (1.26 ± 0.05 vs. 0.24 ± 0.03 ng/ml) but, the in vivo activity was considerably increased up to 1.58×10^5 IU/ml in normocythemic mice assay. Pharmacokinetic animal studies revealed strongly 15.6-fold plasma half-life extension for the PASylated EPO (83.16 ± 13.28 h) in comparison to epoetin α (8.5 ± 2.4 h) and darbepoetin α (25.3 ± 2.2h).

Keywords   Biological activity · Erythropoietin · Molecular dynamic · PASylation · Plasma half-life · Pharmacokinetic properties

Abbreviations

PAS	Proline, alanine, serine
rHuEPO	Recombinant human erythropoietin
PEG	Poly ethylene glycol
CD	Circular dichroism
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
DMSO	Dimethyl sulfoxide
$t_{1/2}$	Terminal half-life
RMSD	Root-mean-square deviation
CZE	Capillary zone electrophoresis

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1 Introduction

Endogenous human erythropoietin (EPO) is produced by special cells in kidney and liver. It is an essential hormone that regulates erythropoiesis through a natural mechanism

[1, 2]. EPO also has protective and antiapoptotic actions on some differentiated tissues that were associated with non-hematopoietic functions of this vital hormone [2, 3]. The protein is specially delivered because of decreasing in red blood cells or an insufficiency in the accumulation of oxygen in circulation. As a result, those who have any deficiency in this characteristic mechanism may need to take an erythropoiesis stimulating drug to moderate the resulting anemia [3]. As shown, the matured human recombinant erythropoietin (rHuEPO) consists of 165 amino acids, that construct a 30.4 kDa single glycoprotein chain [4] Moreover, three N-linked (Asn^{24}, Asn^{38}, Asn^{83}) and one O-linked (Ser^{126}) consensus residues are involved in creating the normal glycosylation pattern of the protein [4, 5]. Actually, this carbohydrate moiety affects the plasma stability and the function of this therapeutic protein [6, 7] .The rHuEPO has many clinical applications. The most popular use is in people with anemia caused by chronic kidney dysfunction. Erythropoietin may use in another case of anemia associated conditions, including AIDS and cancer therapy [8]. Low plasma stability of EPO results in rapid clearance of the protein. So, rHuEPO has a short half-life of 4–13 h after i.v. administration. Accordingly, a variety of strategies have been used to extend the serum half-life of this protein during the past few years. This goal was achieved by a couple of genetic or chemical manipulations. Among these methods, the optimization of the hydrodynamic volume of the molecule was one of the most efficient technique. In this approach, the kidney filtration of the protein could be precisely regulated. Consequently, the pharmaceutical properties of the proteins will be improved by fewer dose administration during therapeutic applications [9].

Therefore, several attempts were made to produce new derivatives of erythropoietin during the last decade with some advantages and disadvantages. Darbepoetin-alfa (Aranesp®), was a hyperglycosylated analogue of erythropoietin that genetically engineered to contain five N-linked carbohydrate chains with a higher molecular weight, and a 3-times longer half-life than epoetin Alfa (Procrit®, Epogen®) in humans [10]. However, the CERA was a PEGylated form of EPO, which has an increased serum half-life depends on the extended hydrodynamic radius and the biological mass of the conjugated protein [11]. Although, the PEGylation technology of a therapeutic protein is an approved method, but often leads to less receptor binding affinity and decreased in vitro efficacy. Moreover, the PEGylation reaction is expensive and requires further downstream processing and purification steps, which raising the total costs of production. In addition, polyethylene glycol (PEG) is non-degradable and causes some side effects such as renal vacuolation upon continuous treatment [12]. Above limitations of the PEGylation strategy encouraged scientists to search for biological alternative polymers

mimetic of the chemical PEG chains. Thus, PASylation technology was developed by XL-Protein GmbH company resulting of Arne Skerra et al. primary research. In this technology, a flexible and uncharged PAS polypeptide chain with a range of 200–600 amino acid residues of proline, alanine and serine can be fused to the N-terminal and/or C-terminal of desired small protein. Consequently, this fragment creates a stable random coil structure and occupies a much larger hydrodynamic volume, which resulting in plasma half-life extension by a factor of 10–100 [13].

In an endeavor to assess the capability of this new technology in the case of the erythropoietin molecule, we attempt to design a preliminary EPO-PAS fusion gene construct to provide a novel human recombinant erythropoietin with enhanced pharmacokinetic properties. We hypothesize that the PASylation of this protein will increase its circulation half-life with an extended in vivo biological activity.

2 Materials and Methods

2.1 Molecular Modeling

Protein sequence of the human EPO was obtained from UniProt knowledge base (Universal Protein Resource) under the IDs of P01588 at http://www.uniprot.org. The PAS sequence, forming random coil conformation, was obtained from WO 2008 155,134 A1(PAS #1; SEQ ID NO: 18). To perform modeling predictions, NCBI protein BLAST tool at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used to achieve a suitable template structure for EPO. The BLAST program was restricted for Homo sapience while protein data bank was set to be the target database; all other parameters were set as default. Modeller software version 9.15 was employed to make 10,000 models using an obtained template (1EER, 1.9 Å, X-Ray Diffraction) for EPO sequence. PAS sequence was modeled using I-TASSER server based on threading fold recognition method. Then, two 3-D structures, EPO and PAS models, were used as new templates to obtain the full length of EPO-PAS model by Modeller software. Finally, a loop refinement procedure was carried out by ModLoop server (http://modbase.compbio.ucsf.edu/modloop/).

2.2 Molecular Dynamics (MD) Simulation

The molecular dynamics (MD) of the model was analyzed by MD simulation using GROMACS 4.5.3 package on RedHat Linux with OPLS-AA force field. First, the system was created by locating the protein in the center of a cubic box with 2.0 nm distance from the edges and next solvated by water molecules using a simple point charge model. The net charge of the system was changed to negative by adding

Cl-ions and, an energy minimization step was subsequently performed using the steepest descent integrator algorithm with a maximum force <1000 kJ mol^{-1} nm^{-1} and 50,000 steps. The periodic boundary condition was applied in x, y, and z directions. The system was equilibrated at 300 K and at 1 bar using Berendsen temperature coupling and Parrinello–Rahman algorithm, respectively. For Coulomb and van der Waals interactions, a 1.0 nm cut off and for long-range electrostatics calculations, particle mesh ewald (PME) was applied. MD run was carried out for 12 ns with a 2 fs time step, and the average structure was then calculated based on root mean square deviations (RMSD) during MD simulation. In addition, the quality of the generated model (φ and ψ angels) was assessed by RAMPAGE server. (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

2.3 Designing the Expression Construct

The gene cassette consisting of the human EPO cDNA (NCBI accession No. NM_000799) with a Kozak sequence at N-terminus and DNA encoding 200 PAS residues was designed and synthesized into pUC57 standard vector by Biomatik gene synthesis service (Biomatik, Inc. Canada). The repeated PAS sequence, consists of ASPAAPAPAS-PAAPAPSAPA (PAS #1; SEQ ID NO:18) as described in WO 2008 155,134 A1 [12] in tandem with six histidine amino acid (C-6×His-tag) at C-terminus. Codon optimization was done for high expression in CHO host cells. The gene was subcloned into the pCMV script expression vector (http://www.genomics.agilent.com). The presence and accuracy of the construction were confirmed by restriction analysis and DNA sequencing.

2.4 Transfection and Selection of High Expressing Cell Clones

CHO-K1 (1×10^5) cells (ATCC No. CCL-61) were grown in a 24-well culture plate (Greiner bio-one) in DMEM-F12 (1:1) medium. The growth medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen Inc.), without antibiotics. The procedure of transfection was carried out by lipofectamine 2000 (Gibco, Invitrogen, Inc.) at the time of 90–95% cell confluency. The cells incubated 48 h post-transfection in the growth medium (without G-418) to express the protein. The minimum concentration of G-418 to be added to the culture medium to prevent the cell growth has been established experimentally with medium containing increasing concentrations of 100–1500 µg G-418 ml^{-1}. The stable transfected cells obtained after 4 weeks under selection pressure. Resistant cell clones were analyzed with the EPO ELISA kit (Roche Diagnostics Gmbh, Mannheim, Germany) with a high-sensitivity sandwich enzyme-linked immunosorbent assay. The human recombinant EPO, calibrated against the second international reference preparation (67/343) was used as an international standard in this assay. The clone with the highest expression level of the EPO-PAS protein, was grown on selection medium for an additional 7 days before the expansion.

2.5 Expression and Purification of rHuEPO-PAS

Stably CHO-K1 transfected cells producing EPO-PAS were cultured in DMEM-F12 containing 10% of FBS and 20 µg gentamicin ml^{-1} (Sigma–Aldrich), in a humidified CO$_2$ incubator with 5% CO$_2$ at 37 °C. Routine subculture was conducted every 3–4 days. Cell Culture supernatants were collected by centrifugation at 180×g for 5 mins and filtered through 0.45 µm filter units to remove cell debris or other particulate materials. An ion immobilized metal affinity chromatography (IMAC) was applied for purification of the EPO-PAS using 5 ml Histrap columns (GE Healthcare Inc, Princeton, NJ). The column was placed in the AKTA Purifier 10, system (GE Healthcare) and a blank run was made using binding buffer (20 mm sodium phosphate, pH 7.4, containing 0.5 M NaCl and 10 mm imidazole). After sample loading and washing of the column with equilibration buffer, anchored His-tagged EPO-PAS fusion protein was eluted with 20 mm sodium phosphate, 0.5 M NaCl and 250 mm imidazole (pH 7.4) at a flow rate of 1 mL min^{-1}. Protein fractions were separated into the defined 2 ml microtubes. All the fractions containing EPO-PAS protein were pooled, buffer exchanged and adjusted to the desired concentration (10 folds) using Amicon centrifugal filter devices (Millipore). Finally, purified protein was aliquoted and stored at −70 °C for further analysis.

2.6 EPO-PAS Protein Analysis

The concentration of EPO-PAS protein at all steps was quantified with a high-sensitivity human EPO ELISA Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Concentrations of total purified proteins were measured using the Bradford method [14]. Protein samples were analyzed using the standard Laemmli SDS–PAGE method [15] on a 12.5% separating gel and visualized by staining the gel with Coomassie Brilliant Blue R-250 [16]. Western blot analysis was performed by transferring the proteins from the SDS–PAGE gel to Nitrocellulose blotting membrane (Sartorius) using a trans-blot apparatus (Bio-rad) according to the method described in the manual accompanying the unit. The membrane was incubated in 2% nonfat dry milk for 2 h at room temperature and was detected using the Anti-6X tag® antibody (HRP) (Abcam) with (1:2000 titers) overnight at 4 °C.

The bands were developed using DAB chromogenic substrate (Sigma–Aldrich) after three consecutive washes in PBS containing 0.1% Tween 20 (5 min/wash) at room temperature.

2.7 Size Exclusion Chromatography

Size exclusion chromatography (SEC) was performed using a waters 600 E multi solvent delivery system with a Dual λ Absorbance Detector Waters 2487 (USA). Operation and acquisition were performed using the millennium^{32} chromatography manager. Separation of the EPO-PAS fusion protein was carried out on a TOSOH TSK G3000PW column (7.5 mm×60.0 cm L); TOSOH Biosciences, Stuttgart, Germany) with a TSK guard column SW (7.5 mm×7.5 cm); TOSOH Biosciences, Stuttgart, Germany). 200 µl of 0.15 mg purified samples ml^{-1}were eluted in a 60 min run at a flow rate of 1 ml/min. Detection was performed at 280 nm. The mobile phase consisted of 1.7 mm potassium dihydrogen phosphate, 7.9 mm disodium hydrogen phosphate, 2.7 mm potassium chloride and 0.25 mm sodium chloride, pH 7.4, and was filtered through a 0.45 µm filter prior use. The apparent molecular weight of EPO-PAS fusion protein was estimated by interpolation from a calibration line obtained from the reference proteins in analytical SEC on Superdex 200 HR 10/300 GL column (Sigma–Aldrich) at a flow rate of 1 ml/min using single pump-based configurations.

2.8 Circular Dichroism (CD) Spectroscopy

Determination of protein secondary structure was performed using a J-810 spectropolarimeter (Tokyo, Japan) with a concentration of 0.25 mg sample ml^{-1} in 50 mM NaH$_2$PO$_4$ and 100 mM NaCl at room temperature. Data were collected at 1 nm intervals over the range of 250–190 nm for the far-UV spectra in a cell with a path length of 0.1 mm. The spectra were corrected for buffer contributions. The Quartz cuvettes of 1 mm in path length at 22 °C were used. The secondary structure of PASylated and not PASylated erythropoietin were quantified and compared by the spectra manager software (JASCO Corporation).

2.9 In Vitro Cell Based Assay

In vitro biological activity of EPO-PAS fusion protein was carried out on UT-7 (Acc137; DMSZ, Braunschweig, Germany) cells proliferation assay. The cells were grown in α-MEM medium supplemented with 10% (w/v) FBS, 20 µg gentamycin ml^{-1}, 2 mm L-glutamine and 1 ng GM-CSF ml^{-1} (Sigma–Aldrich). For bioassay, the cells were suspended in the medium without GM-CSF to a final concentration of 2×10^5 cells/ml. Serial two folds dilutions of EPO-PAS, and standard reference (NIBSC 88/574) were prepared in the assay medium. At first, 50 µl of the diluted protein sample was added to each well of a 96-well microplate in triplicate, and then 50 µl of the cell suspension was added to the wells. Negative and positive cell controls without EPO and 1 IU of standard ml^{-1} were included in the plate respectively. The plate was incubated for 48 h at 37 °C in a humidified 5% CO$_2$ incubator. The proliferation of the cells was evaluated by a modified colorimetric MTT of Mosmann briefly, 20 µl of a sterilized 5 mg MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] ml^{-1} solution was added to each culture well containing a final volume of 0.1 ml of cell suspension. After 3–5 h of incubation, the mitochondrial dehydrogenases of viable cells will cleave the tetrazolium ring, yielding dark purple formazan crystals, which are insoluble in aqueous solutions. 100 µl of dimethyl sulfoxide (DMSO; Sigma–Aldrich) was added to the wells and mixed thoroughly in microplate shaker to enhance dissolution crystals. The resulting purple solution is spectrophotometrically measured at a wavelength of 570 nm, using a microplate reader (TECAN, Sunrise) which is proportional to the cell's growth number [17, 18].

2.10 In Vivo Efficacy Assay

The biological activity of EPO-PAS fusion protein was estimated based on the measurement of stimulation of reticulocyte production in normocythaemic mice as prescribed in the European Pharmacopoeia monograph erythropoietin concentrated solution. The sample and standard groups of 8 weeks old, healthy B6D2F1 mice (18–20 g) were distributed into 6 cages. The animals were injected subcutaneously with three defined (10, 20 and 40 IU/mL) dose values of EPO-PAS test solutions and reference preparations (erythropoietin BRP batch 4, EDQM, E1515000). After 4 days, blood samples were collected from the animals. The samples were stained with 0.1 µg thiazole orange ml^{-1} in PBS1X (pH 7.2) and incubated for 30 min in the dark place at room temperature. The percentage of reticulocyte count of red blood cells (Ret%) was determined using flow cytometry (Beckman Coulter, DU 800, USA.) as described by B.H. Davis et al. [19]. The biological activity of the PASylated erythropoietin was calculated by the usual statistical methods for a parallel line assay compared with that of erythropoietin BRP and expressed in international units (IU).

2.11 In Vivo Half-Life Determination Of EPO-PAS

Animal research procedures were performed with the approval of the National Ethics Committee of the Ministry

of Health and Medical Education of Iran. All facilities, including food, waters, ventilation, space and unnecessary stress were conformed to the regulations, policies and guidelines regarding personnel, supervision and veterinary care. The five female balb-c mice in two groups (test and control) were received a single intravenous injection at a dose of 1.5 µg EPO Kg^{-1}. The human recombinant erythropoietin produced by Pasteur Institute of Iran was used as a control in this assay. At selected intervals after injection, blood samples (100 µl) were collected from orbital venous sinus of mice. The blood serum was separated by centrifugation at 550×g for 5 min, after leaving the tube in a standing position for about 20–30 min at room temperature. Serum samples were isolated by aspiration, stored immediately at −80 °C for further analysis. The circulating concentration of PASylated erythropoietin at different times were determined using human EPO ELISA kits (Roche Diagnostics GmbH, Germany) according to the method described in the manual accompanying the kit. The rate of disappearance of both EPO and EPO-PAS proteins were plotted versus related sampling times. The terminal half-life (t$_{1/2}$), and the other pharmacokinetic parameters were measured using a non-compartmental method (linear trapezoidal calculation of AUC) in Microsoft Excel 2013 [20].

2.12 Statistical Analysis

Statistical analysis was performed with Microsoft Office Excel 2013 and GraphPad prism 6.0 to draw the dose–response graphs. PLAS 3.0 software (Stegmann systems GmbH, Germany) was used to calculate the relative potency by parallel line analysis method. Data are expressed as mean ± SD. The level of statistical significance was set at $p < 0.05$.

3 Results

3.1 Molecular Modeling

ModLoop server was used to rectify the errors for the model built by Modeller software [21]. Loop remodeling was executed on two loops spanning residues between 110 and 120 and 355–366 residues of the model. That apparently matches to QMEAN residue error plot which, indicates a high residue error peak. Table 1 lists the estimated quality scores before and after refinement. Figure 1 shows the elimination of high residue error peaks of the model. The final achieved structure after refinement was shown in Fig. 2.

3.2 Molecular Dynamics (MD) Simulation

The measured root mean deviation of simulated EPO-PAS$_{200}$ indicates the stability of designed model in which the plot shows an equilibrium versus time around 6000 ps (Fig. 3). The final three-dimensional structure of EPO-PAS$_{200}$ was demonstrated in Fig. 4. The torsion angle values of the final model which predicted by Ramachandran plot exhibited that a lot of residues (99.2%) are staying inside the low energy allowed sections (Fig. 5).

Table 1 Quality scores of predicted and refined models

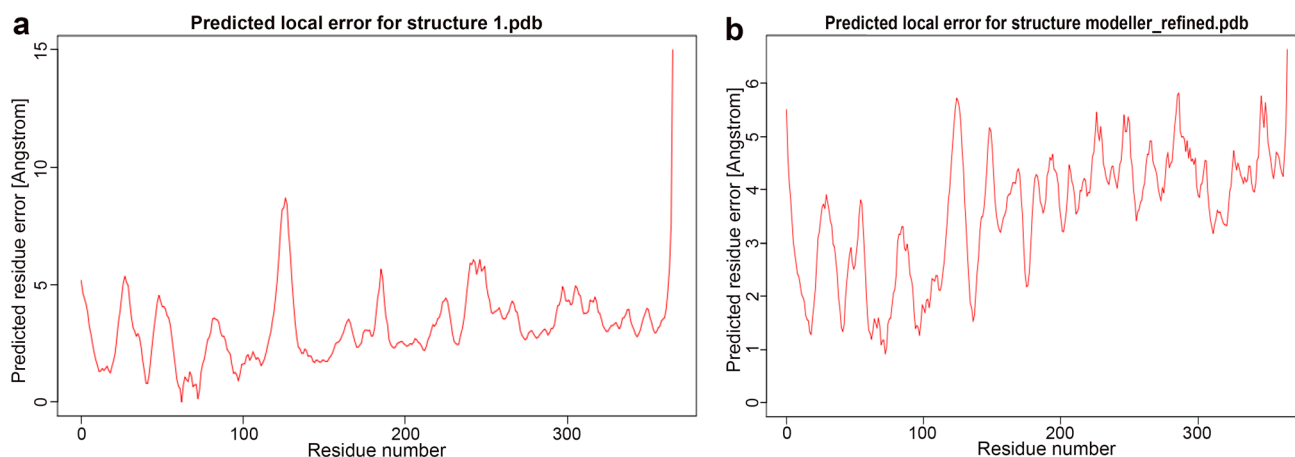| Tools	Z-score	
	Before refinement	After refinement
Modeller	−0.25	−0.04
I-TASSER	−1.55	−0.34

Fig. 1 QMEAN residue *error plots* before and after refinement of models
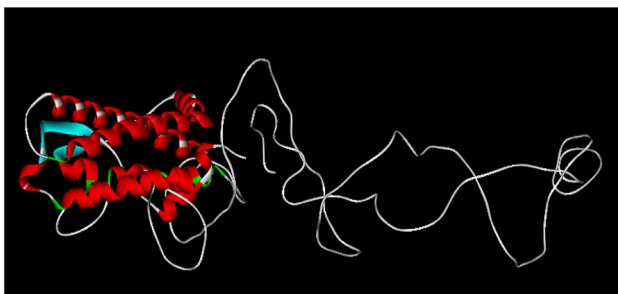
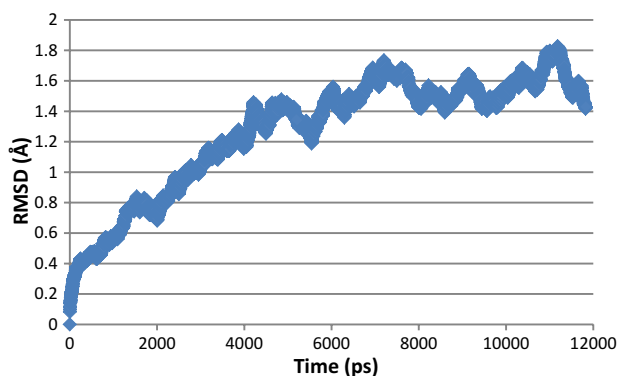**Fig. 2** Schematic representation of 3D-model after refinements by ModLoop server

Fig. 3 RMSD graph for EPO-PAS$_{200}$ residues after molecular dynamic simulation. The *plot* shows fluctuations and plateau states (6000 ps) of applied model
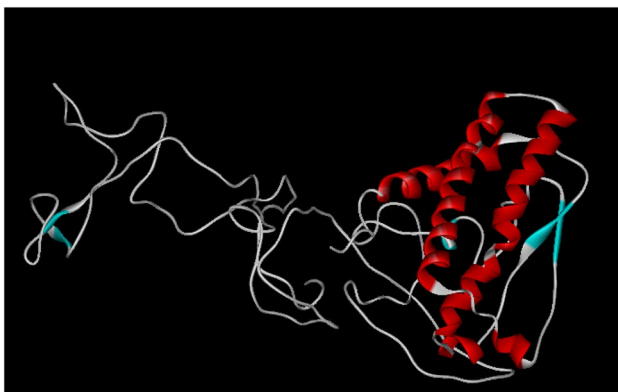

Fig. 4 Finally achieved model after 12 ns MD simulations

3.3 Construction of EPO-PAS Fusion Protein

The pCMV-Script expression vector (Agilent Technologies) including the PAS building blocks and the applied restriction sites were schematically demonstrated in Fig. 6. This vector has appropriate cloning sites, a strong CMV promoter and neomycin-resistance marker for selective optimal expression in CHO mammalian cells. The Kozak sequence as an optimal substitution of translation initiator has incorporated in the inserted DNA. The sequencing alignment results of the whole construct was consistent with the target and showed correct reading frame requirement. Restriction digestion (*Xho*I/*Not*I) analysis confirmed the size of the inserted fragment with no contaminated bands.

3.4 CHO Cell Cultivation and Production of EPO-PAS Fusion Protein

Transfected CHO cells were cultivated in a 175 cm^2 culture flask and harvested every 3 days with a mean cell density of 15×10^6 cells/flask and a viability of 90–95%. The average EPO-PAS$_{200}$ fusion protein cell-specific productivity of 0.01–0.02 mIU/cells/days was obtained by ELISA method.

3.5 Characterization of Purified EPO-PAS Fusion Protein

The specific band of approximately 70 kDa was detected by SDS–PAGE and Western blot analysis methods, which corresponded to the EPO-PAS$_{200}$ fusion protein (Fig. 7). The exact molecular weight and glycosylation characteristics of PASylated erythropoietin need to be determined by the more reliable analytical technique as mass spectrometry (MS) and capillary zone electrophoresis (CZE) using an established appropriate standard protein.

3.6 Size Exclusion Chromatography

The main peak of the EPO-PAS$_{200}$ fusion protein followed by the not PASylated EPO was determined by analytical SEC. Results showed a good level of purity and a distinct higher molecular weight of the EPO-PAS$_{200}$ corresponding to their related retention times. This observation indicated an increased hydrodynamic volume of protein through PASylation with significantly greater apparent size around 230 kDa. The peaks that were eluted at the approximately 15 and 19 ml of elution volume belong to the PASylated and unmodified erythropoietin molecules respectively (Fig. 8).

3.7 Circular Dichroism (CD) Spectroscopy

A circular dichroism spectrum was recorded for the PASylated and not PASylated EPO (Fig. 9). The far-UV spectrum of the unfused EPO exhibited the typical feature of the α-helical protein. However, the addition of PAS sequences of 200 amino acid residues to the C-terminal region of the EPO led to reveal a predominant negative
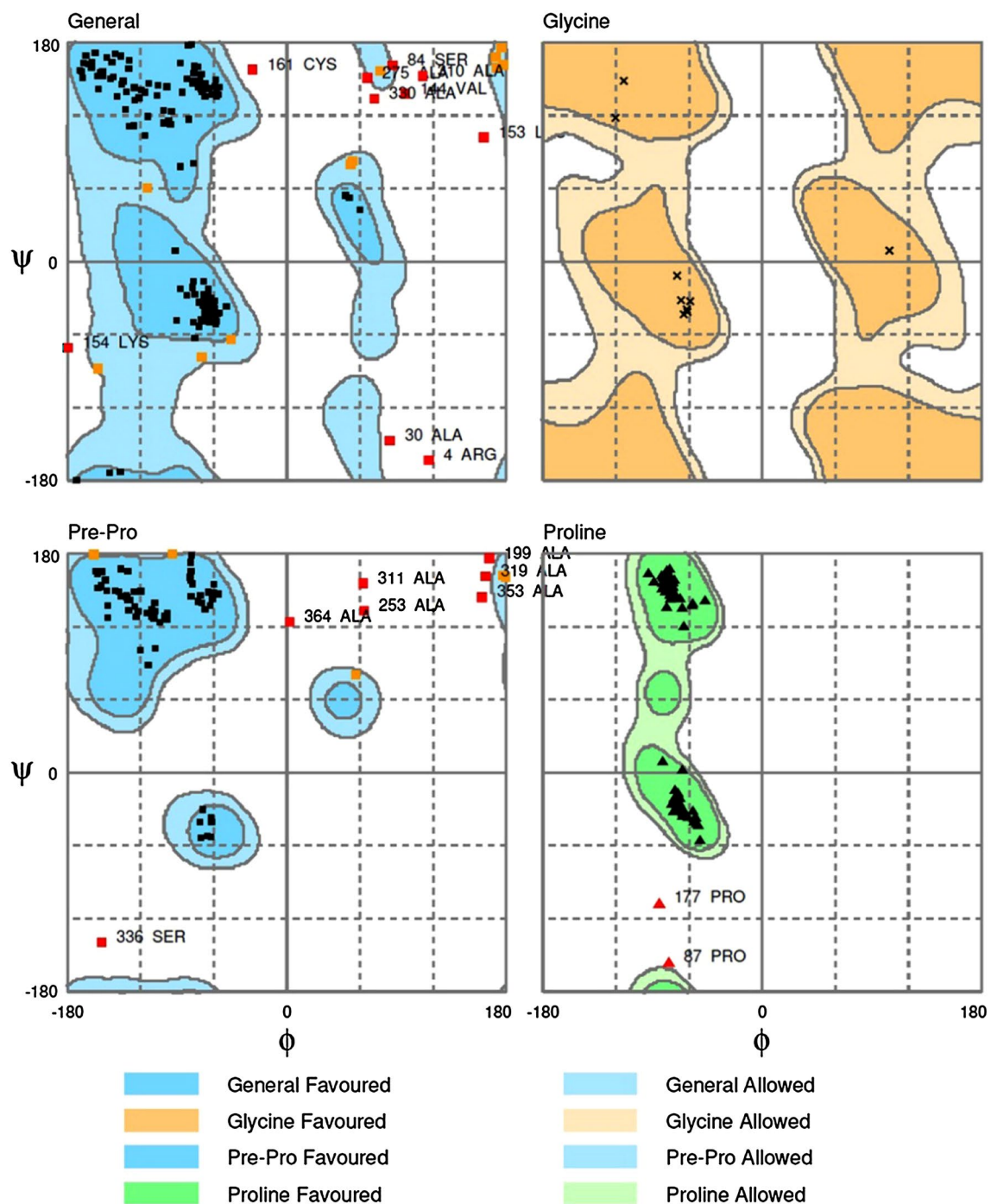
Fig. 5 Ramachandran plot of the final 3D structure of EPO-PAS$_{200}$. The *graph* illustrates the optimal conformation as more than 99.2% of residues are located in the allowed regions

minimum around 200 nm which is clearly indicative of increasing the percent of random coil structure in EPO-PAS fusion protein (60.5%) compared to the corresponding conventional EPO molecule (23.2%).

3.8 In Vivo Biological Activity of EPO-PAS Fusion Protein

The bioactivity of the EPO-PAS$_{200}$ fusion protein and the confidence limits of estimated potency (P = 0.95) were

Fig. 6 The schematic construction of expression vector for production of PASylated EPO in CHO cell line. EPO cDNA in tandem with PAS sequence repeats for defined PAS$_{200}$ fusion tag (PAS #1; SEQ ID NO:18), were cloned in between the two appropriate restriction sites (*Not*I and *Xho*I) in the pCMV-Script vector
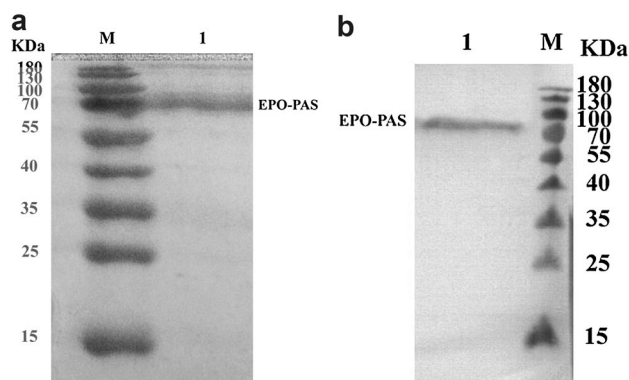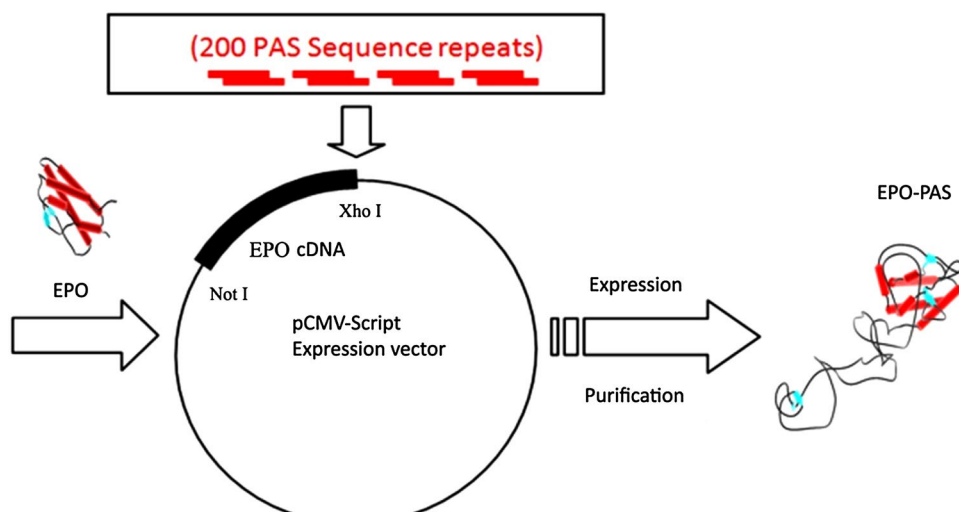

Fig. 7 Characterizing the purity of EPO-PAS$_{200}$ fusion protein. **a** SDS–PAGE analysis of EPO-PAS$_{200}$ stained with coomassie blue dye, after IMAC (*lane 1*). **b** Identification of EPO-PAS$_{200}$ fusion protein with 6×-His Tag® Antibody (HRP) (Abcam) in *lane 1* by western blot method. M; Page Ruler ^{TM} Prestained Protein Ladder (Thermo scientific)

calculated by the PLAS 3.0 software (Stegmann systems GmbH, Germany) for biostatistical linear regression analysis of the dose–response curves. The biological activity of EPO-PAS was estimated as 1.58×10^5 IU/mL relative to Erythropoietin BRP batch 4, EDQM, E1515000 as the reference standard. The dose–response relationship plot of the assay and the representative flowcytometry (FC) reticulocytes counting results for the fusion proteins and standard are shown in Fig. 10. The figures demonstrate the gating region utilized for accurate measurement of reticulocyte counts, separated from dead cells and debris. The gated percent of reticulocytes was obtained 23.68% for EPO-PAS$_{200}$ which, shows 4.45-fold increase related to the EPO standard (5.32%).

3.9 In Vitro Biological Activity of EPO-PAS Fusion Protein

In vitro potency of the EPO-PAS fusion protein in comparison of unmodified EPO standard was measured using UT-7 cell proliferative assay. Data analysis and calculating the EC$_{50}$ values was performed by GraphPad prism 6.0 software using non-linear regression model on each normalized dose–response curve. The estimated in vitro biological activity of EPO-PAS$_{200}$ fusion protein was 1.26 ± 0.05 ng/ml, which represented significantly reduction related to EPO standard (0.24 ± 0.03 ng/ml) protein (Fig. 11).

3.10 In Vivo Half-Life Determination

The mean terminal half-life ($t_{1/2}$) for not PASylated human recombinant erythropoietin was obtained to be 5.33 h from the plot of elimination rate. In contrast, PASylated erythropoietin was cleared much more slowly, with a half-life of 83.16 h in our experience that indicates 15.6 fold longer plasma half-life (Fig. 12). All calculated pharmacokinetic parameters, including AUC, volume of distribution and elimination rate (CL) were improved in EPO-PAS$_{200}$ comparing to EPO standard. (Table 2).

4 Discussion

Inadequate plasma durability of a protein is one of the most important factors that can result in prolonged and undesirable treatment. This limitation may cause insufficient accumulation of drugs at the targeted sites due to rapid kidney elimination time. For that reason, extensive researches have been done to improve the pharmacokinetic properties of drugs using various strategies. In the last decade, several
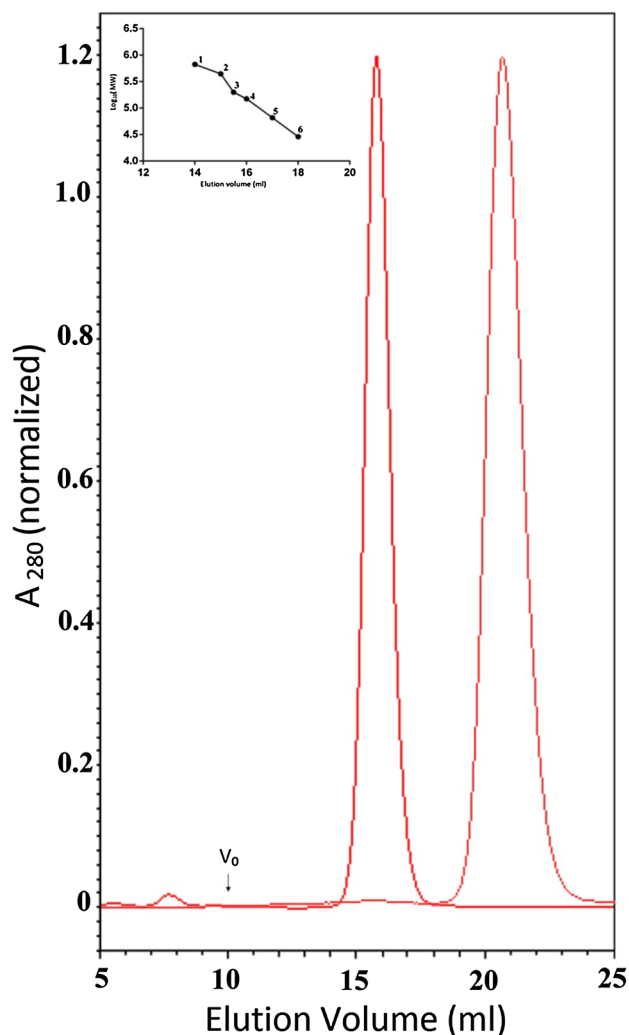
Fig. 8 Analytical size exclusion high-performance liquid chromatography of PASylated (*left* the peak at approximately 15 ml of elution volume) and unmodified EPO (*right* the peak at approximately 19 ml of elution volume) after injection. Gel filtration marker kit (MWGF1000, Sigma–Aldrich) including of erythrocytes carbonic anhydrase, serum albumin and thyroglobulin of bovine with a molecular mass of about 29, 66 and 669 kDa respectively), yeast alcohol dehydrogenase (~150 kDa), β-amylase of sweet potato (~200 kDa) and horse spleen apoferritin (~443 kDa). The apparent molecular mass of EPO-PAS fusion protein was estimated about 230 kDa after calibration with above standard proteins

types of proteins have been created through genetic engineering or chemical modifications to decrease the rapid clearance and limited effectiveness of these biopharmaceuticals. Human recombinant erythropoietin as a small diameter and low molecular mass protein has a very short circulating half-life (5–13 h). Since the efficiency of blood filtration through kidney glomeruli increases by size reduction of the molecule so, frequent administrations require in order to maintain a therapeutically effective dose over a prolonged period of medical applications. In recent years,
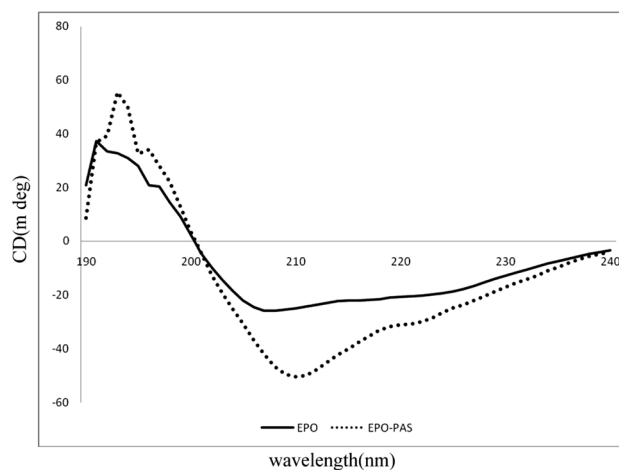

Fig. 9 Far-UV CD spectroscopy of normal (*up*) and PASylated EPO (*down*) to determine secondary structure contents of the proteins. All results were recorded at room temperature in phosphate buffer pH 7.5 using a 1 mm quarts cuvette. The random coil structure in PASylated protein was increased up to 60.5%, compared to the corresponding not PASylated EPO molecule (23.2%)

a large number of technologies have been developed to increase the plasma survival time of the proteins [22]. Based on different mechanisms, which influence half-life, these strategies comprise of several approaches such as dimerization [23, 24], N-glycosylation modifications [25, 26], Fc based fusion [27, 28], fusion to the C-terminal end of gonadotropin hormone [29] or serum albumin [9], PEGylation [30–32] and more recently by peptide addition or fusion to natural and recombinant PEG mimetic polymers [33–35].

PEGylation is considered as a strategy of choice to increase the effective pharmacokinetics and pharmacodynamics properties of a variety of therapeutic proteins. Previous experiments with a PEGylated erythropoietin showed an increase in plasma half-life by influencing receptor binding affinity and in vivo biological activity of the protein [36].

Chemical coupling of the recombinant biopharmaceutical protein with synthetic PEG has the negative influence on protein function. Random Lys side chains PEGylation with N-hydroxyl succinimide (NHS) leads to heterogeneous products with partially obstructed active sites. Site-specific PEGylation with maleimide PEG often leads to decrease protein yields. Additional expensive coupling and purification steps during production of PEGylated proteins, high costs of the pharmaceutical grade of the PEG raw materials and their chemical instability are some drawbacks of PEGylation reactions. Furthermore, PEG has poor bioavailability, immunogenic properties and is not biodegradable, which can cause vacuolation of the kidney epithelium [37–39].
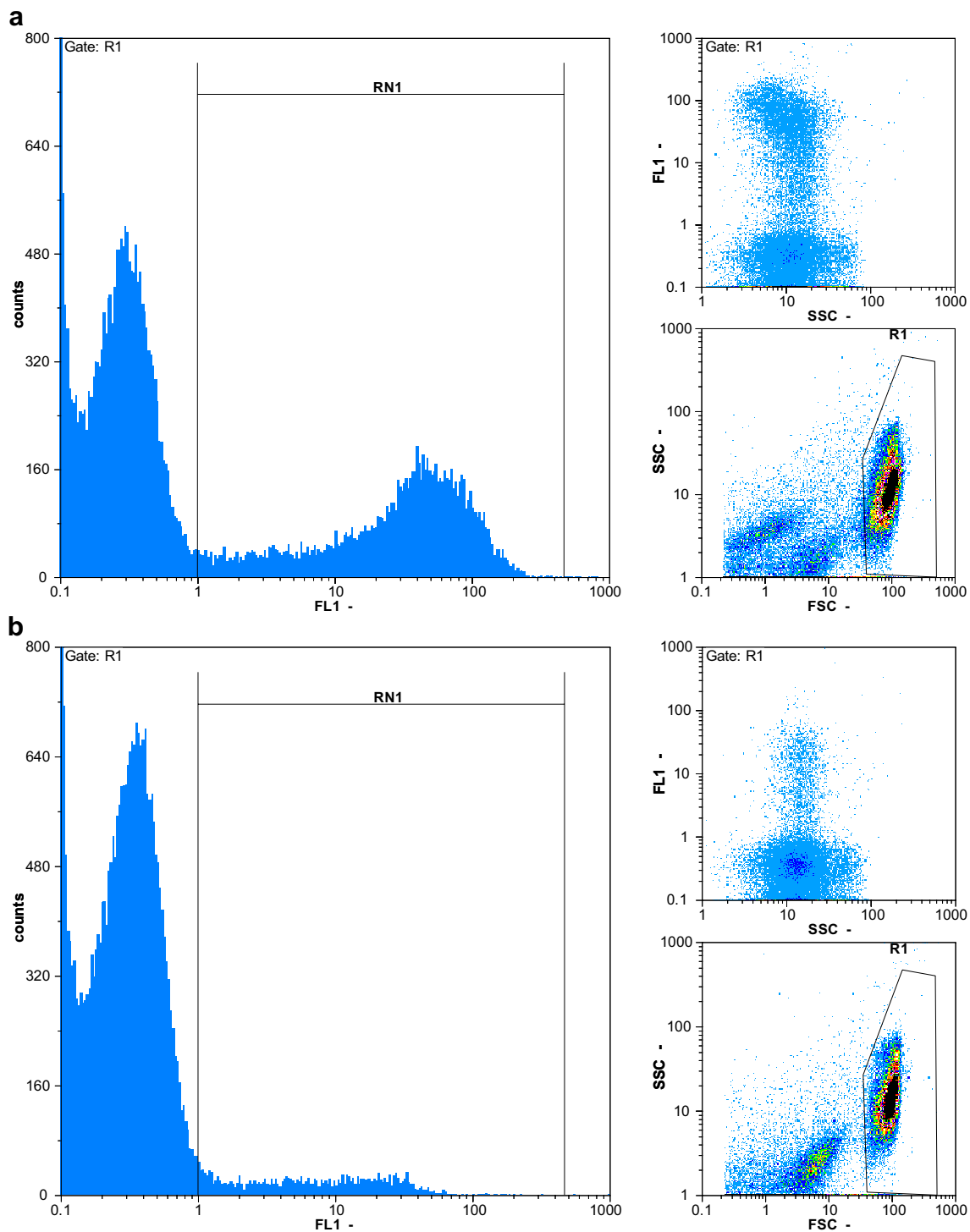
a

b

Fig. 10 Evaluations of in vivo activities of EPO-PAS$_{200}$ and rhEPO using the normocythemic mice assay. Reticulocytes counting was performed by FC using thiazole orange as a fluorescent dye (**a**). RN1 gates represent the percentage of reticulocyte for the EPO-PAS$_{200}$ (**a** 23.68%) relative to EPO EDQM (**b** 5.32%) as standard control following i.v administration. In vivo bioactivity of EPO-PAS (test) was estimated as 1.58×10^5 IU/mL by the PLA 3.0 software (**c**). The Erythropoietin BRP batch 4, EDQM, E1515000 was used as standard

we employed PASylation technology, which has developed by Arne Skerra et al. to improve the in vivo efficacy of human recombinant erythropoietin. This technology utilizes three small amino acids; proline, alanine and serine as PAS sequence. Oligomerization of these sequences can adopt a stable random structure like as PEG with large
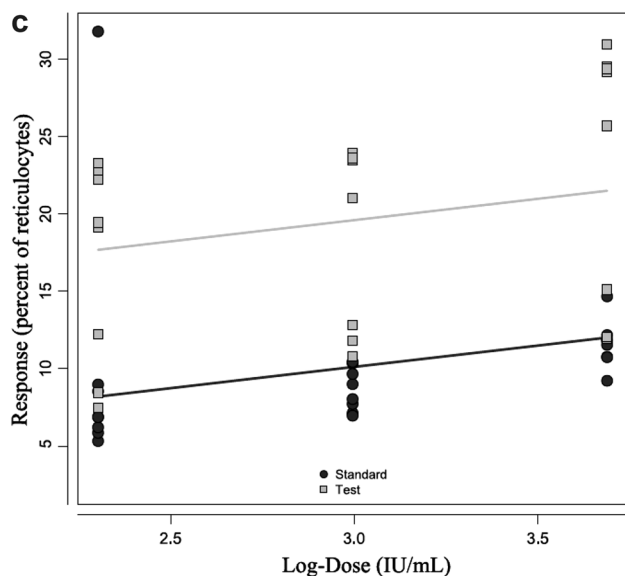
c
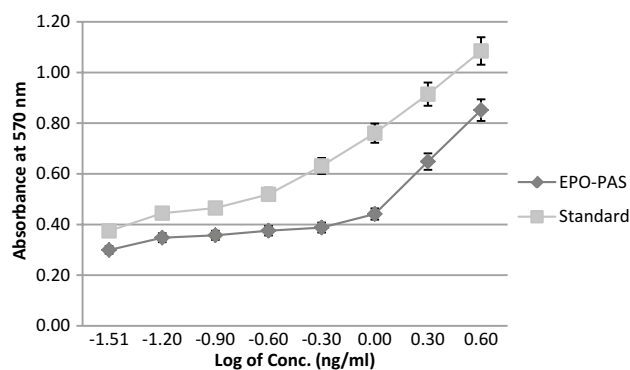

Fig. 10 (continued)

Fig. 11 The proliferation of UT-7 cells in response to the concentration of PASylated erythropoietin. MTT viability assay was used to determine the EC_{50} values of EPO-PAS (1.26 ± 0.05 ng/ml) in comparison to EPO standard sample (0.24 ± 0.03 ng/ml)

apparent hydrodynamic volume under physiological conditions. Decreased renal filtration due to increasing in size leads to enhance the serum longevity of the PASylated protein. In particular, the PAS sequences do not stimulate the immune responses in animals. In addition, it has a very low effect on the biological activity and plasma stability of pharmaceutical drugs [40]. In order to express the EPO-PAS fusion protein, the coding sequence of the PAS polypeptide chain, was attached to the C-terminal end of the protein by genetic engineering. We hypothesized that the presence of this terminal polymer would adopt a stable random coil structure and introduce a much larger hydrodynamic and apparent molecular size. Also, this technology could help us to produce a novel derivative of erythropoietin with satisfactory plasma half-life and in vivo efficacy.
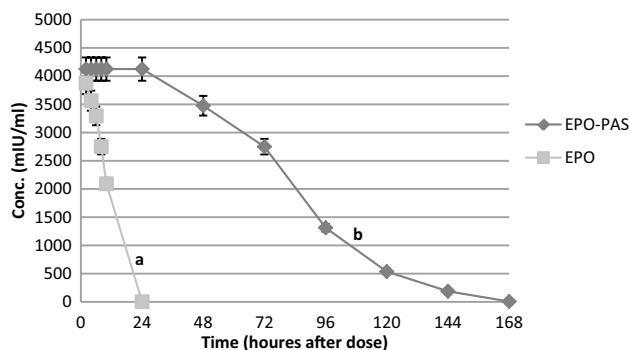
Fig. 12 In vivo pharmacokinetic properties of EPO and EPO-PAS fusion proteins in BALB/c mice. In vivo plasma elimination of normal EPO (*a*) in 24 h and EPO-PAS (*b*) after 7 days (168 h) of i.v. administration. Results are represented as means $\pm$ SD for 5 Balb-c mice per group

Table 2 Mean pharmacokinetic parameters for EPO and EPO-PAS$_{200}$ after i.v. injection of a single dose in Balb-c mice

Pharmacokinetic parameters	EPO-PAS$_{200}$[a]	EPO[b]
Elimination rate constant (h^{-1})	0.008 ± 0.0003	0.13 ± 0.02
$T_{1/2}$ (h)[c]	83.16 ± 13.28	5.33 ± 0.63
AUC $(0-\infty)$[d] (mIU/h/ml)	247872 ± 2974.46	116886 ± 1753.3
Vd (ml/Kg)[e]	0.78 ± 0.25	0.85 ± 0.21
CL[f] (ml/h/Kg)	0.006 ± 0.0005	0.11 ± 0.02

[a]CHO-derived rhEPO received from Pasture Institute of Iran

[b]CHO-derived EPO-PAS fusion protein

[c]Terminal half-life

[d]Area under the concentration time

[e]Volume of distribution

[f]Clearance. Results are represented as means $\pm$ SD for five Balb-c mice per group

In our experiments, the structure and the conformational stability of designed protein were initially confirmed by in silico homology modeling and molecular dynamic simulation studies. Characterization of purified EPO-PAS fusion protein by SDS–PAGE determined that the molecular weight of protein was increased to 70 kDa, which was an approximately 2.3-fold greater than that of not PASylated EPO (30.4 kDa). Moreover, the results of analytical SEC indicated that the apparent size of EPO-PAS fusion protein was approximately 230 kDa as a consequence of increasing the whole diameter of the fusion protein.

The high bioactivity of the PASylated erythropoietin was confirmed by the obtained molecular size as well as the hydrodynamic volume of the fusion protein (more than 60 kDa) and the result of our pharmacokinetic animal experiments.

In the present study, we employed a single dose of intravenous administration in mice for the pharmacokinetic investigations. As expected this experiment represented that the EPO extended with PAS polypeptide remarkably boosted the bioactivity and prolonged its circulating duration (15.6-fold) comprising to EPO reference standard.

Estimations of the area under the concentration time (AUC), terminal half-life, elimination rate constant and clearance (CL) of EPO-PAS$_{200}$ were significantly improved comparing to that of the EPO after i.v. injection (Table 2). The clearance for EPO-PAS$_{200}$ was approximately 18-fold shorter than that for EPO, and the estimated $t_{1/2}$ for EPO-PAS was almost 15.6-fold more than that calculated for EPO (83.16 ± 13.28 vs. 5.33 ± 0.63 h; Table 2). Consequently, the survival and biological properties of the EPO-PAS$_{200}$ fusion protein have successfully extended for comfortable clinical utilization. The obtained plasma elimination time for EPO-PAS$_{200}$ (83.16 ± 13.28 h) was slower than epoetin α (8.5 ± 2.4 h) and Darbepoetin α (25.3 ± 2.2 h). In contrast, it was more than MIRCERA (methoxy PEG-epoetin beta) with 134 ± 56 h circulating half-life after i.v. administration. Due to its long half-life, and higher in vivo potency, a given dose of EPO-PAS$_{200}$ can be administered less frequent than the same dose of epoetin α to achieve the equal biological response.

Results of Schlapschy et al. studies demonstrated that the PASylation of human growth hormone, IFNα-2b, and anti-HER2 antibody 4D5, significantly increased their circulating half-life [40]. Moreover, Mendler et al. in their experiments indicated that fusing of humanized αHER2 and αCD20 Fabs by PAS sequences elevated their tumor concentration. These results were similar to the outcomes that previously described for PEGylated and albumin associated antibodies due to tailored plasma half-life [41]. Taken together, previous findings highlighted the role of PASylation strategy to improve bioactivity and durability of drugs fused to PAS polymer tags with different length.

The major advantages of the PASylation are simultaneous designing and manufacturing of chimeric molecules. In this approach, some difficult purification steps and additional chemical optimizations were excluded. Moreover, the attached polypeptide chain is non-immunogenic and completely biodegradable [41].

Since, this work was the first attempt to employ PASylation technology in the case of erythropoietin so, further analysis of the stability and other physicochemical properties of EPO-PAS protein remained to be characterized. In addition, the influence of different routes of administration and dosage forms should be investigated in the subsequent studies. The effect of the PAS polypeptides on the hydrodynamic volume and in vivo efficacy of erythropoietin should be analyzed with increasing the length of the attached polymers at least up to 600 PAS amino acid residues. Enhanced

pharmacokinetic properties of EPO-PAS with reduced frequency of administration are worthy of consideration for later human's therapy.

In this work, we could design a PASylated human recombinant erythropoietin, in which the PAS amino acid polymer was genetically ligated to the C-terminal end of protein. Our data demonstrated that the EPO-PAS fusion protein can be functionally expressed in CHO cells, with substantially extended circulation half-life, and in vivo bioactivity.

Compliance with Ethical Standards

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