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Development of Simple and Fast Method for Preparation and Purification of Monopegylated Recombinant Human Granulocyte Colony-stimulating Factor (rhG-CSF, Filgrastim) with High Efficiency

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ABSTRACT

Background: PEGylation is a valuable strategy for enhancing the pharmacokinetic properties of recombinant methionyl human granulocyte colony-stimulating factor (rh-Met-G-CSF, filgrastim) which is used to treat chemotherapy induced neutropenia. So development of a cost-effective method is required to attain monoPEGylated G-CSF form. Here it is focused on the PEGylation reaction engineering and purification processes that should be optimized to reduce the costs. Methods: In this study methoxy polyethylene glycol propionaldehydes (mPEG-ALD) 20 kDa MW was utilized to produce monoPEGylated rhG-CSF. PEGylation reaction was carried out at 4 °C and pH 4.5 in the presence of sodium cyanoborohydride and three mPEG-ALD: protein molar ratios (3:1, 5:1 and 10:1). The PEGylation reaction was monitored with SDS-PAGE. Subsequently, isolation of the monoPEGylated form was achieved by cation exchange chromatography (CEC) method. The PEG attachment site was assessed by FTIR and structural characteristics of purified products (unPEGylated and PEGylated rhG-CSF) were investigated by CD and intrinsic fluorescence techniques. Results: The results showed optimal yield of monoPEGylated protein (60%). Also a purity around of 99% achieved for pegylated rhG-CSF. Assay by FTIR disclosed that PEGylation precisely was performed in N-terminus of rhG-CSF. Structural analysis by CD and intrinsic fluorescence indicated that rhG-CSF maintained secondary structure after modification by PEGylation. Conclusion: Overall, this study has expanded a pilot method to purify monoPEGylated rhG-CSF which is simple, fast, and economical method with high efficiency.

Keywords: PEGylation, rhG-CSF, Filgrastim, Purification, Ion exchange chromatography

INTRODUCTION

In the past decades the field of biopharmaceuticals employed the process of covalent bonding PEG to active biomacromoleculs (PEGylation) [1-3]. PEGylation now plays a significant role to elevate the potentials of therapeutic agents [4,5] and improve drug delivery [6-8] because of several advantages: a prolonged residence in body [9-13], a decreased degradation by metabolic enzymes and a reduction or removal of immunogenicity [14-16]. Unfortunately, the PEGylation of proteins reduces biological activity but this is compensated by improved pharmacokinetics including the extended body residence time, as a result of the increased stability and higher hydrodynamic volume [17,18,3]. PEGylation is a versatile technique for pharmaceutical agents that presents a particularly wide range of conjugation chemistry and polymer structure [19]. PEG polymer is non-toxic, non-immunogenic, highly soluble in water and FDA approved [20-23]. Many PEGylated proteins are already available for clinical use [21,24-26]. The progress of PEGylation chemistry [7] permits site-specific amino conjugation, because they generally exposed to the solvent and could be changed with many chemical methods [27,28]. Site-specific modification might lead to a better preservation of the native protein activity in the conjugated form and also helping the purification and characterization procedures, because mixtures of PEGylated products are avoided [29-35]. Alkylation conjugation maintains the positive charge of the primary amino group because of formation a secondary amine [36-38]. PEGylation method which takes advantage of the lower pKa of the N-terminal α-amino groups compared with that of the α-amino group in lysines, was described by Kinstler [39,40]. This conjugation type was

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utilized for granulocyte colony-stimulating factor (G-CSF) by a reductive alkylation with PEG aldehyde, leading to the marketed Pegfilgrastim®(Neulasta) [41,42], used to treat granulocyte depletion during chemotherapy [43].

Granulocyte colony-stimulating factor (G-CSF) is a glycoprotein, growth factor and cytokine produced by monocytes, endothelial cells, and fibroblasts to stimulate the bone marrow to produce granulocytes and stem cells [44,45]. The G-CSF gene is detected on chromosome 17, locus q11.2-q12, [46]. Filgrastim is an analog of almost human G-CSF [47,48]. Commercial therapeutics of filgrastim are employed for decrease of duration of post-chemotherapy neutropenia, periodical neutropenia, neutropenia related with HIV and *etc*. [49-51]. In 1991, the first G-CSF approved by FDA was Neupogen (Amgen). Neupogen has a 175-amino-acid sequence (18800 Da) that is same to endogenous G-CSF except methionine residue at the N-terminal. Because of expression in *E. coli*, filgrastim is non-glycosylated [46,49]. A PEGylated type of G-CSF is pegfilgrastim that a 20 kDa monomethoxypolyethylene glycol polymer covalently bound to the methionyl residue of N-terminal [52]. Filgrastim and pegfilgrastim are relatively non-immunogenic in therapeutic use [53]. Subcutaneous injection once per chemotherapy cycle of pegfilgrastim has as effective as daily filgrastim in treatment of neutropenia [54]. In patient, optimal dosage of injectable is 5 μg kg^{-1} of filgrastim and 100 μg kg^{-1} of pegfilgrastim [55,56].

Pegfilgrastim, because of prolonged circulatory half-life, needs to be injected less frequently therefore, it will improve the quality of life of cancer patients and their families [57,58]. So production of PEGylated form of rhG-CSF (pegfilgrastim) is required. Since the production of pharmaceutical proteins is costly, it is essential that the process of PEGylation be optimized by PEGylation reaction engineering and subsequent purification processes [59,7]. The present study was designed to develop and validate of a simple, fast and inexpensive method for optimization preparation and purification of chemical PEGylation of N-Terminal site-specific rhG-CSF.

MATERIALS AND METHODS

Materials

RhG-CSF solution was provided by Arya Tina Gene Company (Gorgan, Iran) and linear 20 kDa methoxy polyethylene glycol propionaldehydes (PEG_{20KDa}-ALD) was purchased from NOF Company (Japan). SP Sepharose Fast Flow resin was obtained from Amersham Bioscience Company. Fermentase protein molecular weights ladder (Broad Range Fermentase # 7720S), all salts, organic solvents and other chemical reagents for sodium dodecylsulfate polyacrilamide gel electrophoresis (SDS-PAGE) and Bradford test were analytical grade and acquired from Sigma Aldrich-Fluka and Merck (Milan, Italy).

rhG-CSF PEGylation

In this study, chemical modification by N-terminal site-directed mono-PEGylation of rhG-CSF was accomplished according to methods which have been described previously in articles [60-63], with a few differences. In the first step, solution of rhG-CSF (4.5 mg ml^{-1}) in 20 mM sodium acetate buffer, pH 4.5, at 4 °C, was added to the vials containing powders of PEG_{20KDa}-ALD. Then solutions were stirred to dissolve the PEGs. So three different mPEG-ALD: protein molar ratios 3:1, 5:1 and 10:1 were obtained. After that, a 20-fold molar sodium cyanobrohydride as a reducing agent was added to every vial. Finally the reaction mixtures were incubated at 4 °C for 20 h with shaking at 120 rpm.

Purification of Mono-PEG-GCSF

Mono-PEGylated rhG-CSF was purified on SP Sepharose FF column using ion exchange chromatography by step-wise gradient. The washing buffers include buffer A (sodium acetate 20 mM, pH: 4.5) and buffer B (sodium acetate 20 mM, NaCl 1 M, pH: 4.5), were exchanged as gradient. First, before applying the sample, column was equilibrated using buffer A. Thereafter, the obtained sample from previous step, was loaded on cation exchange chromatography column (1 cm × 20 cm) at 4 °C temperature. In elution step, the column was washed with different proportions of buffers A and B (buffer B to buffer A ratio is from 0-100 to 100-0) during 20 column volumes as gradient volume. Each washed fraction of chromatography was collected and then analyzed with SDS-PAGE. After that, buffer of the fractions of consisting monoPEGylated rhG-CSF was exchanged with 10 mM sodium acetate buffer, pH 4.5 and also concentrated to desired concentration by centricon (10 KDa cut-off

membrane).

Characterization and Structural Analysis of MonoPEGylated rhG-CSF

The discontinuous system of SDS-PAGE was employed to analyze the reaction mixtures under different reaction conditions and detection of species having molar mass consistent with mono-PEGylated rhG-CSF as well as the purity of fractions after purification steps. The separating and stacking gels contained 12% and 4% (w/v) acrylamide respectively. Composition of running buffer was 0.1% SDS, 0.05 M Tris, and 0.38 Mglycine buffer with pH 8.3. Before electrophoresis, samples were incubated at 100 °C for 5 min in loading buffer (2% SDS, 5% mercaptoethanol, 25% glycerol, 0.01% bromophenol blue, 0.01 M Tris-HCl, pH 8.8).

Bradford assay. Concentration of the purified PEGylated rhG-CSF was measured according to the Bradford method by using protein BSA (Bovine Serum Albumin) as the standard protein for obtaining the standard curve. The monoPEGylated rhG-CSF concentration was determined according to equation obtained from the standard curve.

Fourier transform infrared (FTIR) measurement. The PEGylation site on of monoPEGylated rhG-CSF was investigated by FTIR. For FTIR measurement in an attenuated total reflection (ATR), 100 µl of rhG-CSF and PEG-GCSF (2 mg ml^{-1}) in 10 mM sodium acetate buffer pH 4.5, were deposited on the crystal area of the single reflection ATR device (FTIR Thermo Nicolet, NEXUS 870 model, USA). Spectra were recorded under the following conditions: 4 cm^{-1} spectral resolution, 25 kH scan speed and in the 4000-1000 cm^{-1} spectral ranges.

Circular dichroism (CD) measurement. Circular dichroism measurement (CD) is a usual method to analyze the secondary structural conformation of native and PEGylated proteins. Circular dichroism (CD) spectra in the far-UV region (190-260 nm) were obtained on AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), using a 0.1 cm path length quartz cell at room temperature. The protein concentration of non-PEGylated rhG-CSF and PEGylated rhG-CSF was 0.2 mg ml^{-1} in 10 mM sodium acetate buffer pH 4.5.

Intrinsic fluorescence measurement. A 400 µl sample of rhG-CSF and PEGylated form of rhG-CSF (0.1 mg ml^{-1}) in 10 mM sodium acetate buffer pH 4.5 was placed in a 1 cm path length quartz cell. Fluorescence emission spectra were then taken in the 300-400 nm range using excitation at 295 nm. The excitation and emission slit widths were set as 5 nm and 10 nm, respectively. All fluorescence experiments were carried out on a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia) at room temperature.

RESULTS

Preparation of PEGylated rhG-CSF

In order to obtain a more prolong half-life PEGylated rhG-CSF, Conjugation reactions were performed using an amine reactive PEGylation reagent, with monomethoxy polyethylene glycol propionaldehyde (mPEG-ALD) 20000 Da MW. PEGylation reactions were carried out at 4°C temperature and pH 4.5 in the presence of sodium cyanoborohydride and three mPEG-ALD: protein molar ratios (3:1, 5:1 and 10:1). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to detect the presence of species having molar mass consistent with mono-PEGylated rhG-CSF. Also to determine efficiency of each PEGylation reaction, a sample of each reaction was run on a SDS-PAGE. The results showed that a reaction with 5:1 mPEG-ALD: protein molar ratio was optimal to direct the reaction toward optimal yields of monoPEGylated protein (60%) (Figs. 1A and B).

Purification of Mono-PEGylated rhG-CSF

Subsequently, PEGylated rhG-CSF was purified from reaction mixture (unreacted rhG-CSF and PEG) by optimized step-wise cation exchange chromatography on a SP Sepharose FF column (1 cm × 20 cm) at 4 °C temperature. After equilibration of column, in elution step, a salt gradient of 0-1 M NaCl in buffer A was used during 20 column volumes as gradient volume. Fractions of chromatography was collected and then analyzed with SDS-PAGE. The high purified protein (with more than 99% purity) was obtained in the fractions after washing with solutions of 15A+5B and 14A+6B (Fig. 2 and Table 1).

Analysis of MonoPEGylated rhG-CSF

Bradford assay. After buffer exchange of the fractions consisting monoPEGylated rhG-CSF in 10 mM sodium

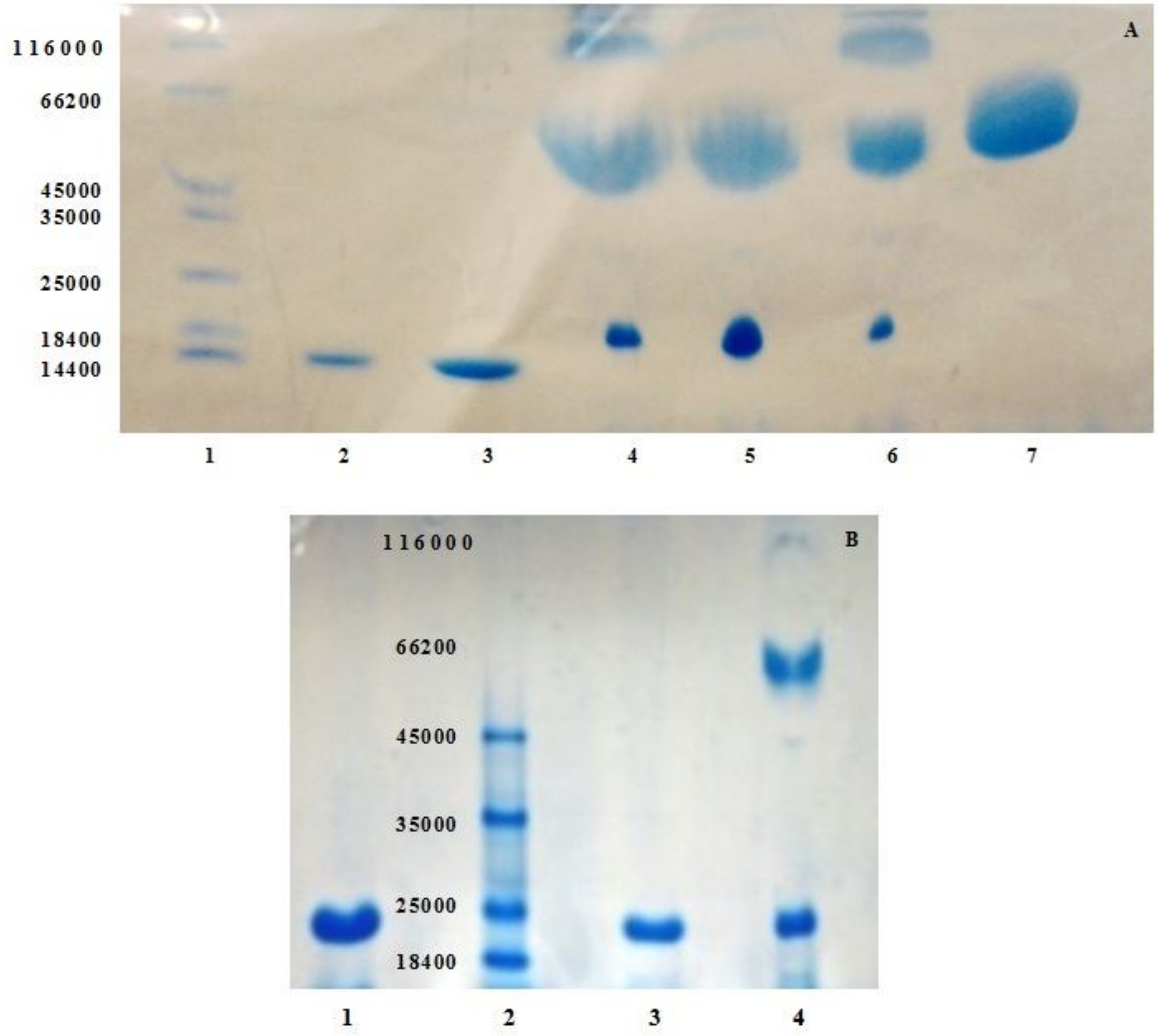


Fig. 1. SDS-PAGE of PEGylation of rhG-CSF. (A) PEGylation of rhG-CSF under different conditions of three mPEG-ALD: protein molar ratios (3:1, 5:1 and 10:1), lane1, protein marker, lane 2, standard rhG-CSF, lane 3, rhG-CSF before PEGylation and lane 4, PEGylation of rhG-CSF under conditions of mPEG-ALD: protein molar ratio 3:1, lane 5, PEGylation of rhG-CSF under conditions of mPEG-ALD: protein molar ratio 5:1, lane 6, PEGylation of rhG-CSF under conditions of mPEG-ALD: protein molar ratio 10:1, lane 7, standard PEGylated rhG-CSF, (B) PEGylation of rhG-CSF under optimal conditions. Lane 1, standard rhG-CSF, lane 2, protein marker, lane 3, rhG-CSF before PEGylation and lane 4, reaction mixture after 20 h PEGylation.

acetate buffer, pH 4.5, its concentration was determined using Bradford assay method. The concentration of PEGylated rhG-CSF was determined (2.5 mg ml^{-1}) using obtained equation (Y = 0.655X + 0.06) from the standard curve.

Fourier transform infrared (FTIR) measurement. FTIR was applied to verify the occurrence of mono-PEGylation at the N-terminus of rhG-CSF. Non-PEGylated and PEGylated rhG-CSF were characterized by FTIR in the 3800-2700 cm^{-1} and 1900-1400 cm^{-1} spectral ranges.

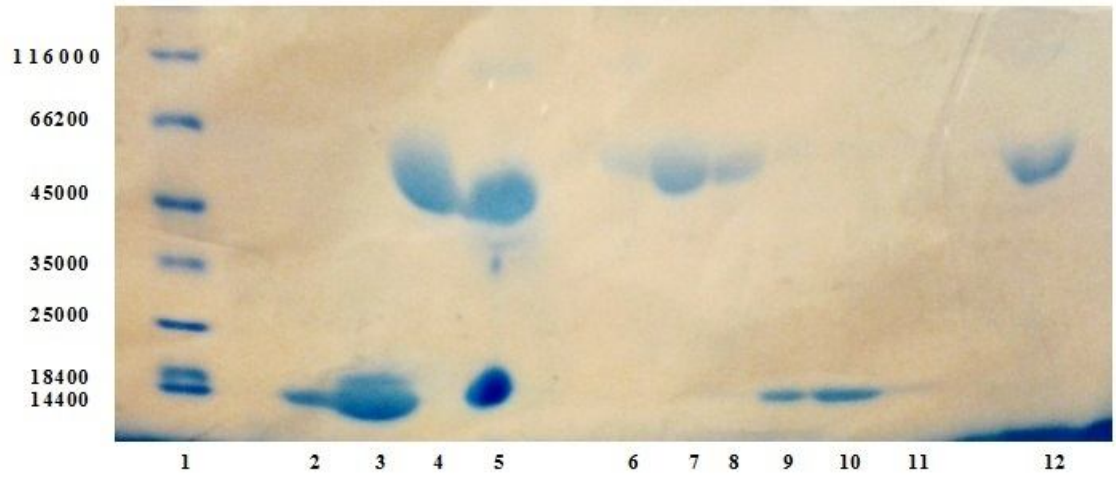


Fig. 2. SDS-PAGE of PEGylation of rhG-CSF and purification of PEGylated and unPEGylated rhG-CSF by SP Sepharose cation exchange step-wise elution chromatography. Lane 1, protein marker, lane 2, standard rhG-CSF, lane 3, rhG-CSF before PEGylation, lane 4, standard PEGylated rhG-CSF, lane 5, rhG-CSF after PEGylation, lane 6, fraction of 16A+4B gradient, containing PEGylated rhG-CSF, lane 7, fraction of 15A+5B gradient, containing PEGylated rhG-CSF, lane 8, fraction of 14A+6B gradient, containing PEGylated rhG-CSF and unPEGylated rhG-CSF, lane 9, fraction of 13A+7B gradient, containing PEGylated and unPEGylated rhG-CSF, lane 10 & 11, fractions of 12A+8B gradient and 11A+9B gradient, respectively containing unPEGylated rhG-CSF, lane 12, form of PEGylated rhG-CSF after buffer exchange.

Table 1. Percentage of Obtained PEGylated and unPEGylated rhG-CSF from Stages of PEGylation and Purification

Sample	Percentage (%)	
	Non-PEGylated rhG-CSF	PEGylated rhG-CSF
Standard rhG-CSF	100	0
rhG-CSF before PEGylation	100	0
rhG-CSF after PEGylation	46.4	53.6
15A+5B Gradient	0	100
14A+6B Gradient	14.3	85.7
13A+7B Gradient	85.5	14.5
12A+3B Gradient	100	0
PEGylated rhG-CSF after buffer exchange	0	100

Results displayed the same Amide I profile for the two form of purified rhG-CSF. Also data showed a higher absorption between 2950 and 2850 cm^{-1} in purified monoPEGylated rhG-CSF compared to the non-PEGylated rhG-CSF confirmed the success of the PEGylation reaction (Fig. 3).

Circular dichroism (CD) measurement. The CD spectra of 250 µl (0.2 mg ml^{-1}) rhG-CSF in 10 mM sodium acetate buffer pH 4.5 and equimolar quantity of PEGylated rhG-CSF in the far UV (190-260 nm) was recorded. Structural studies of circular dichroism (CD) spectroscopy indicated that the spectrum of the PEGylated rhG-CSF was very comparable with rhG-CSF (Fig. 4). Furthermore, deconvolution of CD data by CDNN software also showed that there were mostly same secondary structures in both non-PEGylated and PEGylated rhG-CSF (Table 2).

Intrinsic fluorescence measurement. Assessment of the intrinsic fluorescence intensity was performed using a 400 µl sample of rhG-CSF and PEGylated rhG-CSF (0.1 mg ml^{-1}) in 10 mM sodium acetate buffer pH 4.5 and in a 1 cm path length quartz cell. Fluorescence emission spectra were then taken in the 300-400 nm range using excitation at 295 nm. The extracted data from fluorescence

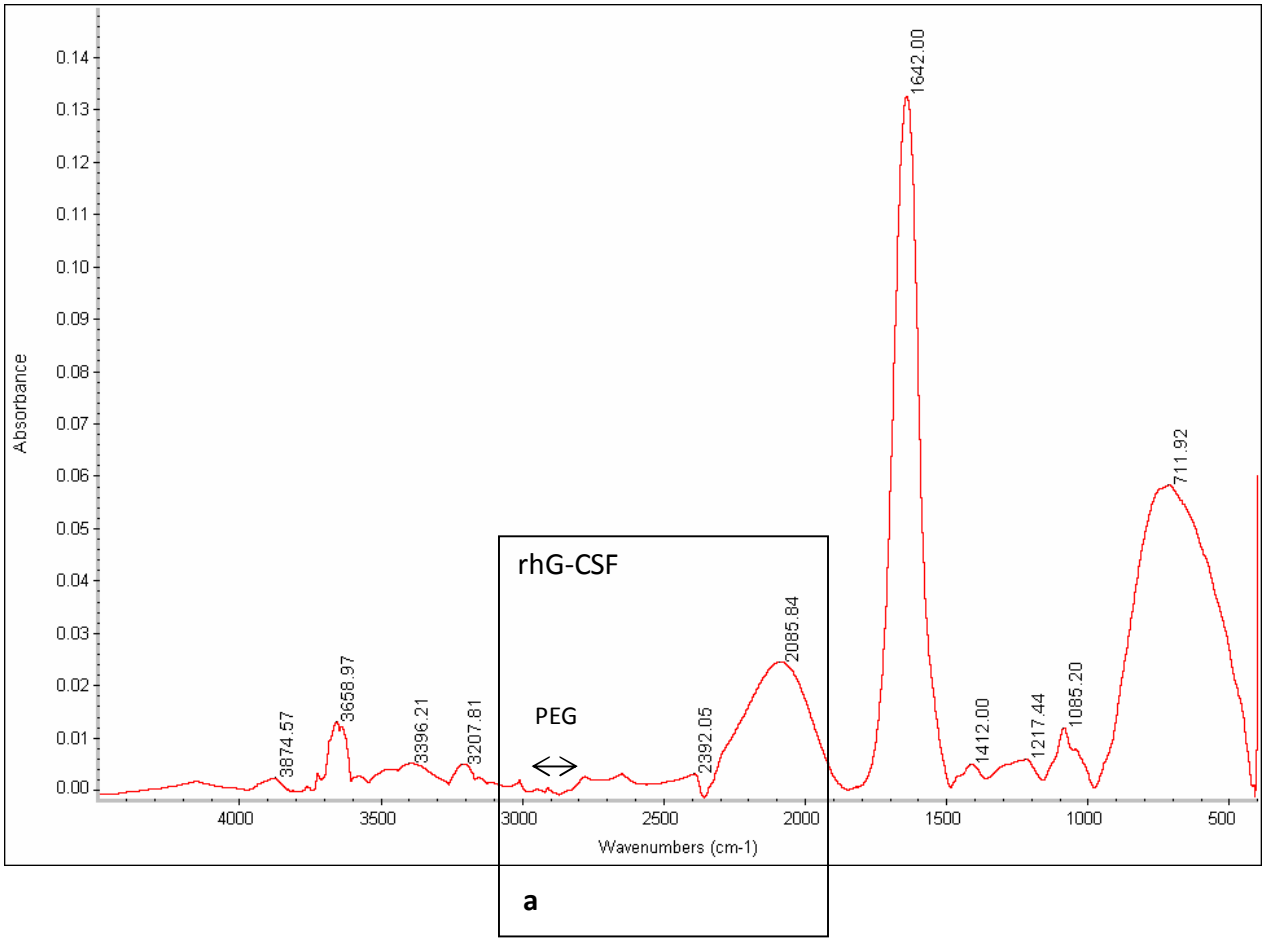
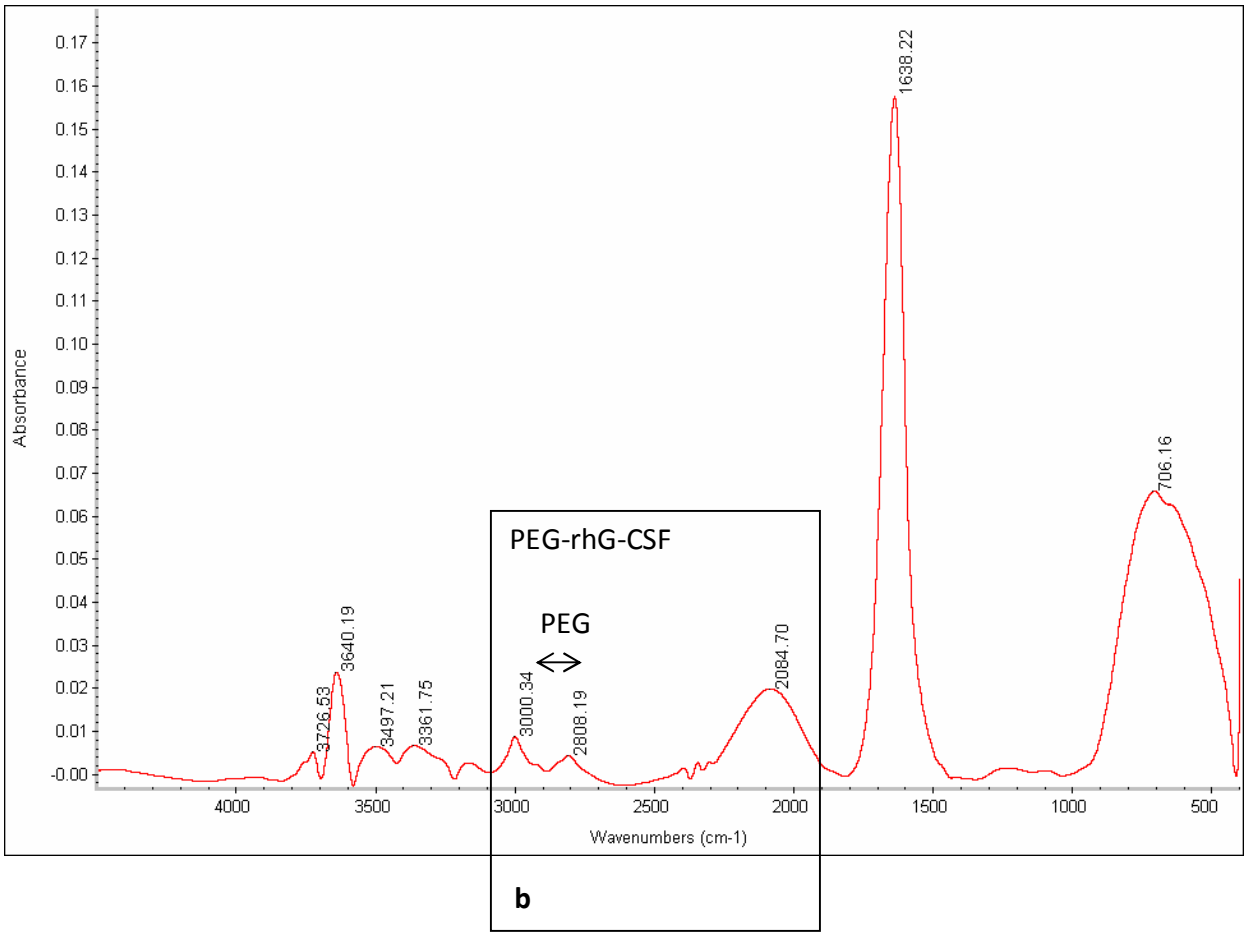
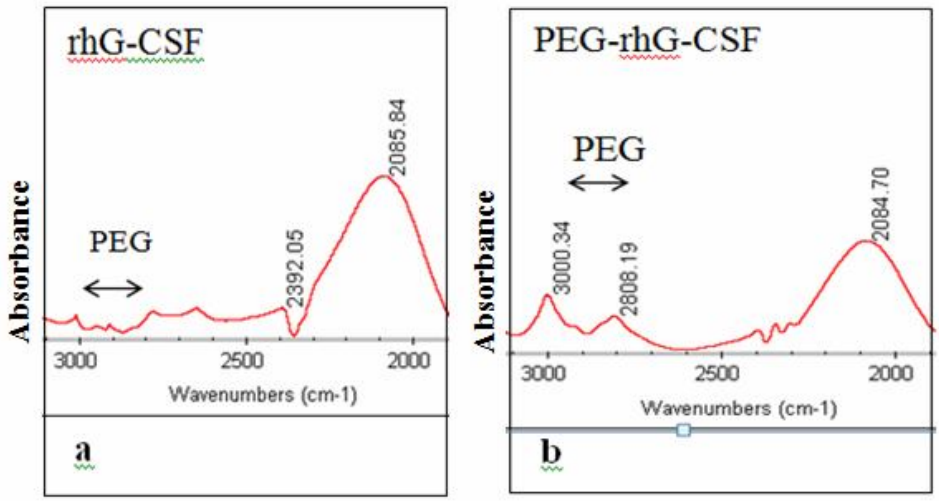


Fig. 3. Analysis of FTIR spectroscopy of PEGylated and unPEGylated rhG-CSF. Concentration of rhG-CSF and PEGylated rhG-CSF were 0.2 mg ml^{-1} in 10 mM sodium acetate buffer, at pH 4.5 and 4 °C. (a) Spectra of FTIR of rhG-CSF (b) Spectra of FTIR of PEGylated rhG-CSF (c) And a comparative image of the FTIR spectrum related to PEGylation of PEGylated and unPEGylated rhG-CSF.





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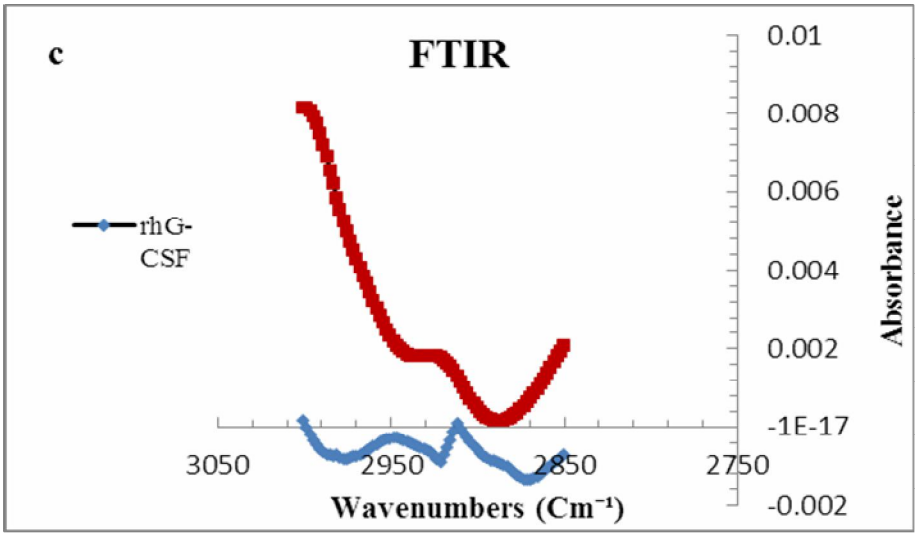

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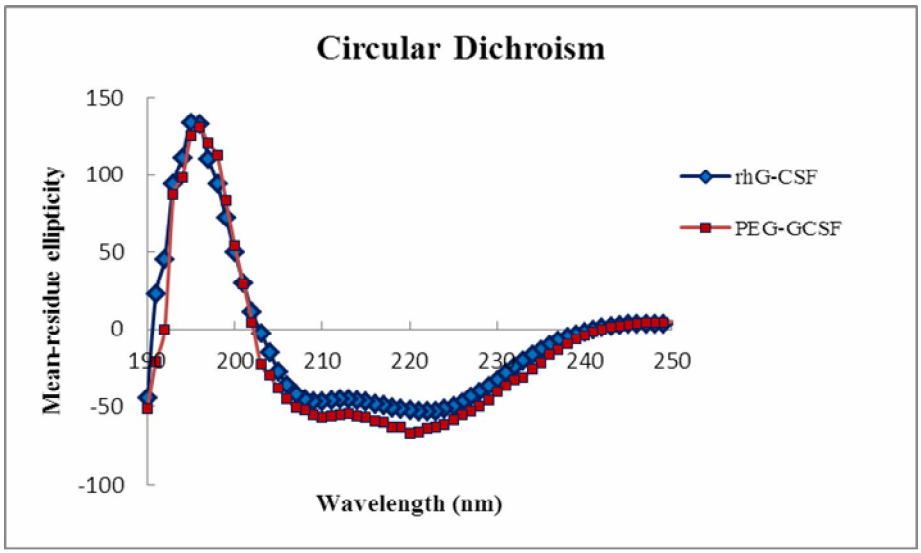

Fig. 4. Spectra of far-UV circular dichroism of rhG-CSF and PEGylated rhG-CSF. Concentration of rhG-CSF and PEGylated rhG-CSF were 0.2 mg ml^{-1} in 10 mM sodium acetate buffer, at pH 4.5 and 4 °C. Spectra of CD of rhG-CSF (♦) and PEGylated rhG-CSF (■).

spectroscopy suggested that the PEGylated form of rhG-CSF and rhG-CSF have identical tertiary structures (Fig. 5).

DISCUSSIONS

G-CSF, a cytokine produced by different tissues, is a physiological regulator of both neutrophil production and function and is used to treat chemotherapy induced neutropenia. Two type of approved G-CSF for clinical applications are filgrastim (Neupogen) and pegfilgrastim (Neulasta). Filgrastim, rhG-CSF, is administered daily from 24 h after chemotherapy and continuing until a target

Table 2. Secondary Structures Percentage of rhG-CSF and PEGylated rhG-CSF

Secondary structures	Percentage (%)	
	rhG-CSF	PEGylated rhG-CSF
Helix	84.47	93.49
Antiparallel	0.19	0
Parallel	1.23	0.29
Beta-Turn	7.3	4.7
Random Coil	6.72	1.44
Total Sum	100	100

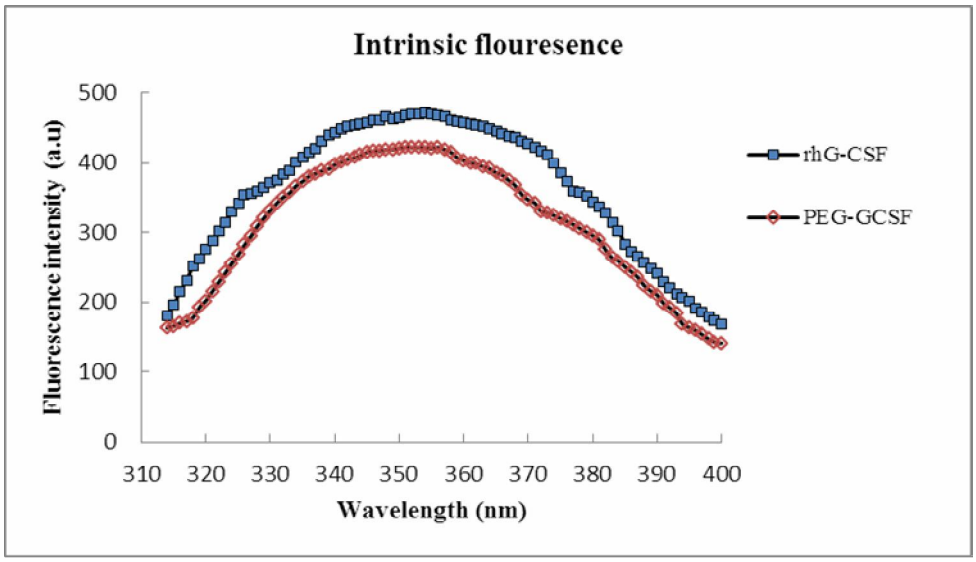

Fig. 5. Structural stability analysis of rhG-CSF and PEGylated rhG-CSF by intrinsic fluorescence spectroscopy. Concentration of rhG-CSF and PEGylated rhG-CSF were 0.1 mg ml^{-1} in 10 mM sodium acetate buffer, at pH 4.5 and 4 °C. Fluorescence emission spectra of rhG-CSF (■) and fluorescence emission spectra of PEGylated rhG-CSF (◊).

absolute neutrophil count (ANC) is reached (approximately 5,000/µl). The important disadvantage of filgrastim is its short half-time (3 to 4 h) therefore, it should be injected daily for up to 10 days during chemotherapy. Since the injections are painful and inconvenient for patient may result in reduced trend to the remedy. Pegfilgrastim is formed by attaching a PEG to the N-terminal residue of filgrastim to increase its half-life. The molecular weight of Pegfilgrastim is too high for kidneys clearance and so amount of pegfilgrastim in blood decreases by a self-regulation mechanism dictated by neutrophil uptake and utilization, as neutrophil counts increase. Since neutrophil-mediated clearance takes longer than renal clearance, so that its half-life is almost 24 h. As a result, only one 100-µg kg^{-1} subcutaneous injection is needed per chemotherapy cycle. Also bone pain was side effect that very often reported with

either pegfilgrastim or filgrastim treatment [64,65].

In order to improve pharmacokinetic feature of rhG-CSF and to reduce the frequency of its administration, the present work was carried out to develop a practical method which is described as follows: chemical modification by N-terminal site-directed mono-PEGylation of rhG-CSF was carried out using mPEG-propionaldehyde derivative (Mw = 20 kDa) under slightly acidic pH conditions (pH 4.5) with reducing reagent of sodium cyanoborohydride. Also one-step cation exchange chromatography was used to purify the monoPEGylated rhG-CSF. To determine efficiency of each PEGylation reaction and assay the presence and purity of mono-PEGylated form of rhG-CSF, SDS-PAGE assessment was done. Afterwards, Bradford assay was accomplished to define the concentration of PEGylated rhG-CSF. After characterizing the purity using SDS-PAGE and measuring concentration by Bradford assay, the following analysis were done on native and PEGylated rhG-CSF. FTIR was applied to verify the occurrence of mono-PEGylation at the N-terminus of rhG-CSF and then the secondary and tertiary structures of PEGylated rhG-CSF were evaluated using intrinsic fluorescence and circular dichroism (CD) measurements.

In previous articles [66,63,60,67,61,68], researchers have studied some of various factors that affect properties of PEGylated protein including the molecular weight, structure and the number of PEG chains attached to the protein, the PEGylation site on the protein, PEGylation chemistry, pH, temperature and incubation condition of reaction, and protein to PEG molar ratio. Here, we examined different protein to PEG molar ratio. The molar ratio of PEG to protein affects the number of modified amine groups. The number of PEG equivalents was optimized in order to acquire maximal conjugation with the minimal permissible PEG. It is determined that optimalest polymer/protein molar ratio is 5:1. Furthermore, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to investigate the modification rates and homogenicity of the final reaction mixtures, (3:1, 5:1 and 10:1 polymer/protein molar ratios), and the results (Figs. 1A and B) indicated that the mono-pegylated rhG-CSF was chemically well-defined and was molecularly homogeneous and showed that a reaction with 5:1 mPEG-ALD: protein molar ratio was sufficient to direct the reaction toward optimal yield of monoPEGylated protein (60%). According to Kinstler *et al.*, mPEGpropionaldehyde, such as other aldehydes, under acidic conditions (approximately pH 5) mainly prefer to connect to the α-amine of N-terminal due to its lower pKa than the other nucleophiles. To attain the N-terminally monoPEGylated rhG-CSF, PEGaldehyde should be coupled to primary α-amino group of the N-terminal residue of protein through a reductive alkylation, using sodium cyanoborohydride, a mild reducing agent, which give a stable secondary amine linkage *via* Schiff base reaction.

Additionally, to isolate and purify PEGylated proteins, different methods of chromatography are used including ion exchange chromatography (IEC) [69,70], size exclusion chromatography (SEC) [71,72], reversed phase chromatography (RP-HPLC) [73-75], hydrophobic interaction chromatography (HIC) [76], and hydrophobic interaction membrane chromatography (HIMC) [77] because of the repeatability of their results. Properties of the protein and PEG should be taken into account in order to achieve high-resolution products. Since PEGylated protein using mPEG-ALD has been proved to preserve the charge of protein, which means that there were no net charge differences between PEGylated and unPEGylated forms, thus among the chromatographic methods listed above IEC was selected as a result of the number of binding sites of protein to the column's matrix. Given pI value of rhG-CSF is 5.6, it is positively charged at pH values around 4, so cation exchange chromatography using SP Sepharose FF resin, was suitable method to separate unPEGylated and PEGylated rhG-CSF. Separation is based on electrostatic charges differences in ion exchange chromatography. PEGylation on N terminal residue does not significantly change the net charge of protein, so PEGylated protein has the same charge of non-PEGylated form. But shielding characteristic of PEG chain and weakening the interaction of PEGylated species with the resin, could contributed to separation of PEGylated protein from non-PEGylated one in ion exchange chromatography [78]. This is also the reason why the separation of PEGylated rhG-CSF from resin is much easier and hence leads to one-step purification procedure in this research. Also, results of SDS-PAGE of fractions of purification stage suggested that monopegylated rhG-CSF had purity of around 99 (Fig. 2 and Table 1).

Non-PEGylated and PEGylated rhG-CSF forms were characterized by FTIR in the 3800-2700 cm^{-1} and 1900-1400 cm^{-1} spectral ranges to verify the occurrence of mono-PEGylation at the N-terminus of rhG-CSF. The reason for investigating N-terminal PEGylation site by FTIR is that it allows to assay the secondary structure of proteins by the Amide I band between 1700 and 1600 cm^{-1}, and the CO stretching vibration of the peptide bond, is sensitive to the backbone conformational change. Also since each functional group has a special peak in FTIR. Results of FTIR analysis of purified monoPEGylated rhG-CSF compared to the non-PEGylated rhGCSF showed a higher absorption between 2950 and 2850 cm^{-1} that confirmed the achievement of the PEGylation reaction. Moreover, the both forms of purified rhG-CSF displayed the identical Amide I profile, indicating the preservation of secondary structure of forms of unPEGylated and PEGylated rhG-CSF.

Furthermore, structural studies by circular dichroism (CD) spectroscopy demonstrated that the spectrum of both purified forms had similar ellipticities with minimums at ~208 nm and ~222 nm and a positive ellipticity at ~193 nm, typical of α helical proteins, which indicated that the structure of monoPEGylated rhG-CSF was very comparable with the rhG-CSF. To estimate the percentage of secondary structure in non-PEGylated and PEGylated form of rhG-CSF, the result was deconvoluted by CDNN software. Deconvolution of CD data between 195 nm and 260 nm also exhibited that there were mostly similar secondary structures in both form of rhG-CSF and few differences between Helix of forms of non-PEGylated and PEGylated rhG-CSF. Therefore, PEGylation didn't exert significant effect on their secondary structure and given the protein function is strictly related to the preservation of its native structure, this subject is crucial (Fig. 4 and Table 2).

In analysis fluorescence spectroscopy, the fluorescence emission intensity at 330-360 nm after excitation of the two tryptophane (Trp59 and Trp119) of non-PEGylated and PEGylated rhG-CSF at 295 nm were assessed. Similar emission of both form of rhG-CSF at 345 nm proved that the purified monoPEGylated rhG-CSF have alike tertiary structure with rhG-CSF. Therefore, the extracted data from fluorescence spectroscopies confirmed that tertiary structure of the purified monoPEGylated rhG-CSF was not influenced by PEGylation (Fig. 5).

CONCLUSIONS

The subject of this research was to develop an effective, simple and inexpensive method to prepare and purify monoPEGylated rhG-CSF. Therefore it was revealed that the optimized reaction condition for the highest PEGylation degree of the monoPEGylated rhG-CSF was PEG to protein molar ratio 5:1 respectively. As well as, one-step cation exchange chromatography method was efficient and reproducible process to purify both the rhG-CSF and PEGylated rhG-CSF. The FTIR absorption spectra of unPEGylated and PEGylated rhG-CSF confirmed the success of N-terminal PEGylation reaction. Also the data of extracted from CD and fluorescence spectroscopies indicated that the secondary and tertiary structure of rhG-CSF did not considerably changed due to PEGylation. In conclusion, this study has developed an experimental method to purify mono PEGylated rhG-CSF which is simple, fast, and low cost method with high efficiency.

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