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Chemical and Enzymatic Site Specific PEGylation of hGH

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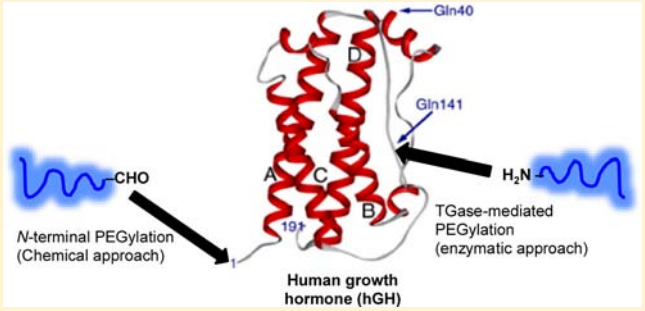
Supporting Information

ABSTRACT: Several strategies for site-specific PEGylation have been successfully exploited to conjugate poly(ethylene glycol) (PEG) to pharmaceutical proteins. The advantages sought are those of improving efficacy and increasing the half-life of conjugated proteins while achieving a higher degree of homogeneity. Recombinant human growth hormone (hGH) was thus PEGylated exploiting two site-specific strategies: *N*-terminal PEGylation using the $PEG_{20\ kDa}$–aldehyde polymer and microbial transglutaminase (mTGase) mediated enzymatic PEGylation using $PEG_{20\ kDa}$–NH_2. *N*-Terminal PEGylation of hGH was carried out by covalent attachment of PEG to the α-amine residue of Phe1 that yielded the monoconjugate PEG-Nter-hGH with a mass of 44152.2 Da, as measured by MALDI-TOF mass spectrometry. The mTGase mediated PEGylation, performed in a water/ethanol solution mixture, allowed a PEG coupling reaction only at the level of hGH Gln141, yielding the single monoconjugate PEG-Gln141-hGH with a mass of 44064.9 Da. Circular dichroism studies showed that both conjugation strategies preserved the native-like secondary structures of hGH. It is vital to maintain the structural integrity of hGH if PEGylated hGH is to be used in therapeutic applications. As expected, the pharmacokinetic profile in rats of PEG-Nter-hGH and PEG-Gln141-hGH revealed a significant increase in systemic exposure with respect to unmodified hGH. The conjugates showed a half-life increase of 4.5-fold with respect to hGH. These results demonstrate that both chemical and enzymatic site-selective PEGylation of hGH generates conjugates with a prolonged half-life.



INTRODUCTION

Recombinant hGH was first introduced in 1985 and it has been used to treat disorders of short stature caused by growth hormone deficiency in children and adults. The protein has also been used to treat other diseases not related to hGH deficiency, such as Turner's syndrome and growth failure due to chronic renal insufficiency.[1] hGH treatment protocols are nearly always long-term and characterized by daily subcutaneous (s.c.) injections. The frequent dosing schedule is necessary because hGH, just as other small proteins (i.e., with a molecular weight below the kidney clearance threshold, approximately 60 kDa), is rapidly cleared from the body and has a low bioavailability. According to surveys, more than 50% of patients prescribed growth hormone fail to comply with some aspect of the treatment regimen[1] and this can be attributed to its complexity. It is assumed that simplifying the medication regimen of hGH would improve patient compliance.[1,2]

Several strategies aiming to prolong the half-life of therapeutic proteins have been investigated. PEGylation, defined as the coupling of PEG chains, is considered one of the most successful techniques to enhance the therapeutic and safety profile of peptides and proteins. PEG is a synthetic, nontoxic, non-immunogenic, amphiphilic, highly flexible polymer. Until now, most PEG–protein conjugates approved by the FDA have been obtained by coupling strategies that involve the random conjugation of PEG chains, mostly to lysine residues. The overall feasibility of these coupling strategies appears to be limited given the heterogeneity and decreased bioactivity of the final product.[3−6]

In order to increase the homogeneity of conjugates and thus to simplify the purification and characterization steps, researchers have attempted to develop procedures for site-specific PEGylation. Effective approaches such as *N*-terminal conjugation[7,8] and thiol conjugation of free cysteine (Cys) using genetic engineering and a new enzymatic method employing transglutaminase (TGase) have been developed. The *N*-terminal PEGylation was achieved by performing the coupling at slightly acidic pH values to lead to protonation of the ε-amino group of lysines and therefore directing the PEG coupling at the α-amino group at the *N*-terminal protein which, thanks to its lower pK_a, was still available as a nucleophile. This approach produces better results if it is performed with less reactive PEGs, such as the aldehyde derivatives like PEG-proprionaldehyde. Briefly, the reaction involves the formation of an unstable Schiff base coupling bond which in turn is reduced to a stable secondary amine.[9] This conjugation method has been successfully exploited

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to prepare Neulasta, a *N*-terminal mono-PEGylated granulocyte colony-stimulating factor.[10]

Thiol conjugation requires a single free Cys residue in the protein sequence to achieve a site-specific modification. Cys can be modified selectively, rapidly, quantitatively, and even in a reversible or irreversible fashion.[11] A FDA-approved example of thiol PEGylation is PEGylated TNF α-inhibitor Fab antibody fragment, which is presently used clinically under the name of CIMZIA (certolizumab pegol) to treat rheumatoid arthritis and Crohn's disease.[12,13] An alternative approach to thiol PEGylation, called bridging PEGylation, has been introduced by Brocchini. In this case, a specific bifunctional PEGylating agent is coupled to a reduced disulfide bridge within the protein, thus forming a three-carbon bridge that links the two cysteins' sulfur atoms.[14]

A transglutaminase (TGase) enzyme has recently been used to covalently link PEG chains at the γ-carboxamide group of glutamine (Gln) protein residues.[15,16] TGase(s) are a class of enzymes capable of catalyzing an acyl transfer between two proteins involving the glutamyl group of a glutamine (acyl donor) and a primary amine (acyl acceptor), usually the ε-amino group of a lysine.[17] Microbial TGase (mTGase) from *S. mobaraense* has been investigated for bioactive drug application and in particular for site-specific PEGylation of pharmaceutical proteins when a PEG-NH_2 derivative is used as an acyl acceptor. mTGase-mediated PEGylation makes it possible to attain highly homogeneous conjugates because not all glutamines are enzyme substrates. Due to the strict requirements of the mTGase then, in most cases only one or two glutamines per protein are mTGse-substrate.[18,19]

Site-selective PEGylation might be the right strategy to overcome the problem of compliance linked to hGH treatment. The study presented here aimed to improve the pharmacokinetic profile, stability, and therapeutic efficacy of hGH by developing and testing PEGylated conjugates. In order to select the best conjugation approach, two PEG-hGH monoconjugates were studied: (i) an *N*-terminal derivative, PEG-Nter-hGH, obtained by using $PEG_{20\ kDa}$–aldehyde; and (ii) a Gln141 derivative, PEG-Gln141-hGH, obtained by performing mTGase-mediated PEGylation with $PEG_{20\ kDa}$–NH_2 in a water/ethanol buffer. The conjugates were systematically characterized and compared and their pharmacokinetic profiles were evaluated in rats. To our knowledge, this study directly compares for the first time two conjugates of the same protein obtained with two different site-selective coupling approaches.

EXPERIMENTAL PROCEDURES

Materials. mTGase from *S. mobaraensis* was purchased from the Ajinomoto Co (Tokyo, Japan), hGH from Bioker SpA (Cagliari, Italy), and methoxy-PEG-propionaldehyde 20 kDa ($PEG_{20\ kDa}$–aldehyde) and methoxy-PEG-amino 20 kDa ($PEG_{20\ kDa}$-NH_2) from the IRIS Biotech (Marktredwitz, Germany). The hGH ELISA kit was from Tema ricerca Srl (Bologna, Italy). All the other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich (Milan, Italy).

Analytical Methods. Electrophoresis (SDS-PAGE) was performed as described elsewhere and the gel was stained with Blue Coomassie.[20]

Far-UV CD spectra were measured on a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control unit at 20 °C. Solutions of hGH, PEG-Nter-hGH, or PEG-Gln141-hGH were prepared in 50 mM phosphate buffer at pH 7.0 The concentration of protein in all the samples was 0.1 mg/mL as spectrophotometrically determined at 280 nm (absorption coefficients of native protein: 0.794 mL cm^{-1} mg^{-1}). The contribution of PEG was negligible, as it did not have absorbance at that wavelength.

The spectra were collected over the wavelength range of 190–250 nm. The sample cell path length was 1 mm. The CD data were converted to mean residue ellipticity, expressed in deg cm^2 $dmol^{-1}$ by applying the following formula:

$$\Theta = \Theta_{obs}(MRW)/10L[C]$$

Where Θ is the observed ellipticity in degrees, MRW is the mean residue weight of the peptide (molecular weight divided by the number of residues), [C] is the peptide concentration in mg/mL, and L is the optical path length in centimeters.

Thermal denaturation of the free and conjugated hGH was monitored on the same samples used for CD analysis by recording the decrease of the ellipticity signal at 208 nm as a function of the temperature. Denaturation experiments were carried out in a 0.1 cm cell path length heated from 15 to 90 °C.

MALDI-MS spectra were performed on a REFLEX time-of-flight instrument (4800 Plus MALDI TOF/TOF, AB Sciex, Framingham, Massachusetts, USA) equipped with a SCOUT ion source operating in positive linear mode. Ions generated by a pulsed UV laser beam (nitrogen laser, λ 337 nm) were accelerated to 25 kV. A saturated solution of sinapinic acid in water/ACN (1:1, v/v) was used as a matrix and mixed with the samples dissolved in 0.1% TFA aqueous solution at a v/v ratio 1:1.

Determination of mTGase Activity. The mTGase activity was determined by using the specific substrate, CBZ-n-glutaminylglycine (Z-Gln-Gly), as described elsewhere.[21] Briefly, for each tube containing 500 μL of 0.1 M phosphate buffer (pH 7.0) 0.1 M hydroxylamine and 30 mM of Z-Gln-Gly, a predetermined volume of ethanol 50% was added in order to achieve a final volume of 1 mL. 50 μL of mTGase (30 mg/mL) was added, and after 10 min of incubation at 37 °C, 0.5 mL of ferric chloride (5% w/v)–trichloracetic acid (12% w/v) was then added. The absorbance at 525 nm was measured and the residual enzymatic activity was calculated considering the enzymatic activity of mTGase in a phosphate buffer as 100%.

***N*-Terminal PEGylation of hGH.** An hGH solution (1.0 mg/mL) in 0.1 M acetate buffer (pH 5.0) was prepared. $PEG_{20\ kDa}$–aldehyde (5-fold molar excess) was dissolved in the solution. After 1 h, $NaCNBH_3$ (50-fold molar excess) was added. The mixture was left to stir at room temperature for 5 h and then analyzed by SEC-HPLC using a Zorbax GF-250 column (250 × 4.6 mm; Agilent Technologies, Palo Alto, CA) eluted with 0.1 M phosphate buffer, 0.2 M NaCl (pH 7.2) containing 20% (v/v) of ACN, flow rate 0.3 mL/min. The effluent was monitored by measuring the absorbance at 280 nm. The conjugate was purified by SEC-HPLC using a Zorbax GF-250 column (250 × 9.4 mm; Agilent Technologies, Palo Alto, CA) eluted under the condition described above with a 1.0 mL/min flow rate. The peak corresponding to the conjugate PEG-Nter-hGH was collected and the solution was concentrated under vacuum and dialyzed against phosphate buffer 50 mM (pH 7.0). The protein concentration was determined by UV absorption at 280 nm. The conjugate was characterized by SDS-PAGE, RP-HPLC, CD, and MALDI-TOF mass spectrometry.

mTGase-Mediated PEGylation of hGH. An hGH solution (1.0 mg/mL) in 0.1 M phosphate buffer (pH 7.0) containing 50% of ethanol (v/v) was prepared. $PEG_{20\ kDa}$–NH_2 (10-fold molar excess) was dissolved in this solution and mTGase was

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FPTIPLSRLF DNAMLRAHRL HQLAFDTYQE FEEAYIPKEQ KYSFLQNPQT SLCFSESIPT
PSNREETQQK SNLELLRISL LLIQSWLEPV QFLRSVFANS LVYGASDSNV YDLLKDLEEG
IQTLMGRLED GSPRTGQIFK QTYSKFDTNS HNDDALLKNY GLLYCFRKDM DKVETFLRIV
QCRSVEGSCG F

Figure 1. Amino acid sequence of human growth hormone (hGH). The sites for amino PEGylation are underlined (Phe at the N-terminus and Lys residues) and the Gln residues, potential sites for TGase-PEGylation, are written in bold.

added at E/S ratio of 1:20 (w/w). The mixture was stirred at room temperature for 18 h and then analyzed by RP-HPLC on a Phenomenex Jupiter C18 column (250 × 4.6 mm; 5 μm) with a flow-rate of 1.0 mL/min, detection at 280 nm, and eluted with a solvent gradient of water/ACN both containing 0.1% TFA. Gradient 0 min − 5% ACN, 5 min − 40% ACN, 25 min − 80% ACN, 27 min − 90% ACN, 30 min − 5% ACN. The reaction was stopped by acidifying the solution to pH 3 with a few drops of acetic acid and then centrifuging it at 4000 × *g* for 10 min. The supernatant was purified by RP-HPLC using a C18 column (Vydac 218 tp 250 × 10 mm; 12 μm) eluted with a solvent gradient of water/ACN both containing 0.1% TFA. Gradient: 0 min − 5% ACN, 5 min − 40% ACN, 25 min − 80% ACN, 27 min − 90% ACN, 30 min − 5% ACN. The effluent was monitored by measuring the absorbance at 280 nm. The peak corresponding to the conjugate PEG-Gln141-hGH was collected and the solution was concentrated under vacuum. The solution obtained was dialyzed against phosphate buffer (50 mM pH 7.0) and the protein concentration was determined by UV absorption at 280 nm. The conjugate was characterized by SDS-PAGE, RP-HPLC, CD, and MALDI-TOF mass spectrometry.

Proteolytic Digestion of PEG-Nter-hGH. Native and PEG-Nter-hGH (0.5 mg) were dissolved in 6 M Gdn-HCl, 50 mM Tris-HCl (pH 9.0) to reach a protein concentration of 1.0 mg/mL. In order to reduce disulfide bridges, tris(2-carboxyethyl)-phosphine was added to the protein solution at the final concentration of 5 mM and the reaction mixture was kept for 1 h at 37 °C. The reduced protein was then reacted by adding iodoacetamide to the final concentration of 25 mM, and the S-alkylation was allowed to proceed for 30 min at 37 °C in the dark. Protein samples were purified by RP-HPLC on a Phenomenex Jupiter C18 column (250 × 4.6 mm; 5 μm) at a flow rate of 1.0 mL/min, detection at 280 nm, and eluted with a solvent gradient of water/ACN both containing 0.1% TFA. Gradient 0 min − 5% ACN, 5 min − 40% ACN, 25 min − 80% ACN, 27 min − 90% ACN, 30 min − 5% ACN, and the collected peaks were lyophilized. The reduced and S-carboxamidomethylated samples of hGH and PEG-Nter-hGH were dissolved in 8 M urea and diluted in 50 mM phosphate buffer (pH 7.9) to reach 0.5 mg/mL and the urea concentration of 0.8 M. An aliquot of trypsin was then added at an E/S ratio of 1:50 (by weight), and the proteolysis was allowed to proceed at 37 °C overnight. The digestion mixtures were purified by RP-HPLC on a Phenomenex Jupiter C18 column (250 × 4.6 mm; 5 μm) flow rate 1.0 mL/min, detection at 226 nm, eluted with a solvent gradient of water/ACN both containing 0.1% TFA. Gradient 0 min − 5% ACN, 5 min − 5% ACN, 115 min − 80% ACN, 120 min − 95% ACN, 125 min − 100% ACN, 130 min − 5% ACN. The chromatographic peaks of native and PEGylated hGH were collected, lyophilized, and analyzed by ESI-MS.

Ethics Statement. The study protocol was approved by the Ethics Committee of the University of Padova and the Italian Ministry of Health, and the animals were handled in compliance with national (Italian) Legislative Decree 116/92 guidelines and with the "Guide for the Care and Use of Laboratory Animals" by the National Research Council of the National Academies.

Pharmacokinetic Study in Rats. The pharmacokinetics of hGH and of the PEG-Gln141-hGH and PEG-Nter-hGH conjugates were assessed in female Sprague−Dawley rats (250−300 g). The rats were randomly divided into four groups of 3 animals per group. A dose of 0.1 mg/kg hGH (equiv.) was administered via tail vein to the rats anesthetized with isoflurane gas. Blood samples were collected (200 μL) at predetermined times from the tail vein and centrifuged at 1500 × *g* for 15 min. The hGH content in serum samples was quantified using the hGH ELISA kit (Tema ricerca Srl). The pharmacokinetic data elaboration was performed by GraphPad *Prism* software applying a bicompartmental model.

RESULTS AND DISCUSSION

***N*-Terminal PEGylation of hGH with $PEG_{20\ kDa}$−aldehyde.** hGH contains 10 primary amines (Figure 1) that can theoretically react with $PEG_{20\ kDa}$−aldehyde: the α-amine of Phe1 and the ε-amino groups of nine Lys residues. The reaction was performed at pH 5.0 to obtain the specific PEG-Nter-hGH conjugate. The site selective conjugation at acidic pH takes advantage of the different pK_a values of the α-amino group versus the ε-amino groups.[10] All the ε-amino groups are protonated at pH 5.0, whereas a significant percentage of the α-amine of Phe1 is still present as free base and is thus available for coupling with $PEG_{20\ kDa}$−aldehyde. Analysis of the reaction mixture after 5 h by SEC resulted in the following peak percentages: 60.8% of PEG-Nter-hGH (T_R = 6.367 min), 28.6% of native hGH (T_R = 9.08 min), and 10.6% of diPEGylated hGH (T_R = 10.638 min) (Figure 2, panel A).

The purified monoconjugate was lyophilized and characterized by SDS-PAGE 12% and MALDI-TOF. The SDS-PAGE analysis showed a single band around 66 kDa (Figure 3). PEGylated proteins have a large hydrodynamic volume. As a result, those conjugates usually display apparent MW values via SDS-PAGE that do not correspond to the MW of the standards and cannot provide an accurate estimation of their exact value.[10] PEG-Nter-hGH, hGH, and PEG-aldehyde were thus analyzed by MALDI-TOF mass spectrometry. The results showed that a monoPEGylated product was present with a MW centered at 44152.2 Da corresponding approximately to the sum of one chain of PEG-aldehyde conjugated to hGH (Figure 2, panel B). The MALDI spectra of hGH (MW 22162.4 Da) and PEG-aldehyde (MW 22 kDa) are described in the Supporting Information (Figures S1−S2).

mTGase-Mediated PEGylation of hGH with $PEG_{20\ kDa}$−NH_2. To optimize the preparation of PEG-Gln141-hGH by mTGase-catalyzed PEGylation, the reaction was investigated at different values at E/S ratios 1/100, 1/75, 1/50, and 1/20. The conjugation yield was significantly improved at E/S 1/20. Under this condition, a monoPEGylated conjugate PEG-Gln141-hGH was obtained. The analysis of the reaction mixture after 18 h by RP-HPLC showed the following peak percentages: 63.3% of

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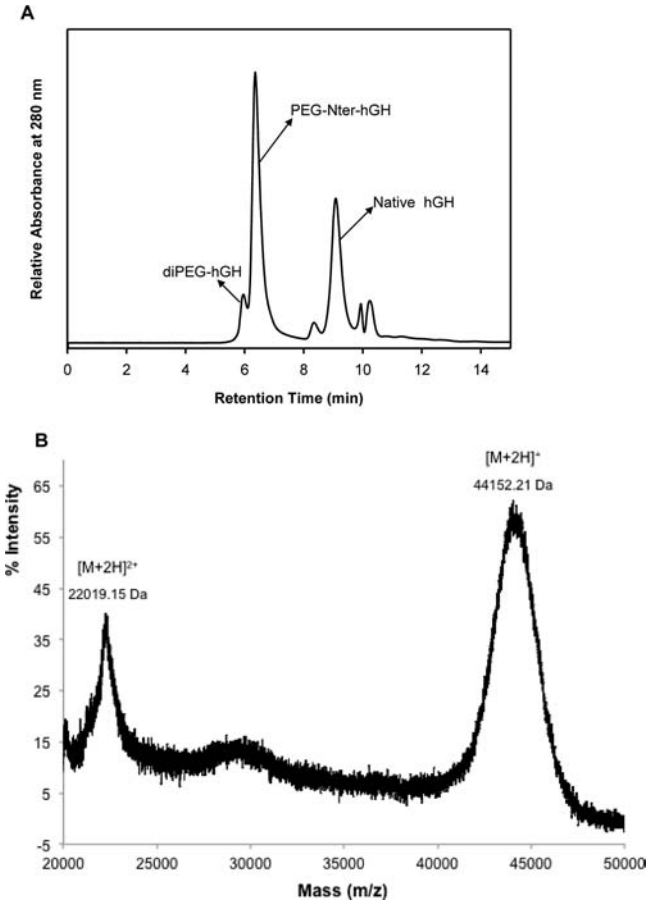

Figure 2. Panel A: SEC analysis of the reaction mixture of PEG-Nter-hGH. The chromatogram was obtained by monitoring the effluent at 280 nm and using a Zorbax GF 250 column (250 × 4.6 mm) eluted with the conditions described in the materials and methods. The peak marked as diPEG-hGH corresponds to a diPEGylated-hGH eluting at 6.01 min; PEG-Nter-hGH corresponds to the monoconjugate eluting at 6.8 min. The free protein elutes at 9.2 min. Panel B: PEG-Nter-hGH was purified and analyzed by MALDI-TOF mass spectroscopy. The MW of the product is centered at 44152.2 Da.

PEG-Gln141-hGH (T_R = 21.220 min), 25.5% of native hGH (T_R = 23.453 min), and 11.2% of diPEGylated conjugate (T_R = 20.017 min) (Figure 4, panel A).

The purified PEG-Gln141-hGH was characterized by SDS-PAGE 12% (Figure 3) showing a single band at about 66 kDa. The accurate MW of PEG-Gln141-hGH was determined by MALDI-TOF mass spectrometry. The spectrum confirmed the monoconjugate nature of PEG-Gln141-hGH with a m/z centered at 44064.9 Da (Figure 4, panel B), consistent with the sum of the MWs of hGH and PEG-NH_2 (see Figure S3 in Supporting Information for MALDI spectrum of PEG-NH_2). mTGase-mediated PEGylation sites of hGH were identified previously. In accordance with Mero et al.,[19,22] out of the 13 Gln(s) of hGH, only two—Gln40 and Gln141—are substrates of mTGase. When the reaction was conducted in 50% (v/v) ethanol, mTGase mediated PEGylation was direct only to Gln141.[22]

Both SDS-PAGE and MALDI-TOF mass spectrometry analyses comparatively showed that these conjugates are monoderivatives with similar MWs and great homogeneity. Both methods showed a comparable selectivity with only small

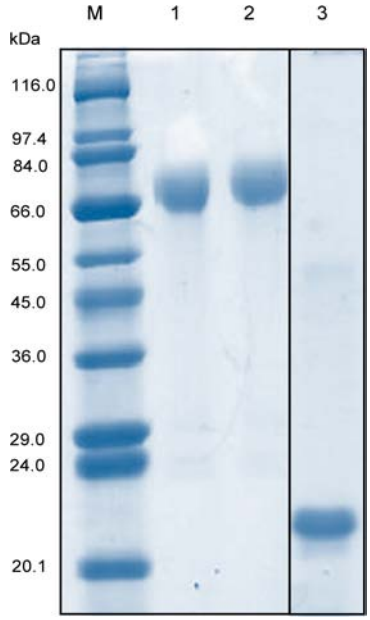

Figure 3. SDS-PAGE gel (12%) of monoPEGylated hGH: **M**, Marker; **1**, PEG-Nter-hGH band at around 66 kDa; **2**, PEG-Gln141-hGH band at around 66 kDa; **3**, native hGH band at around 22 kDa.

percentages (about 10−11%) of diPEGylated conjugates in the reaction mixtures.

Identification of the Sites of *N*-Terminally PEGylated hGH. The selectivity of hGH *N*-terminal PEGylation reaction was investigated by comparing the fingerprint of native and PEGylated hGH. Reduced *S*-carboxymidomethylated hGH and PEG-Nter-hGH were digested with trypsin. The digested samples were analyzed by RP-HPLC (Figure 5) and the peptides were analyzed by ESI-TOF mass spectrometry. Most of the tryptic fragments were identified and all the amine-containing peptides were detected in the digested samples. In accordance with Clark et al.,[2] the most reactive amino groups include the α-amine of Phe1 (F1) and the ε-amino group of Lys140 (F11), followed by Lys145 (F11), Lys38 (F4), Lys70 (F5), Lys41(F5) Lys158 (F12), Lys168 (F14), Lys172 (F14), and Lys115 (F8). The native hGH fragments containing the amino groups are shown in Figure 5. Each peak of the digests of native hGH and PEGylated hGH was analyzed by ESI-TOF and compared (Figure 5). The peak which eluted at 34.5 min exhibited a mass of 929.9899 Da, corresponding to the native *N*-terminal hGH fragment (F1) containing Phe1 (Table 1). The masses of all the other amino-containing fragments of native hGH were determined experimentally (Table 1). The values recorded in native and PEGylated hGH were very similar to the theoretical ones expected.[23] It was found nevertheless that the peak corresponding to fragment 1−8 (F1) was not present in the RP-HPLC chromatogram of PEG-Nter-hGH (Figure 5), and no peak corresponding to its molecular weight was detected by ESI-TOF. As the peak disappeared following conjugation with $PEG_{20\ kDa}$−aldehyde, the PEGylated fragment could not be detected by ESI-TOF, indicating that α-amine of Phe1 was selectively modified.

Circular Dichroism (CD) Analysis. As development of PEGylated biotech drugs requires preservation of the protein secondary structure after conjugation, conjugates were prepared for CD analysis, which also offered interesting insights with regard to the comparison between the two PEGylation strategies.

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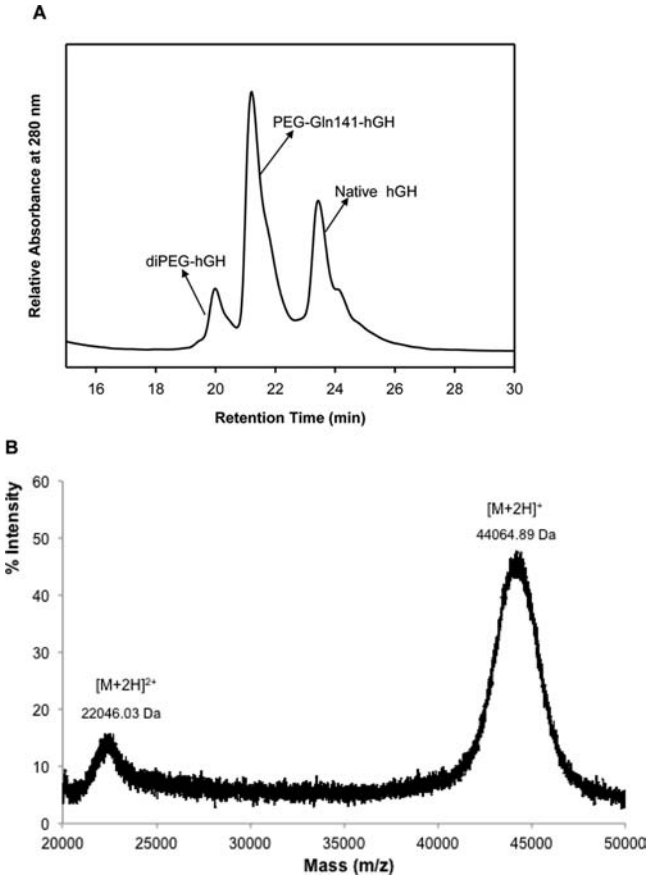

Figure 4. Panel A: RP-HPLC profiles of the reaction mixture of PEG-Gln141-hGH mTGase mediated conjugation. The chromatogram was obtained by monitoring the effluent at 280 nm and using a Phenomenex Jupiter C18 column (250 × 4.6 mm; 5 μm) eluted with the conditions reported in the Experimental Section. The peak marked as diPEG-hGH corresponds to a diPEGylated-hGH eluting at 20.1 min, PEG-Gln141-hGH corresponds to the monoconjugate eluting at 21.7 min. The free protein elutes at about 23.8 min. Panel B: PEG-Gln141-hGH was purified and analyzed by MALDI-TOF mass spectroscopy. The MW of product is centered at 44064.9 Da.

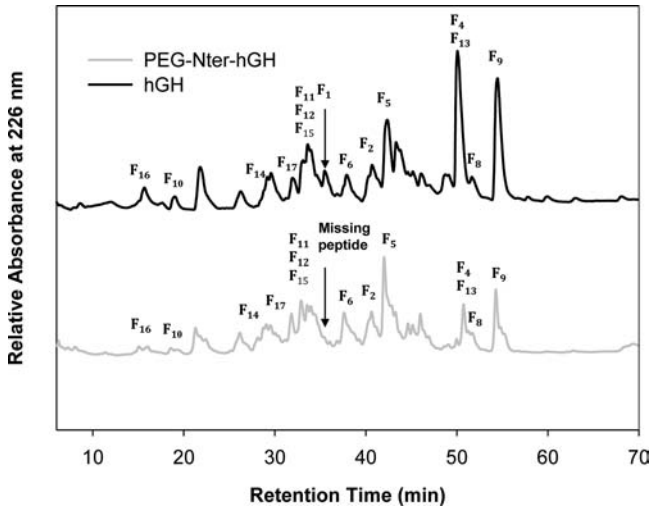

Figure 5. RP-HPLC chromatographic profiles of tryptic digests of hGH and PEG-Nter-hGH. The chromatograms were obtained by monitoring the effluent at 280 nm and using a Phenomenex Jupiter C18 column (250 × 4.6 mm; 5 μm) eluted with the conditions reported in the Experimental Section. The peptide F_1 (FPTIPLSR), corresponding to the fragment containing the *N*-terminal amino group, is missing in the digest of PEG-Nter-hGH.

Native hGH has a predominantly α-helical structure in physiological conditions with two characteristic, strong bands at 222 and 208 nm, as revealed by the far UV-CD spectrum shown in Figure 6.[24] It was found then that both PEGylation approaches of hGH, at the *N*-terminus or at the Gln141, preserved the protein's secondary structures. The conjugates exhibited the same ellipticities at 222 and 208 nm and profiles superimposable with that of hGH. These results are consistent with previous studies showing that the protein's secondary structure is not influenced by PEGylation.[25−27]

The thermal denaturation investigation of hGH and conjugates, measured by evaluating the changes in the ellipticity at 208 nm, moreover, showed that all the samples were very stable at the studied temperature range (15−90 °C; data not shown). These data further confirm that PEGylation did not have detrimental effects on protein conformation and stability, and indicate that there are no evident differences linked to the site of PEG coupling.

Pharmacokinetic Study in Rats. The pharmacokinetics of PEG-Nter-hGH and PEG-Gln141-hGH after i.v. administration were evaluated in rats. As shown in Figure 7, high plasma levels of hGH were recorded immediately for both the native and PEGylated hGH. The hGH level for the native protein decreased rapidly 1 h after the injection and fell to below the detection limit after 7 h. The conjugates showed a marked prolongation of their half-lives, with detectable levels of hGH up to 24 h. Consistent with what has been reported concerning other cytokines of a similar size modified with a 20 kDa PEG, a sufficient pharmacokinetic prolongation was ensured.[11] The principle pharmacokinetic parameters are reported in Table 2.

The half-life of native hGH was similar to that reported elsewhere.[2,28,29] The half-lives of PEG-Nter-hGH and PEG-Gln141-hGH were 7.57 and 7.15 h, respectively, corresponding to about a 4.5-fold increase with respect to hGH (1.63 h). In agreement with longer half-lives, the clearance and the apparent volume of the distribution of the conjugates were significantly reduced with respect to those of the native protein. In the latter case, the site of PEG coupling did not affect the pharmacokinetic performance of the conjugates. In a previous study, Cho et al. made reference to hGH mutants modified with a 30 kDa PEG. In that case, the pharmacokinetic and pharmacodynamic profiles were significantly affected by the site of polymer coupling.[30] Other studies presently ongoing in our lab are investigating if PEG-Nter-hGH and PEG-Gln141-hGH possess a different in vivo potency. This comparison study demonstrates that both approaches of site-selective hGH PEGylation—if administered intravenously—yield conjugates that cause a significant increase in the systemic exposure of hGH compared to the unmodified counterpart (Table 2), a result also noted with PEG-hGH conjugates obtained using other PEGylation strategies.[31,32]

CONCLUSIONS

Given the strict regulatory requirements for approval of PEGylated proteins and the intrinsic difficulty involved in characterizing them in vitro and in vivo, a well-defined site-specific PEGylation approach is necessary to ensure success. Deciding which PEGylation method is best for a given protein when a conjugation project is being designed is not an easy task, because unexpected setbacks might occur affecting the in vivo

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Table 1. Fragments Obtained upon Digestion of Native hGH and PEG-Nter-hGH with Trypsin[a]

sample	fragments	sequence	molecular mass (Da) calculated[b]	molecular mass (Da) experimental[c]
hGH	F1 [1−8]	FPTIPLSR	930.5	929.9
PEG-Nter-hGH	F1 [1−8]		2293.0	*d*
hGH	F2 [9−16]	LFDNAMLR	979.5	979.5
PEG-Nter-hGH	F2 [9−16]			979.5
hGH	F4 [20−38]	LHQLAFDTYQEFEEAYIPK	2342.1	2342.1
PEG-Nter-hGH	F4 [20−38]			2342.2
hGH	F6 [39−70]	EQKYSFLQNPQTFSESIPTPSNREEQK	3801.8	3801.7
PEG-Nter-hGH	F6 [39−70]			3801.3
hGH	F7 [71−77]	SNLELLR	844.4	844.4
PEG-Nter-hGH	F7 [71−77]			844.4
hGH	F8 [95−115]	SVFANSLVYGA	2262.1	2262.1
PEG-Nter-hGH	F8 [95−115]	LLK		2261.9
hGH	F9 [116−127]	DLEEGIQTLMGR	1361.6	1361.6
PEG-Nter-hGH	F9 [116−127]			1360.7
hGH	F10 [128−134]	LEDGSPR	773.3	773.3
PEG-Nter-hGH	F10 [128−134]			773.3
hGH	F11 [135−145]	TGQIFKQTYSK	1300.6	1300.9
PEG-Nter-hGH	F11 [135−145]			1300.6
hGH	F12 [146−158]	FDTNSHNDDALLK	1489.6	1489.7
PEG-Nter-hGH	F12 [146−158]			1489.6
hGH	F13 [159−167]	NYGLLYCFR	1205.5	1205.5
PEG-Nter-hGH	F13 [159−167]			1205.4
hGH	F14 [168−172]	KDMDK	636.3	636.3
PEG-Nter-hGH	F14 [168−172]			636.3
hGH	F15 [173−178]	VETFLR	764.4	764.4
PEG-Nter-hGH	F15 [173−178]			764.3
hGH	F16 [179−184]	IVQCR	675.3	675.3
PEG-Nter-hGH	F16 [179−184]			675.3
hGH	F17 [184−191]	SVEGSCGF	842.3	842.3
PEG-Nter-hGH	F17 [184−191]			842.3

[a]The peptide F1 [1−8] (FPTIPLSR) was obtained only in the case of native hGH. The molecular mass was determined by ESI-TOF mass spectrometry. [b]Theoretical weight of the fragments obtained by web.expasy.org/peptide_mass. [c]Experimental molecular masses determined by ESI-TOF mass spectrometry. [d]Fragment not found.

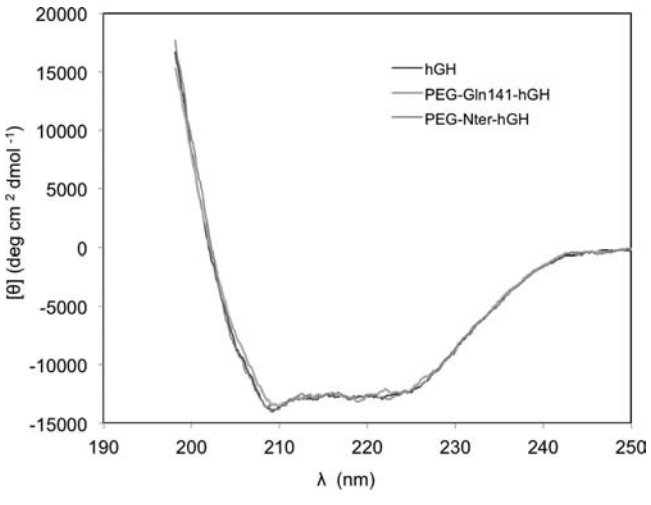

Figure 6. Comparison of the secondary structure of native hGH, PEG-Gln141-hGH, and PEG-Nter-hGH by far-UV CD. The spectra were recorded at the protein concentration of 0.1 mg/mL, as outlined in the Experimental Procedures.

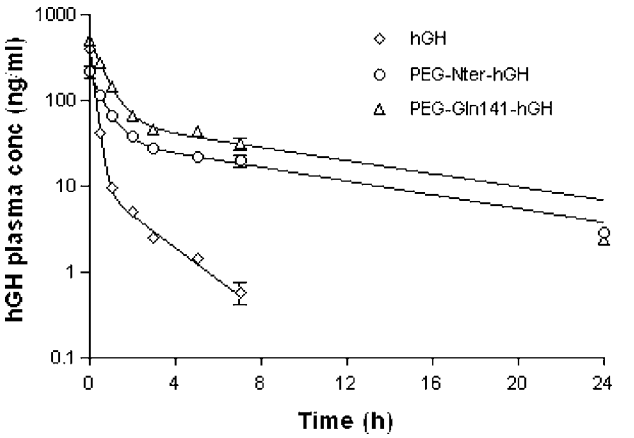

Figure 7. Pharmacokinetic best-fit profiles of hGH, PEG-Nter-hGH, and PEG-Gln141-hGH calculated by GraphPad *Prism* software. The products were administered at a dose of 0.1 mg/kg hGH (equiv) to female Sprague−Dawley rats (250−300 g). The hGH/PEG hGH levels in the blood were monitored by immunoassay. Data are presented as mean ± SEM $n = 3$. The *Y*-axis is presented as a logarithmic scale.

performance of the conjugate. In our case, the pharmacokinetic performance of the conjugates was determined by the molecular mass of the PEG and not by the site where polymer coupling was carried out, as outlined in another study.[30] It can in any case be presumed that the site of polymer coupling has an important

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Table 2. Main Pharmacokinetic Parameters of hGH, PEG-Nter-hGH, and PEG-Gln141-hGH after i.v. Administration of 0.1 mg/kg hGH (equiv) to Female Sprague-Dawley Rats (250−300 g)[a]

sample	$t_{1/2}$ (h)	AUC (ng h/mL)	V_D (mL)	Cl (mL/h)
hGH	1.63 ± 0.19	103.49 ± 3.24	2872.23 ± 663.57	290.08 ± 9.19
PEG-Nter-hGH	7.57 ± 2.71[c]	468.67 ± 17.83[c]	970.71 ± 439.68	64.07 ± 2.39[c]
PEG-Gln141-hGH	7.15 ± 2.63[c]	928.13 ± 234.52[b]	480.74 ± 29.89[b]	33.77 ± 8.70[c]

[a]Data are means ± S.E.M. $n = 3$. $t_{1/2}$: elimination half-life. AUC: area under the curve. Cl: clearance. V_D: apparent volume of distribution. [b]$p < 0.05$. [c]$p < 0.01$ versus hGH (significance was calculated using ANOVA).

effect on the steric entanglement of polymer chains during the protein/receptor recognition process.

According to our data, the *N*-terminal and mTGase-mediated PEGylation exhibited comparable results in terms of their selectivity, preservation of the protein secondary structures, and pharmacokinetic profiles. Neither conjugate, in fact, clearly prevailed over the other, meaning that the selection can be made simply on the basis of reaction stability.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF spectra of hGh, PEG-Nter-hGH, PEG-aldehyde, PEG-Gln141-hGH, and PEG-NH_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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