original article

JPHS

Site – specific PEGylation of rHuEPO and evaluation of its biological activity and stability

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ABSTRACT

Despite the critical role of erythropoietin (EPO) as therapeutic agent in treatment of anemia, its consumption is limited due to several disadvantages including the product short half-life, immunogenicity and susceptibility to proteolytic degradation. To overcome these drawbacks efficient methods such as site-specific PEGylation have been developed among witch N-terminal PEGylation has found more interest due to its capability to preserve the protein native structure. In this study a site-specific PEGylation of rHuEPO is conducted and the biological activity and the stability of the modified protein are evaluated. rHuEPO was N-Terminally PEGylated with PEG-propionaldehyde through Schiff base reaction in acidic pH conditions. The product was then purified to yield in mono N-terminally PEGylated EPO. The resultant PEG-EPO and unmodified EPO was then compared to evaluate the consequence of the pegylation process. The results showed that despite the lower activity of PEG-EPO relative to the unmodified EPO, the earlier gained improved stability as a consequence of site-specific PEGylation. This observation provides further indication for the usefulness of PEGylation approach to enhance characteristics of erythropitein.

Key words: N-terminal PEGylation , *in vitro* biological activity, stability

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1. Introduction

Recombinant proteins has become of a great importance as human therapeutic agents as a consequence of the prominent development in the expression systems that allows for better preservation or enhancement of the substance biological activity compared to the native proteins (Walsh, 2000). Among them can be mentioned erythropoietin (EPO), a 30 KDa glycoprotein composed of 165 amino acids (Markam *et al.*, 1995) which plays a critical role in formation of red blood cell. Among EPOs rHuEPO is known as one of the most essential pharmaceutical drugs in the treatment of various blood disorders and different kinds of anemia (Fukuda *et al*., 1989). EPO is produced through of DNA recombinant technology (Fukuda *et al*., 2002) which not only let the protein have biological activity close to that of the native protein but also allows for the large scale bioproduction of the agent (Fukuda *et al*., 1987).
Due to the short half-life of the natural EPO, the protein is needed to be administered three times weekly to leave an optimal therapeutic effects (Flaherty *et al*., 1990). To make EPO a more robust therapeutic agent, several approaches have been suggested and implemented including manipulation of amino acid sequence (Flaherty *et al*., 2000), conjugation to serum proteins (Lyczak *et al*., 1994) and PEGylation. (Abuchowski *et al*., 1977). PEGylation which is the covalent modification of PEG with protein is extensively used for decreasing the half-life and immunogenicity of the drug and at the same time increasing its solubility and resistance to proteolytic degradation (Harris *et al*., 1992). The enhanced properties of the PEGylated proteins such as PEG-Introne, PEGasys and PEG-GCSF which has induced a significant market demand have provided motivation for applying the technology to modify other therapeutic proteins. Despite its stabilizing effect the PEGylation process also involves some disadvantages with regarding to the drug properties including the lack of homogeneity and inadequate biological activity of the products (Veronese, 2001; Bentley *et al*., 1999). Therefore the ultimate advantage of modifying therapeutic protein with PEGylation can be realized only when elimination of these drawbacks can be approached. To address this challenge a number of site-specific PEGylation methods have been suggested providing a framework for both increasing the stability of the proteins and conserving their pharmacological properties. Current site-specific PEGylation methods include cystein-specific PEGylation, oxidation of carbohydrate residues and N-terminal PEGylation among which the latter has gained more interest regarding its capability to preserve the intact protein structure thereby conserving protein activity (Kinstler *et al*., 1996; Edwards *et al*., 1999; Bentley *et al*., 1999; Zalipsky *et al*., 1986; Goodson *et al*., 1990).
In this study we aimed at modification of rHuEPO using a site-specific PEGylation approach and characterizing the properties of the resulting proteins. It is speculated that in an acidic pH, PEG-aldehyde can bind to N-terminal amine group of EPO due to its lower pKa relative to the other amine groups of this protein.
To investigate the effect of PEGylation on both stability and pharmacologic properties of CHO-cell expressed rHuEPO, the protein was undergone N-terminal PEGylation followed by purification process to obtain a mono-PEGylated EPO. The natural and PEGylated EPO were then compared with respect to their *in vitro* activities using UT-7 cell lines. After keeping in 4°C for a period of 2 weeks, the stability of both unmodified EPO and PEGylated EPO were determined and compared.

2. Materials and Methods

Linear methoxy-PEG-Propionaldehyde of 20 KDa was purchased from JenKem (China). Recombinant Human CHO-cell expressed EPO was obtained from Pasteur institute of Iran, UT-7 cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). All other chemicals were purchased from Sigma (USA).

2.1. Preparation of N-Terminally mono-PEGylated EPO

PEG-EPO conjugate was prepared via reductive alkylation of the protein with linear monofunctional methoxy-PEG-propionaldehyde of 20 KDa. To a solution of 0.3 mg/ml rHuEPO in 20 mM phosphate buffer containing 150 mM NaCl in pH of 5, a ten- fold molar excess of linear methoxy-PEG-propionaldehyde 20KDa was added and the mixture was stirred in 50 rpm at 4°C. Then 20 mM of the reductive agent sodium cyanoborohydride was added and the reaction continued for 15 hours. Finally the mixture was diluted with 90 ml Tris buffer 20mM with pH adjusted at 8 by HCl 37% to make the reaction mixture suitable for the next step, the anion exchange chromatography.

2.3. Anion exchange chromatography

Hi Trap Q-sepharose chromatography column (Bio-Rad, FPLC system) was used for separation of poly-PEGylated, mono-PEGylated and unmodified EPO based on their charge difference. After equilibration of the packed column with 20 mM of Tris buffer at pH of 8, the solution mixture in Tris buffer at pH of 8 was loaded on the column and washed with 20 Mm Tris buffer in order to let the free PEG leave the column. Then solutions of 50, 300, 1000 mM NaCl in 20 mM Tris buffer was used to separate poly PEGylated, mono-PEGylated and unmodified EPOs respectively.

2.4. Size exclusion High Performance Liquid Chromatography

A final protein purification step of to remove excess amount of salts and any other undesired EPO-complexes was carried out by size-exclusion chromatography (HiLoad Superdex 75 prep grade, 16/60 columns, Amersham Biosciences ,GE Healthcare) using a column buffer of 50 mM sodium phosphate pH=7, 150 mM sodium chloride, 10% glycerol.

The purity of purified mono -N-Terminally PEGylated EPO determined by size exclusion HPLC column (PROTEIN KW – 802.5, 80 mmID X 300 mm length , 5μm particle size, Shodex, Japan). Mobile phase was 20mM sodium phosphate buffer containing 150 mM NaCl, pH=7, with a flow rate of 1 ml/min at 214 nm. Elution of 20 μL injected sample was monitored by UV absorbance.

2.5. Sodium dodecyl sulfate – poly acrylamide gel electrophoresis

2.5.1. Sample preparation for SDS-PAGE analysis

The samples were concentrated by microcone (Millipore, system) to a final volume of 20 μL, then mixed with the same volume of Treatment buffer (Stacking buffer 2.5 ml, SDS10% 4 ml, Glycerol 20% 2ml, Bromophenol Blue 2mg, Dithiothreitol 0.2M) in a microtube and placed in a boiling water bath for 4 min. SDS- PAGE was carried out according to standard Laemmli conditions. The Hoefer SE 260 apparatus (Amersham Biosciences, GE Healthcare) with a 12.5% separating gel was used (Kurfurst, 1992). The un-reacted EPO and PEG-protein conjugate were loaded running at 110 V. The gel dimension was 10 ×6×0.5. Running buffer included 0.025 M Tris, 0.19 M glycine and 1% SDS. Gels were silver stained for analyzing the purity of PEGylated EPO as well as the yield of reaction with the aid of densitometer. Barium iodide staining was used to show the efficiency of ion exchange chromatography (IEC) in removing un-reacted free PEG through making complex between barium iodide and PEG (Kurfurst, 1992).

2.6. Silver staining protocol

Among various available silver staining methods, we used the one that is based on the method of Heukeshoven and Dernick (Heukeshoven and Dernick, 1985) regarding its convenience, sensitivity and reproducibility (Hames, 1998). The gels were kept in fixing solution (Ethanol, absolute 40% Acetic acid glacial 10% and distilled or deionized water to 1 L) for 30 min. Then the sensitizing solution (30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, and 0.125% (v/v) glutaraldehyde, distilled or deionized water to 1 L) was used for 30 min and the gel went through three steps of washing with distilled or deionized water, each step lasting 5 min. Then a staining solution (0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde, distilled or deionized water to 1 L) was used for staining the gels for 20 min. Finally the gels were washed twice with distilled or deionized water for 2 min. The last step of staining which caused the stain to be developed was carried out using a developing solution (2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde, distilled or deionized water to 1 L) and silver-stained the gels were obtained.

2.7. Barium iodide staining protocol

After soaking the gel in 20 ml Glutaraldeyhde 5% for 15 min, the gel was put in 20 ml perchloric acid 1M for 15 min. 10 ml of Barium chloride 1 M and 8 ml of iodine solution 0.5 M was added simultaneously to form a complex between PEG and iodine. After 10 min the gel was stained. (Kurfurst, 1992)

2.8. In vitro bioassay

UT-7 cell lines were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and maintained in αMEM medium supplemented with 10% FBS, 40 mg/mL Gentamycin, 2 mM glutamine and 5 ng/ml GM-CSF (Sigma-Aldrich, U.S.A). For bioassays, the cells were washed twice by centrifugation in the assay medium (10% FBS, 40 mg/mL Gentamycin, 2 mM glutamine). After determining cell number and trypan blue viability, the cells were resuspended in the assay medium to reach the concentration of 105/ml. 50 μL of the cell suspension (5000 cells) were dispensed into each well of a 96-well tissue culture plate (except the blanks). Serial 2-fold dilutions of unmodified EPO and PEGylated EPO were prepared in the assay medium. 50 μL of the diluted protein samples were added in triplicate to the test wells and the plates incubated for 3 days at 37° C in a humidified 5% CO2 atmosphere. The positive control contained 200ng/ml rHuEPO and the negative control did not contain any form of EPO. Cell growth rate was evaluated on the basis of the Mossman colorimetric MTT assay (Mossman *et al*., 1983). 20 μL of sterilized 5 mg/mL MTT solution (Sigma-Aldrich, U.S.A) was added to each culture well and the plates were incubated at 37°C for 1-5 h. After adding the MTT solubilization solution, the absorbance of the wells was read at 570 nm using a microplate reader. For each test, a blank containing the complete medium without cells was included to measure the background absorbance.

2.9. Stability evaluation

Unmodified EPO and purified mono-PEGylated EPO were kept at 4°C for 2 weeks, then both of them were evaluated in terms of *in vitro* biological activity.

3. Results and Discussion

Covalent attachment of PEG to the proteins is considered as a highly efficient method to increase drug half-life leading to enlargement of the drug hydrodynamic volume thereby decrease of its renal clearance. The PEG chains bind to the protein through covalent attachment. These binding will be destroyed by the action of protease degradation but in a slower rate than that of unmodified EPO. Therefore the total time of drug presence in the body will increased when it is undergone a PEGylation process. Previous studies suggested that the N- terminal end of EPO is most likely non-essential for biological activity of the substance (Biossel *et al*., 1993), hence the PEGylated EPO is predicted to have the standard biological activity as well. In comparison to the first-generation PEGylated reagents, second generation acts more selectively leading to decrease of the heterogenicity of the products which in turn can influence their biological activities. Attachment of PEG to N-terminal amino group of proteins takes place through a Schiff base reaction then the product was reduced to yield in stable secondary amines (Laemmli, 1970).
To decrease the possibility of interaction of other amine residues in the protein, pH was adjusted at 5, which is specific for attachment of PEG to N-terminal amino group. Because the pKa of N-terminal amino group of EPO is lower than that of other existing amine groups, in acidic pH, this amine is largely un-protonated and therefore is more susceptible for conjugation to PEG aldehyde derivatives (Kinstler *et al*., 1999).

3.1. SDS-PAGE analysis results

SDS-PAGE is a popular tool for characterization of the proteins. The efficacy of PEGylation reaction was monitored by SDS-PAGE and silver staining as shown in Fig. 1.

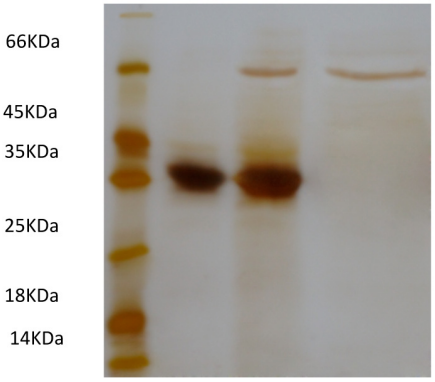


Figure1. SDS-PAGE results of Anion exchange chromatography. From left to right, lane 1: protein size marker SM0431 (Fermentas), lane 2: unmodified EPO, lane 3: PEGylation mixture containing both PEGylated and unmodified EPO, lane 4: mono-PEGylated EPO eluted by 300Mm NaCl in Tris buffer.

There is a band at about 35 KDa which corresponds to unmodified EPO and a band at 66 KDa attributed to mono-PEGylated EPO. The yield of PEGylation reaction was estimated 23% according to densitometer. Due to high flexibility of the PEG chains in absorbing water, the real molecular weight of the PEG is higher than the expected value (Roberts *et al*., 1998).

3.2. Anion exchange chromatography results

Although N-terminal PEGyaltion is a site specific reaction, the formation of other conjugated complexes are unavoidable. Therefore IEC was performed to separate PEGylated conjugates from unmodified EPO as well as free PEG. To this aim, after equilibration of the column with Tris buffer and loading the reaction mixture, the column was washed with Tris buffer to let the free un-reacted PEG leave the column (PEG dose not bind to column). Then increasing gradient concentrations of NaCl was utilized to separate poly-PEGylated, mono-PEGylated and unmodified EPOs. Fig. 2 shows the chromatogram of IEC protocol.

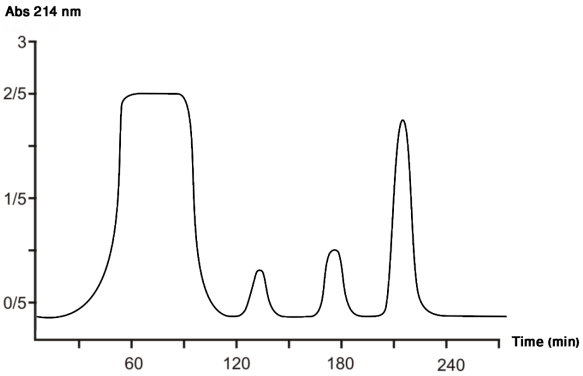


Figure 2. The IEC result of the PEGylation reaction.

SDS-PAGE analysis was perform to evaluate the efficiency of IEC in separating PEG conjugated products from unmodified EPO (Fig. 3)

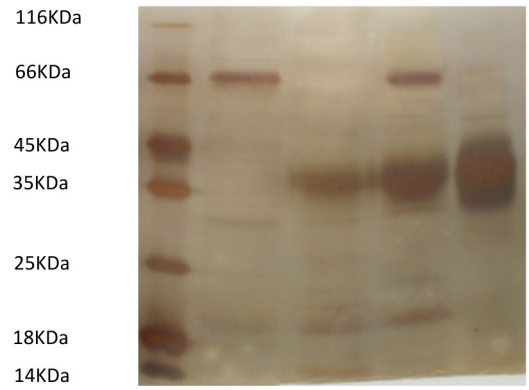


Figure 3. Silver staining SDS-PAGE after PEGylation reaction.

As silver staining is not sensitive enough to show the free un-reacted PEG chain, barium iodide staining was carried out to evaluate the efficiency of IEC in removing free PEG chains from the reaction mixture. Fig. 4 shows the result of SDS-PAGE analysis of IEC followed by barium iodide staining.

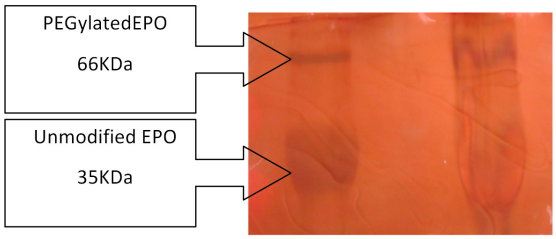
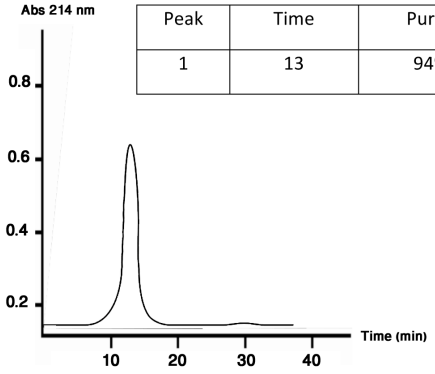


Figure 4. Barium iodide staining.

The right lane is related to the PEGylation reaction mixture containing unmodified EPO in 35KDa, PEGylated EPO in 66 KDa and PEG chain across the gel. The left lane is the result of elution with Tris buffer 20 mM containing NaCl 1M to elute mono-PEGylated and unmodified EPO together. According to the gel staining result, the left lane excluded any free PEG impurities along the gel, so through IEC we could effectively separate free un-reacted PEG chains.

3.3. Size exclusion- HPLC results

As the final step of purification of mono-PEGylated EPO, preparative gel filtration was performed to polish the product and exchange buffer to make it ready for *in virto* bioassay. To this aim, after equilibration of column the third peak of IEC (which was already collected) was loaded on the column, then a single volume of the column of equilibration buffer was passed through the column. By observing the peak, the mono-PEGylated EPO was collected. For determining product purity, analytical gel filtration was performed. 20μL of purified and polished mono-PEGylated EPO was loaded on the column. Result of analytical SE-HPLC is shown in Fig. 5. After 13 minutes a peak appeared and the purity of mono-PEGylated EPO was detected to be 94%.



Peak	Time	Purity
1	13	94%

Figure 5. The result of size-exchange chromatography of mono-PEG-EPOSE-HPLC analysis

3.4. In vitro bioassay results

The increasing concentrations of unmodified EPO and PEGylated EPO were used to stimulate cell lines proliferation. The result of bioassay is shown in Table1.

Table 1. The results of in vitro bioassay

Protein (ng/ml)	Abs of rHuEPO	Abs of PEG-EPO
0	0.08	0.07
0.078	0.12	0.07
0.156	0.16	0.11
0.312	0.31	0.19
0.625	0.56	0.33
1.25	1.01	0.76
2.5	1.07	1.01
5	1.05	1.05
10	1.06	1.06

Based on the absorbance values of both EPO and PEGylated EPO (which are proportional to efficiency), dose-response curves were obtained (Fig. 6).

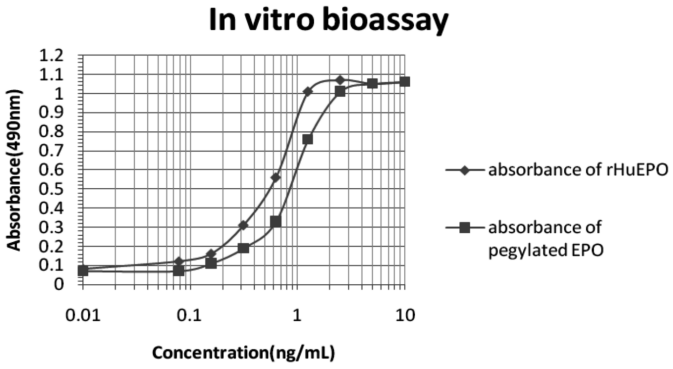


Figure 6. Dose - response curve of EPO and PEGylated EPO.

The OD50 of both EPO and PEGylated EPO is about 0.5 corresponding to rHuEPO EC50 concentration of 0.55 mg/ml and PEGylated EPO concentration of 0.8 mg/ml. It is therefore observable that the in virto biological activity of PEGylated EPO has only marginally decreased in comparison to the unmodified EPO.
According to the structure-action relationship studies on EPO, N-terminal region of EPO is not an active site (Biossel *et al*., 1993) thus the covalent attachment of PEG to it will not significantly decrease the biological activity. For instance the *in vitro* biological activity of PEG-Introne (amine PEGylated interferone) is 1% of the unmodified interferone. Thus in comparison to non-specific PEGylation, N-terminal PEGylation can greatly preserve *in vitro* biological activity.

3.5. Stability results

EPO and PEGylated EPO were kept for 2 weeks at 4°C and during this period their *in vitro* biological activity

was evaluated. Table 2 represents a comparative description of the stability of both EPOs.

Table 2. Stability results

Decrease potency%	Potency IU/ml	CONC mg/ml	SAMPLE
	24655.336	0.38	EPO
	2750.462	0.12	PEG-EPO
20%	19755.220	o.38	EPO (after 2 weeks in 4°C)
12%	2413.360	0.12	PEG-EPO (after 2 weeks in 4°C)

According to the Table 2 the decreased *in vitro* activity of EPO is larger than that of PEGylated EPO suggesting the higher stability of the latter as a result of attachment to PEG (Harris, 1992).

N-terminal PEGylation of EPO in this study resulted in 23% modification of total EPO with PEG. Previous conformational studies on EPO have revealed that the N-terminal amine group of EPO is not completely in exposure (Cheetham, 1998) which justifies the reason for the relative low yield of PEGylation reaction. The goal of this study was not to optimize PEGylation reaction yield, rather it aimed at setting up a lab-scale method for site specific PEGylation of the therapeutic proteins and peptides. Further improvement of the rHuEPO PEGylation yield can be achieved through increasing the reaction time and/or using PEG derivatives with lower molecular weights which are more reactive than the heavier ones. Moreover elimination of the disulfide bind between Cys 29 & 33 which is not crucial for EPO binding to the receptor, may result in increase of N-terminal exposure, thereby increasing the reaction yield as seen in PEGylation process of other EPO proteins (Mattews, 1996).

In this study an appropriate purification process with the aim of characterizing the PEGylated proteins was establish. The approach involves development of a SDS-PAGE analysis method and two staining protocols allowing for measurement of the homogenicity and efficiency of purification process. After final purification of mono-N-terminally PEGylated EPO, the in vito biological activity and stability test was performed. The result showed marginal loss of *in vitro* biological activity of PEG-EPO but the proteins stability increased. This result suggests the usefulness and applicability of our method as a basis for developing industrial process of PEG-rHuEPO.

On the whole our study establishes an initial step for further research with the aim of improving the efficiency of rHuEPO PEGylation process which ultimately can lead to realization of large-scale production of the modified agent.

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