Characterization and Stability of *N*-terminally PEGylated rhG-CSF

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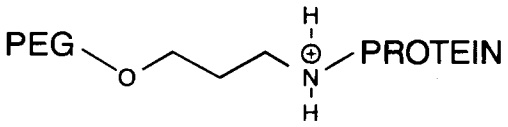
Purpose. The liquid stability of rhG-CSF was investigated after polyethylene glycol (PEG) with an average molecular weight of 6000 daltons was covalently attached to the *N*-terminal methionine residue.
Methods. The conjugation methods chosen for modifying the *N*-terminal residue were alkylation and acylation. The *N*-terminally PEGylated rhG-CSF conjugates were purified by cation exchange chromatography. The physical characterization methods of SDS-PAGE, endoproteinase peptide mapping, circular dichroism and in-vivo bioassay were used to test for differences between the PEG-rhG-CSF molecules.
Results. Physical characterization indicated no apparent differences in the rhG-CSF molecules that were conjugated with either method. Stability, in liquid at elevated temperatures, of these conjugated molecules indicated that the primary pathway of degradation was aggregation. Conjugation through alkylation offered the distinct advantage of decreasing, by approximately 5 times, the amount of aggregation present as compared to acylation.
Conclusions. We suggest, that the increased stability observed for the molecules utilizing the alkylation conjugation method may be due to the preservation of charge on the alpha amino group of rhG-CSF.

KEY WORDS: granulocyte-colony stimulating factor (G-CSF); PEGylation; *N*-terminal; stability; site specific.

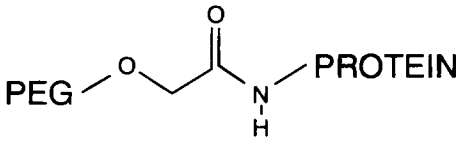
INTRODUCTION

Human granulocyte colony stimulating factor (G-CSF) is a hematopoietic growth factor which regulates the proliferation and differentiation of neutrophilic granulocytes (1–3). Recombinant human G-CSF (rhG-CSF) has found success in treating the myelosuppression associated with cancer chemotherapy treatments (4).

It has been generally observed that the effects of physiologically active proteins such as rhG-CSF are short-lived due to their rapid clearance in the body. The covalent attachment of polyethylene glycol (PEG) is currently being employed to extend the circulation time of proteins in the blood (5–7). Since the introduction of PEG modified proteins (8), a great deal of interest has been generated in this type of macromolecular conjugation. Numerous chemistries are available for conjugating PEG to specific amino acid residues found in proteins. It has been previously shown that a single PEG group attached to the *N*-terminal residue of rhG-CSF increased the proteins circulation time and had no effect on its activity (9). We have investigated alkylation and acylation for the site specific addition of PEG to the *N*-terminal residue of rhG-CSF. Alkylation (10) forms a secondary amine, according to *Scheme I*, with the *N*-terminal methionine of rhG-CSF and thus preserves the charge on the *N*-terminal amino group. Acylation (10) forms an amide linkage, according to *Scheme II*, which removes the charge on the amino group of the *N*-terminal methionine of rhG-CSF.



Scheme I



Scheme II

In this report, we demonstrate that the physical characterization techniques of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism, endoproteinase peptide mapping and *in-vivo* bioassay all indicate no apparent difference in the type of chemistry used for attaching the PEG group to rhG-CSF. However, size exclusion chromatographic analyses, after incubation at elevated temperatures, indicates a greater degree of aggregation associated with the acylation PEGylation conjugation method as compared to alkylation.

MATERIALS AND METHODS

Materials

All reagent grade chemicals and buffer components were purchased from Sigma (St. Louis, MO). Recombinant human Granulocyte-colony stimulating factor (rhG-CSF) was prepared as previously described (3). HPLC grade solvents were obtained from Baxter (Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Pierce Chemicals (Rockford, IL). Lysyl Endopeptidase (EndoLysC) and α-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) were obtained through Wako Chemicals (Richmond, VA). Tributylphosphine (TBP, technical grade) was purchased from Aldrich (Milwaukee, WI). The 300 Å pore size C_4 (214-TP-54) reversed phase column was purchased from the Separations Group (Vydac, Hesperia, CA). α-Cyano-4-hydroxycinnamic acid was purchased from Biomolecular Separations, Inc. (Reno, NV). The 6kDa methoxy polyethylene glycol propionaldehyde was obtained from Shearwater Polymers (Huntsville, AB). The 6kDa methoxy polyethylene glycol carboxymethyl-N-hydroxysuccinimdyl ester was purchased from Union Carbide (Terrytown, NY). All compositional analyses were performed on a Beckman 6300 amino acid analyzer and all sequence analyses were performed on a Hewlett Packard G1005A protein sequencer.

Derivatization of rhG-CSF with 6 kDa Methoxy Polyethylene Glycol Carboxymethyl-N-hydroxysuccinimidyl Ester

A solution of rhG-CSF (30 mg, 1.60 μmol) at 5 mg/ml in 0.1M NaH_2PO_4, pH 6.5, at 4°C was added to a vial containing

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6 kDa methoxy polyethylene glycol carboxymethyl-N-hydroxysuccinimidyl ester (114 mg, 19.2 μmol). Once the PEG was dissolved, the reaction was allowed to stir at 22°C for 1 h. Two molar hydroxylamine (2.6 ml, pH 7.3) was added to the reaction mixture in order to cleave any unstable sites of PEGylation on rhG-CSF. After 1 h of treatment with hydroxylamine, the reaction mixture was diluted to 50 ml with 1 mM HCl (final protein concentration of 0.5 mg/ml) and the pH was adjusted to 3.5 with 1M HCl. The solution was loaded onto a Sepharose HP HiLoad 16/10 (1.6 cm × 10 cm) cation exchange column (Pharmacia, Alameda, CA) pre-equilibrated with 20 mM sodium acetate, pH 4.0, (buffer A) at a flow rate of 30 cm/h using a Pharmacia LCC 501 Plus FPLC system. The column was washed with three column volumes (3 × 20 mL) of buffer A before a linear gradient to 45% buffer B (buffer A + 1 M NaCl) was applied over 25 column volumes. A total of 72-7 ml fractions were collected. The fractions having an absorbance at 280 nm were analyzed by reducing SDS-PAGE. The SDS-PAGE data indicated that the mono PEGylated rhG-CSF was collected in 2 of the 7 ml fractions. No protein was detected in the column wash. Upon purification, we obtained 43% mono PEGylated rhG-CSF (the site of PEGylation was determined by endoproteinase peptide mapping, see below), 42% unreacted rhG-CSF and 15% multi-PEGylated rhG-CSF based on peak areas. The purified mono acyl-PEGylated rhG-CSF conjugate was concentrated to 5 ml in an Amicon (Beverly, MA) stir cell fitted with a YM10 membrane (molecular weight cut off 10 kDa) and buffer exchanged with 10 volumes of 10mM sodium acetate, pH 4.0, containing 5% sorbitol. After exchange, the protein concentration was adjusted to 1mg/ml (using 10mM sodium acetate, pH 4.0, containing 5% sorbitol), sterile filtered through a 0.2 μm Costar filter unit, placed in 3ml sterile injection vials (1 ml/vial), sealed with a sterile rubber septa and crimp capped. A total of six vials were placed at 45°C.

Derivatization of rhG-CSF with 6 kDa Methoxy Polyethylene Glycol Propionaldehyde

A solution of rhG-CSF (20 mg, 1.06 μmol) at 5 mg/ml in 0.1 M NaH_2PO_4, pH 5.0, at 4°C was added to a vial containing 6 kDa methoxy polyethylene glycol propionaldehyde (48 mg, 7.95 μmol). Once the PEG was dissolved, sodium cyanoborohydride (0.082 ml of a 1.0 M solution in water) was added to the reaction mixture. The reaction was stirred at 4°C for 16 h and then diluted to 50 ml with 1mM HCl (final protein concentration of 0.5 mg/ml) and the pH was lowered to 3.5 with 1 M HCl. The solution was loaded onto a Sepharose HP HiLoad 16/10 (1.6 cm × 10 cm) cation exchange column (Pharmacia, Alameda, CA) pre-equilibrated with 20 mM sodium acetate, pH 4.0, (buffer A) at a flow rate of 30 cm/h using a Pharmacia LCC 501 Plus FPLC system. The column was washed with three column volumes (3 × 20 mL) of buffer A before a linear gradient to 45% buffer B (buffer A + 1M NaCl) was applied over 25 column volumes. A total of 72-7ml fractions were collected. The fractions having an absorbance at 280nm were analyzed by reducing SDS-PAGE. The SDS-PAGE data indicated that the mono PEGylated rhG-CSF was collected in 3 of the 7 ml fractions. No protein was detected in the column wash. Upon purification, we obtained 71% mono PEGylated rhG-CSF (the site of PEGylation was determined by endoproteinase peptide mapping, see below), 28% multi-PEGylated rhG-CSF and less than 1% unreacted rhG-CSF based on peak areas. The purified mono alkyl-PEGylated rhG-CSF conjugate was concentrated to 5 ml in an Amicon (Beverly, MA) stir cell fitted with a YM10 membrane (molecular weight cut off 10 kDa) and buffer exchanged with 10 volumes of 10 mM sodium acetate, pH 4.0, containing 5% sorbitol. After exchange, the protein concentration was adjusted to 1mg/ml (using 10mM sodium acetate, pH 4.0, containing 5% sorbitol), sterile filtered through a 0.2 μm Costar filter unit, placed in 3 ml sterile injection vials (1 ml/vial), sealed with a sterile rubber septa and crimp capped. A total of six vials were placed at 45°C.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using a 10–20% ISS MiniPlus gel (Nattick, MA). Samples were diluted with either reducing or non-reducing buffer and 5.0 μg of protein was loaded into each well. The gels were run on a discontinuous buffer system and stained with Coomassie Blue (11).

Size Exclusion HPLC

HPLC was performed on a Waters Liquid Chromatograph (Millford, MA) equipped with a WISP 717 autosampler refrigerated at 6°C, and a 490E multiwavelength UV/Vis detector. Data was recorded by Waters Maxima software on a IBM/PC. The SEC samples were analyzed with an isocratic mobile phase of 0.1 M sodium phosphate, pH 6.9, on a Phenomenex BioSep S3000 column (Torrence, CA).

Ion-exchange HPLC

Analytical cation exchange samples were analyzed on a TosoHaas SP-5PW column (Montgomery, PA) initially equilibrated in 20mM sodium acetate, pH 5.4 (buffer A), and eluted with a linear gradient of 20mM sodium acetate, pH 5.4, containing 0.5 M NaCl (buffer B) at 2% B/min linear gradient over 30 min.

Reversed Phase HPLC

Samples were analyzed on a Vydac C_4 column equilibrated in 60% buffer A (HPLC grade H_2O containing 0.1% TFA) and 40% buffer B (95% CH_3CN, 5% HPLC grade H_2O and 0.1% TFA) and eluted with a 1% buffer B/min linear gradient over 30 min.

Peptide Mapping

Either 0.25 or 0.5 mg of rhG-CSF (standard) or of the purified mono PEGylated rhG-CSF was dried in a speed vac, reconstituted in 100 μL of 8 M urea, and sonicated for 10 min. After sonication, 10 μL of 1 M Tris-HCl, pH 8.5 and 5–10 μg of EndoLysC from a 1mg/mL stock solution in 10 mM Tris-HCl, pH 8.5, were added. The total volume was adjusted to 200 μL with distilled H_2O, and the proteolytic digestion was carried out for 7 h at room temperature.

Following the hydrolysis with EndoLysC, the disulfide bonds were simultaneously reduced with 5 μL of 80 mM TBP and alkylated with 10 μL of 40 mM ABD-F (2 mM final concentration) (12). The reaction mixture was heated at 60°C for 10 min, and then cooled to room temperature.

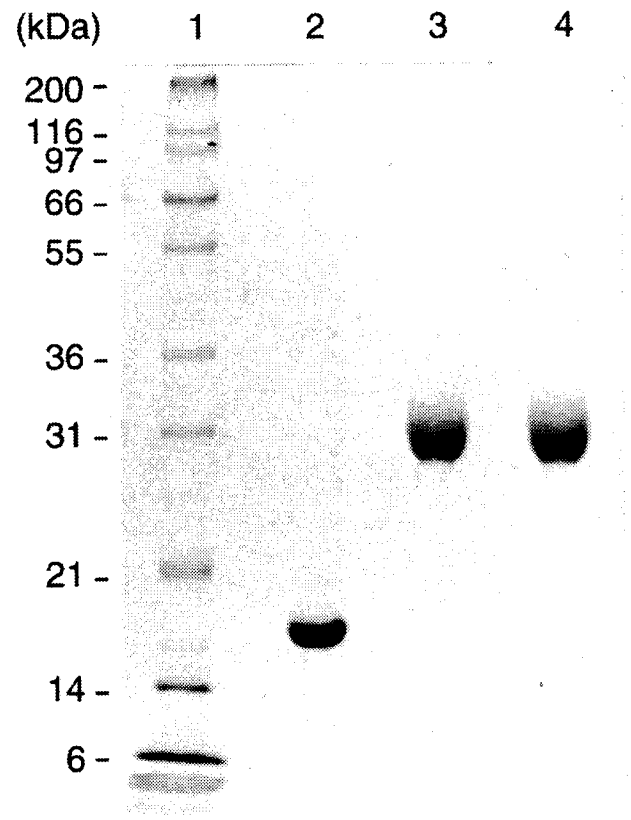

Fig. 1. SDS-PAGE analysis of the purified mono PEGylated rhG-CSF stained with Coomassie Blue. *Lane 1,* Novex molecular weight standards. *Lane 2,* unmodified rhG-CSF. *Lane 3,* mono PEGylated rhG-CSF purified from utilizing the acylation conjugation method. *Lane 4,* mono PEGylated rhG-CSF purified from utilizing the alkylation conjugation method.

Immediately after reduction and alkylation, the generated peptides (200 μL) were acidified and injected directly onto a Vydac C_4 column equilibrated with buffer A (HPLC grade H_2O containing 0.1% TFA). The generated peptides were eluted with a linear gradient of 3–76% solvent B (95% CH_3CN, 5% HPLC grade H_2O and 0.1% TFA) over 115 min. Elution was monitored for absorbance at 215 nm. Individual peptides from the rhG-CSF standard peptide map were collected and identified by amino acid compositional analysis, *N*-terminal sequencing and MALDI/MS (data not shown). All peaks present in the peptide map of both the purified mono PEGylated rhG-CSF conjugates were collected and compared to the rhG-CSF standard for peptide content and molecular weight by MALDI/MS.

Treatment of PEGylated Peptide with CNBr

Approximately 300 μg of the PEGylated *N*-terminal peptide was treated with a 500 molar excess of CNBr in 70% trifluoroacetic acid under nitrogen. The reaction was allowed to proceed for 24 hr at room temperature. After completion, the sample was dried in a speed vac and washed three times with deionized H_2O. The dried sample was reconstituted with 8 M urea, injected onto a Vydac C_4 column and eluted using the same linear gradient previously described under peptide mapping.

Determination of the pI for both N-terminally PEGylated rhG-CSF Conjugates

Isoelectric focusing was performed on an IsoGel agarose IEF plate (precast 12.5cm × 10cm IEF gels) using a pH gradient of 3–7 (FMC, Rockland, ME). The anode and cathode buffers were fluid 3 (anode) and fluid 10 (cathode) from Serva (Paramus, NJ). The purified mono PEG-rhG-CSF conjugates (approximately 3 μg) were loaded near the anode end and the gel was run at 10°C using a Hoffer Isobox (Pharmacia) equipped with a cooler and focused at 1000 volts, 20mA and 15 watts for 60 minutes. Low pI (2.6–6.5) standards (Pharmacia) were run to calibrate the gel. The gel was fixed, stained with coomassie blue and scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA). The pI's were calculated as follows: mono PEG-rhG-CSF generated utilizing the acylation chemistry had a pI of 5.7 and the mono PEG-rhG-CSF generated utilizing the alkylation chemistry had a pI of 6.1.

Circular Dichroism

CD spectra were obtained with a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a 0.020 cm cell. Calculations of mean residue ellipticity was made using a mean residue mass of 107 Da.

In Vivo Biological Activity Assay

Male Golden-Syrian hamsters were randomized and dosed at 100μg of protein/kg of rhG-CSF or PEGylated rhG-CSF by subcutaneous injection. After injection, blood was collected at selected times by cardiac puncture after sacrificing by CO_2 asphyxiation. Collected blood samples were analyzed using a Sysmex F800 microcell counter (Kobe, Japan) for WBC concentration.

Matrix-Assisted Laser Desorption Mass Spectrometry

MALDI/MS was performed with a Kompact MALDI III mass spectrometer (Kratos Analytical, Ramsey, NJ) fitted with a standard 337nm nitrogen laser. The spectra were recorded with the analyzer in the linear mode at an accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Traditional PEGylation at amine residues has occurred in the pH range of 8.5–9.0 (10). As previously described (9), we have found conditions (see materials and methods) where the site specific addition of a PEG moiety at the *N*-terminus can be controlled to a large degree by lowering the pH at which the PEGylation reaction takes place. The lower pH takes advantage of the differences in pKa values of the α-amino (pKa 7.8, (10)) group versus the ϵ-amino (pKa 10.1, (10)) groups on the side chains of lysine residues.

The mono PEGylated rhG-CSF produced in the PEGylation reactions (acylation and alkylation) were separated from higher PEGylated rhG-CSF (rhG-CSF molecules containing more than one PEG group) and the unreacted rhG-CSF molecules by cation exchange chromatography. The purity of the mono PEGylated rhG-CSF isolated by ion exchange chromatography was assesssed by SDS-PAGE (Figure 1). The purified PEGylated protein appeared as a single diffuse band when using either the alkylation (lane 3, Figure 1) or acylation conjugation

methods (lane 4, Figure 1). The estimated molecular weight from SDS-PAGE (data not shown) suggests that each rhG-CSF PEG conjugate contains only a single PEG molecule. The retarded mobility of PEGylated proteins (resulting in higher molecular weight estimates) on SDS-PAGE gels has been previously discussed (13), therefore, the actual molecular weight of the PEG-protein complex is best determined by mass spectrometry. We have used MALDI/MS to determine the molecular weights of the purified mono PEG conjugates. These results are presented in Table I and indicate a molecular weight of 24,860 and 24,816 daltons for the acylation and alkylation conjugates, respectively (the molecular weight of rhG-CSF is 18,800 daltons, as determined previously (3)). The MALDI/MS molecular weight data ranged from approximately 24.6 to 25.0 kDa for each conjugate, however, only the midpoint of the range is reported in Table I. Although the molecular weight determination of PEG modified proteins is complicated due to the inherent polydispersity which is typical of a polymer like PEG, these molecular weights are in good agreement with the rhG-CSF conjugates containing a single 6000 dalton PEG derivative.

The location of the conjugated PEG moeity on the purified mono PEGylated rhG-CSF conjugates was determined by peptide mapping. Peptide fragments were generated from unmodified rhG-CSF or from the purified mono PEG-rhG-CSF conjugates by treatment with EndoLys-C. The peptide fragments were then resolved by reversed phase HPLC (Figure 2: panel A—unmodified rhG-CSF; panel B—mono PEGylated rhG-CSF (acylation); panel C—mono PEGylated rhG-CSF (alkylation)). A peak eluting at approximately 60 min was absent from the PEG-rhG-CSF conjugated materials. The peptide eluting in this peak was determined by amino acid analysis and *N*-terminal sequencing to be the *N*-terminal 17 residues (3) of rhG-CSF. Thus, in the PEG conjugated samples, only the corresponding *N*-terminal peptide fragment appeared to be modified which resulted in a new peak eluting at approximately 77 min. Analysis of each of these peaks from the respective PEG-rhG-CSF conjugates indicated that the peak eluting at 77 min was the *N*-terminal peptide (as determined by amino acid analysis and/or *N*-terminal sequencing, see Table I). Further analysis by MALDI/MS indicated a molecular weight of 7,788 daltons (Table I) for both PEG conjugates (MALDI/MS of the unmodified *N*-terminal peptide indicated a molecular weight of 1,788 daltons, see Table I). All peaks eluting in the EndoLysC peptide maps of the PEG rhG-CSF conjugates (Figure 2, panel B and C) were analyzed by MALDI/MS (data not shown). Only the peak eluting at approximately 77 min in each chromatogram had a molecular weight different (for this peptide the molecular weight was 6kDa greater than expected, see Table I) from the rhG-CSF standard (Figure 2, panel A). The SDS-PAGE (a single broad band), peptide mapping (indicating only a single completely modified peptide) and the MALDI/MS data suggest that the isolated PEGylated rhG-CSF molecules from either conjugation method contain a single 6kDa PEG molecule conjugated at the *N*-terminal portion of the molecule.

Alkylation does not block the *N*-terminus to sequencing as does acylation due to the formation of an amide bond with the α-amino group of the *N*-terminal residue. Therefore, with alkylation it is problematic to determine the residue in the *N*-terminal peptide that is PEGylated. We have shown in Figure 3 that after treating the *N*-terminal PEGylated peptide (from the alkylation conjugation method, Figure 3) with CNBr, we isolated a peptide that had a similar retention time to the unmodified *N*-terminal peptide (see Figure 3). Table I indicates that the modified *N*-terminal peptide collected in panel B of Figure 3 had a molecular weight as determined from MALDI/MS of 7,788 Daltons and a sequence of Met(0)—Lys(16) (the residue numbering is in accordance with the previously published sequence for rhG-CSF (3)). After treating with CNBr, the peptide collected (Figure 3) had a molecular weight as determined by MALDI/MS of 1657 Daltons and a sequence of Thr(1)—Lys(16)(for the full sequence of this peptide see the legend for Figure 3) indicating that the *N*-terminal methionine contains the PEG modification in the alkylation conjugation method.

As shown in Figure 4, a single s.c. injection of the *N*-terminally PEGylated rhG-CSF leads to prolonged elevation of WBC starting within 24 h, however, baseline levels were not achieved until 7 days after injection. Although PEGylation leads to the prolonged elevation of the plasma levels of rhG-CSF as compared to unmodified rhG-CSF (14), there appears to be no selective advantage attributed to either conjugation method. The activity curves (Figure 4) appear to be equivalent for both conjugation methods.

The CD spectra of the PEGylated rhG-CSF conjugates overlay (Figure 5) each other showing the same ellipticities with minimas at 208 and 222 nm. Therefore, the secondary structure is not influenced by PEGylation of rhG-CSF on the *N*-terminus by either conjugation method. These results are consistent with those previously shown for rIL-2 after PEGylation (15).

Table I. The Positive-ion MALDI/MS Data for the Species Indicated

	MALDI/MS Mw (Expected) Whole Protein	MALDI/MS[a] Mw (Observed) Whole Protein	MALDI/MS Mw (Expected) *N*-terminal Peptide[b]	MALDI/MS[a] Mw (Observed) *N*-terminal Peptide	Observed N-terminal Peptide Sequence[c]
Acyl Linked 6 kDa PEG-G-CSF	24,800 Da	24,860 Da	7,788 Da	7,831 Da	Met (0) – Lys (16)
Alkyl Linked 6 kDa PEG-G-CSF	24,800 Da	24,816 Da	7,788 Da	7,781 Da	Met (0) – Lys (16)
G-CSF	18,800 Da	18,804 Da	1,788 Da	1,789 Da	Met (0) – Lys (16)

[a] The mass accuracy of MALDI/MS is ±0.1%.

[b] The calculated molecular weights for the PEGylated peptides were summed from the known peptide sequence and the average nominal polyethylene glycol molecular weight of 6000 Daltons.

[c] Sequences were determined by amino acid analysis for the acyl linked PEG containing peptide and *N*-terminal sequencing and CNBr digestion for the alkyl linked PEG containing peptide.

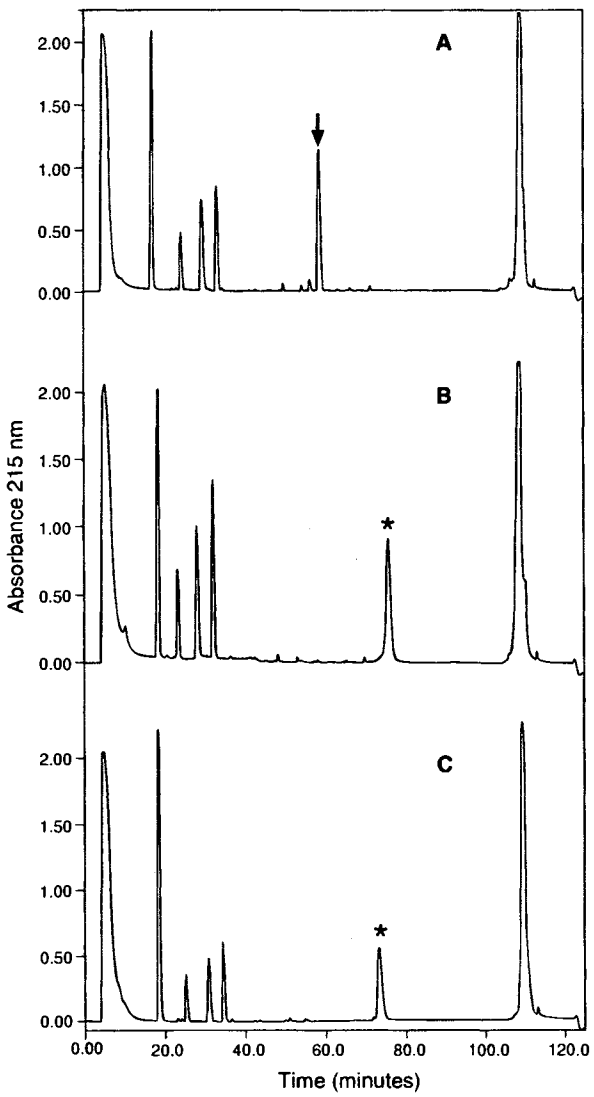

Fig. 2. The reversed phase HPLC chromatograms for the Lysyl Endopeptidase digests of unmodified and each of the purified mono PEGylated derivatives. *Panel A*, unmodified rhG-CSF. The peak labeled with an arrow was identified by both *N*-terminal sequencing and MALDI/MS (Table I) as the *N*-terminal peptide of rhG-CSF. *Panel B*, represents rhG-CSF that has been PEGylated utilizing the acylation conjugation method. The peak labeled with an asterisk (*) was identified by amino acid analysis (Table I) as the *N*-terminal peptide of rhG-CSF. *Panel C*, represents rhG-CSF that has been PEGylated utilizing the alkylation conjugation method. The peak labeled with an asterisk (*) was identified by *N*-terminal sequencing and amino acid analysis (Table I) as the *N*-terminal peptide of rhG-CSF.

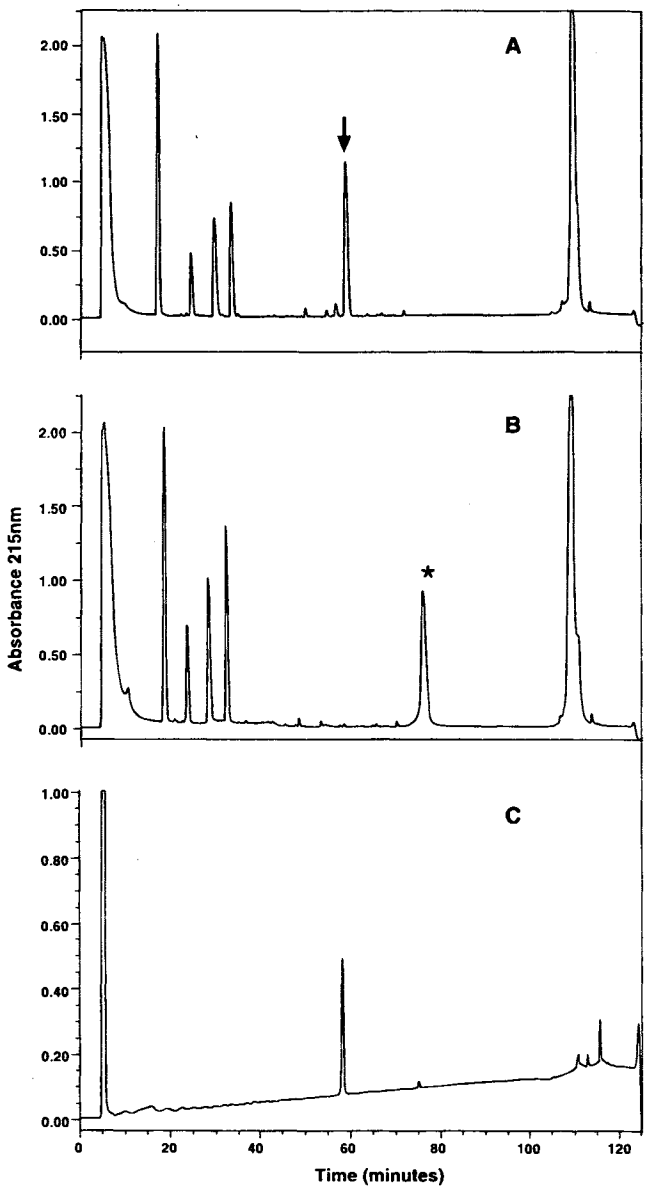

Fig. 3. The Lysyl Endopeptidase reversed phase HPLC chromatograms which compare the standard unmodified rhG-CSF versus the PEGylated rhG-CSF and the PEGylated *N*-terminal peptide after treating with CNBr. *Panel A*, unmodified rhG-CSF. The peak labeled with an arrow is the *N*-terminal peptide. *Panel B*, the peptide labeled with an asterisk (*) is the PEGylated *N*-terminal peptide resulting from the alkylation conjugation method. *Panel C*, is the resulting peptide after treating the labeled (*) peptide in panel B with CNBr. *N*-terminal sequencing gave the following one letter sequence: T-P-L-G-P-A-S-S-L-P-Q-S-F-L-L-K, and MALDI/MS indicated a molecular weight of 1657 Daltons.

The storage effects from modifying proteins through PEGylation are poorly understood and even more so when the degree of PEGylation is limited. Considering that the physical characterization studies did not indicate any differences between conjugation methods, we compared their liquid stability's. The primary degradation pathways for these PEGylated rhG-CSF conjugates were assessed at 45°C to accelerate the aging process. The formulated samples were assayed after 1, 2, 3, 5 and 8 weeks of incubation. Degradation was assayed using SDS-PAGE, size exclusion, reversed phase and cation exchange chromatography.

The results of the accelerated degradation studies are presented in Figure 6 which represents the decrease in the main peak recovery as analyzed by size exclusion. The data indicates that the acylated PEG-rhG-CSF degrades faster than the alkylated PEG-rhG-CSF. For all time points, the primary pathway of degradation is through aggregation. Figure 7 represents size exclusion chromatograms that compare the acylation versus alkylation conjugation chemistries after storage for eight weeks at 45°C. The large aggregates shown in Figure 7 run at the

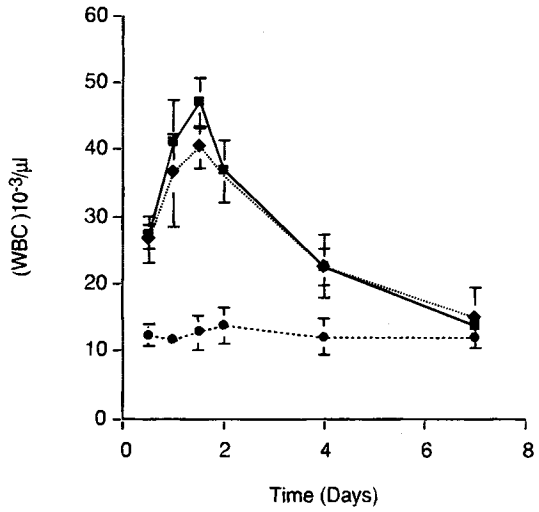

Fig. 4. The induction of peripheral WBC counts in hamsters from the subcutaneous injection of either vehicle (buffer) only consisting of 10mM sodium acetate, pH 4.0, containing 5% sorbitol (●), PEGylated rhG-CSF (◆) from the acylation conjugation method or PEGylated rhG-CSF (■) from the alkylation conjugation method and measured over time. The data represent the mean +/− S.D. for n = 4 hamsters/point.

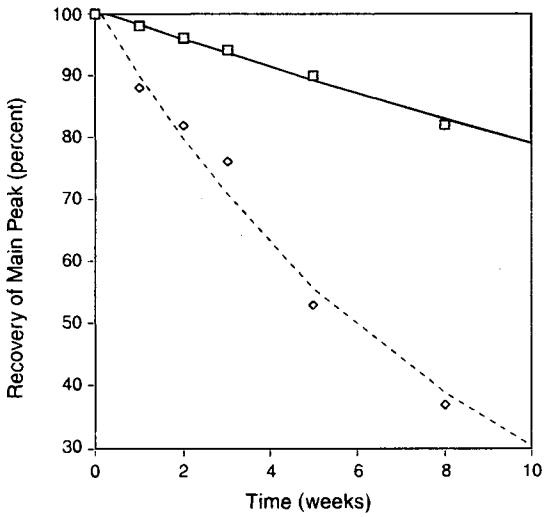

Fig. 6. The kinetics of degradation of *N*-terminally PEGylated rhG-CSF. Degradation was determined by the loss of monomer according to size exclusion chromatography for the alkylated conjugate (□), and for the acylated conjugate (◇). The lines represent calculated curves using the first-order rate equation: $[A] = [A]_o * e^{-kt}$, where [A] is the percent monomer at any given time, $[A]_o$ is the percent monomer at time zero, k is the rate constant, and t is time. The calculated curves were fit to the data using a non-linear least squares analysis and resulted in a k of 0.0242 $week^{-1}$ and $R^2 = 0.9899$ for the alkylated conjugate (—) and a k of 0.1203 $week^{-1}$ and $R^2 = 0.9822$ for the acylated conjugate (---).

exclusion limit (in excess of 700 kDa) of the column used, whereas the mono PEG-rhG-CSF conjugates (using either PEGylation chemistry) have an apparent molecular weight (based on column standards, data not shown) of approximately 60 kDa (the increase in molecular weight, as compared to MALDI/MS, is due primarily to the large Stokes radius of the extended PEG moiety and the accompanying water of hydration). Greater than 95% of the aggregate generated appears to be non-covalent (as determined from the densitometric scan of the non-reducing SDS-PAGE gels, data not shown). These data indicate that, after incubation at 45°C, PEGylation utilizing the alkylation conjugation method produces approximately 5 times less aggregate than acylation (see Figure 6, the rate constant for the alkylated PEG-rhG-CSF was determined to be .0242 versus .1203 for the acylated PEG-rhG-CSF). The loss of main peak by cation exchange and reversed phase chromatography is also due to aggregation (data not shown). However, chemical degradation within the aggregate was not determined.

The only difference between the two types of PEGylation chemistries is in the type of bond formed between the PEG moiety and the protein. PEGylation through acylation removes

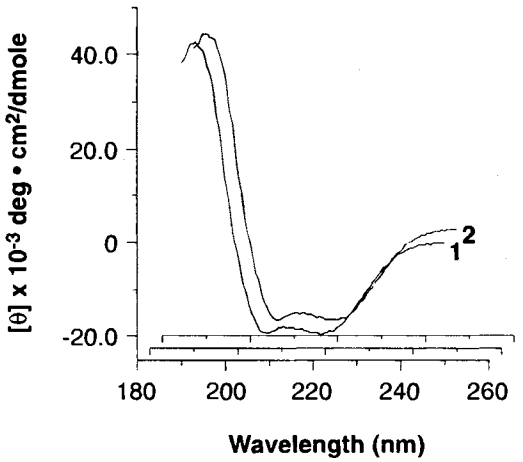

Fig. 5. Circular dichroism spectra of the PEGylated rhG-CSF conjugates. Far ultraviolet circular dichroism spectra of alkylated PEG-rhG-CSF conjugate (spectrum 1) at 0.9 mg/ml, and acylated PEG-rhG-CSF conjugate (spectrum 2) at 1.2 mg/ml. All samples were in 1 mM HCl at 23°C.

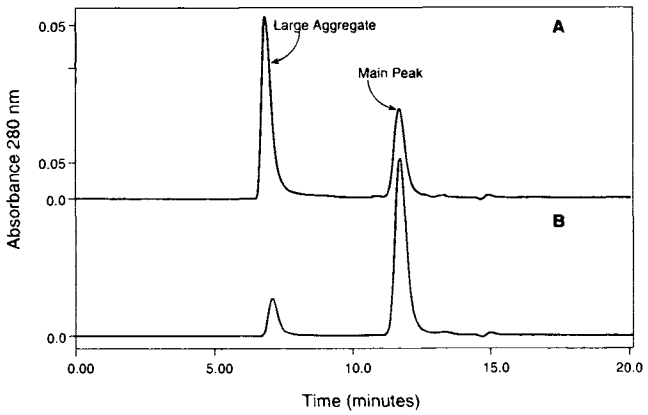

Fig. 7. The size exclusion HPLC chromatograms comparing the two conjugation methods after storage for eight weeks at 45°C. *Panel A*, represents rhG-CSF which was PEGylated utilizing the acylation conjugation method. The aggregate and main peaks are labeled above. *Panel B*, represents rhG-CSF which was PEGylated utilizing the alkylation conjugation method.

the charge on the α-amino group through the formation of an amide bond. This is supported by the experimentally measured decrease in the pI (5.7) for the acylation conjugation method versus the pI (6.1) for the alkylation conjugation chemistry. Therefore, it is suggested that the increased aggregation observed with the acylation conjugation method may result from the charge neutralization of the *N*-terminal α-amino group of rhG-CSF.

CONCLUSION

In this report, we have shown that the type of conjugation method used for covalently attaching a PEG moiety to protein can lead to a substantial increase in protein aggregation. The detrimental effects of aggregation in parenteral formulations of therapeutic proteins affirms the importance of minimizing this type of degradation. Therefore, when protein PEGylation is considered the type of conjugation used should be a major factor in determining how the PEG group is covalently attached to a given protein.

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