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Comparison of Site-Specific PEGylations of the N-Terminus of Interferon Beta-1b: Selectivity, Efficiency, and in Vivo/Vitro Activity

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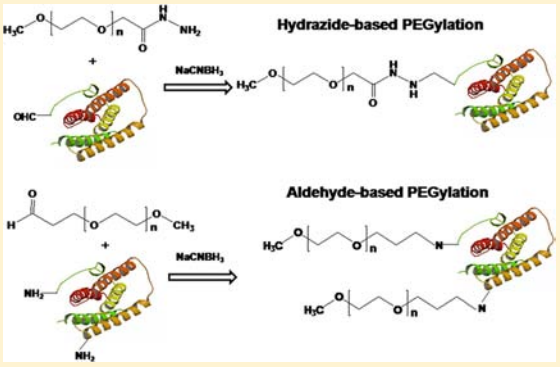
ABSTRACT: PEGylation, including nonspecific and site-directed approaches, is a well-established and validated strategy to increase the stability, in vivo plasma retention time, and efficacy of protein pharmaceutics together with a reduction in immunogenicity and hydrophobicity. Site-directed conjugation by PEG-aldehyde is the most widely used method for N-terminal modification; however, the generation of multimodified products is inevitable because of lysine chemistry, which always leads to difficulties in purification and quantification. In this study, we developed a specific PEGylation strategy through the periodation of the N-terminus of interferon beta-1b (IFN-β-1b) followed by the coupling of PEG-hydrazide. The prolonged elimination half-life and significantly diminished immunogenicity of the PEG-hydrazide-modified protein indicated the development of an effective process for improving the pharmacology and immunogenicity of IFN-β-1b. We further conducted comparisons on the selectivity, velocity, yield, and pharmacokinetics of the two methods. The results demonstrated that the hydrazide-based conjugation was a highly specific coupling reaction that only produced homogeneous N-terminal mono-PEGylated conjugate but also generated heterogeneous multimodified products in the aldehyde-based process. In addition, a better PEGylation yield was found for the hydrazide conjugation compared with that of the aldehyde strategy. These investigations supply a practical approach for the site-specific modification of proteins with an N-terminal serine or threonine to achieve improved homogeneity of the conjugates as well as enhanced pharmacological properties.



INTRODUCTION

PEGylation, covalent attachment of poly(ethylene glycol) to proteins, is a well-established and validated strategy to increase the stability, in vivo plasma retention time, and efficacy of protein pharmaceutics together with a reduction in immunogenicity and hydrophobicity.[1,2] In the early days of PEGylation, attention was paid to amino groups of lysine residues in sequence because they were the most accessible active groups exposed to solvents and could be coupled with multiple available PEG derivatives such as PEG succinimidyl carbonate (PEG-SC) and PEG succinimidyl propionate (PEG-SPA). However, a high number of heterogeneous multimodified products and positional isomers were generated during nonselective couplings between amino groups and PEG derivatives, which led to complicated purification processes for fractionating different PEGylated forms. Moreover, modification of some lysine residue regions around the active center of a protein would result in severe or entire loss of biological activity.[3] Because the homogeneity of the product is a crucial concern for the preservation of bioactivity and medical approval, site-directed PEGylations were devised to overcome these limitations by selecting specific binding sites, including free mercapto groups and the N-terminal amino group.[4,5] Modification of a sulfhydryl site is processed on the unbonded cysteine residue by selective PEG derivatives, such as PEG maleimide (PEG-MAL) and PEG orthopyridyl disulfide (PEG-OPSS).[6] However, there is no free mercapto group in most proteins. In addition, introduction of a free thiol by genetic engineering would result in significant loss of stability because of the formation of protein dimers. Another widely used site-directed modification is the covalent coupling of the N-terminal amino group with PEG-aldehyde (PEG-ALD).[7] Because of the differences in the pK_a values of the N-terminal α-amino group (7.6−8.0) versus the ε-amino group of lysine residues (9.3−9.5), selective PEGylation is achieved by performing the reaction under mildly acidic conditions (e.g., pH 6.0−6.5).[8] However, a completely site-specific modification proved to be challenging because the reaction processed on the ε-amino group was restrained to a certain extent but could not be avoided. It has been reported that heterogeneous multimodified products were also generated by controlling the pH of the solution.[9,10] Moreover, some proteins would be unstable under the pH suitable for this protocol.[9]

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Therefore, researchers have continued to probe more reliable and feasible site-specific methods to solve these problems. In previous studies, a PEGylation strategy with complete selectivity through the N-terminal periodation of a protein followed by site-directed modification by PEG-hydrazide was reported.[11,12] However, few detailed investigations of this reaction have been illustrated. In this study, a comparative analysis of the selectivity, velocity, yield, and pharmacokinetic profiles of two methods were carried out using human interferon beta-1b (IFN-β-1b) as the model protein.

IFN-β-1b was approved for the treatment of multiple sclerosis (MS) in 1993 because of its effectiveness in reducing exacerbations and relapsing−remitting potency of this disease.[13−15] In recent years, IFN-β-1b has also been shown to exert antitumor activity in carcinoma therapy.[16−18] In spite of the excellent bioactivity of IFN-β-1b in disease therapy, the major obstacles in its clinical development have been frequent dosing, formulation, and immunogenicity.[19] The mean serum half-life following subcutaneous administration was less than 2 h, and the mean serum concentration was sustained at a fairly low level because of rapid serum clearance.[20] IFN-β-1b is highly hydrophobic and difficult to maintain at an effective blood−drug concentration in aqueous solution, and its solubility is less than 0.05 mg/mL under physiological pH conditions. In addition, it requires surfactants such as SDS to maintain solubility in aqueous solution.[21] Moreover, the strong hydrophobicity of IFN-β-1b also leads to difficulties in the purification and quantification procedure. On account of the multiple available modification strategies, great progress has been achieved on the research of the PEGylation of IFN-β-1b, especially site-directed PEGylation.[22−24] Basu et al. applied several different PEG polymers in the bioconjugation of IFN-β-1b, such as PEG-SC, PEG-SPA, and PEG-ALD, and comparisons of the structural and functional properties of IFN-β-1b and its PEGylated counterparts were conducted. However, barely any product with significant homogeneity was achieved because of the imperfections of the present strategies.

In this study, two site-specific PEGylation strategies toward IFN-β-1b were utilized, including the conventional aldehyde-based PEGylation[24] and the newly developed (in this article) hydrazide-based strategy. In the latter procedure, the N-terminal serine of IFN-β-1b was stoichiometrically periodated to an aldehyde group to be the sole binding site, which coupled with mPEG-hydrazide (mPEG-HZ) to form a covalent carbon−nitrogen single bond in the presence of sodium cyanoborohydride.[12] Results revealed that this strategy is a truly site-specific conjugation that provided utterly homogeneous mono-PEGylated IFN-β-1b, whereas some multi-modified products appeared in the aldehyde-based process. In addition, the PEGylation yield of the hydrazide-based modification was found to be remarkably increased compared with that of conventional conjugation, indicating a higher PEGylation efficiency. Furthermore, results also confirmed that the hydrazide-modified conjugate (mono-HZ-PEG-IFN-β-1b) presented improved pharmacokinetics and immunogenicity profile compared to that derived from mPEG-ALD or native protein. The results in this study show that hydrazide-based PEGylation is an applicable and efficient strategy for site-specific modification of IFN-β-1b to achieve improved in vivo properties.

EXPERIMENTAL PROCEDURES

Materials. All activated PEG polymers were purchased from Jenkem Technology Co., Ltd. (China). Sodium dodecyl sulfate (SDS), $NaIO_4$, ethylene glycol, and sodium cyanoborohydride were purchased from Sigma-Aldrich (USA). Prepacked HiLoad 16/60 Superdex 200 prep grade columns, analytical Superdex 200 10/30 GL columns, and Sephadex G-25 columns were supplied by GE Healthcare (Sweden). Vesicular stomatitis virus (VSV), Vero cells, and Lewis lung cells (LLC) were from ATCC (USA). ELISA kits were purchased from R&D Systems (USA). HRP-conjugated goat anti-rat IgG was from Abcam (USA). TMB substrate was from Moss (Canada).

Expression and Purification of IFN-β-1b. The expression and purification process of IFN-β-1b was performed as described previously.[25] *Escherichia coli* harboring a plasmid encoding IFN-β-1b was cultured at 37 °C in LB medium and then inoculated in a BIOSTAT-C plus fermenter (Sartorius, Germany) containing 25 L of fermentation medium. Recombinant IFN-β-1b was produced by IPTG induction and isolated by butanol extraction. The inclusion bodies were solubilized in 20 mM sodium phosphate buffer, 0.8% SDS, pH 7.0, containing 10 mM β-mercaptoethanol. An equal volume of 2-butanol was added to the solution and gently agitated for 10 min. The mixture was centrifuged at 5000*g* for 10 min, and the organic phase was collected. Then, 1.5 M ammonium sulfate was added in the organic phase, and the mixture was agitated for 15 min. The collected precipitate was washed three times with deionized water. The purity of the IFN-β-1b was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after freeze-drying.

N-Terminal Periodation of IFN-β-1b. IFN-β-1b (1 mg/mL) was reacted with $NaIO_4$ at a 1:2 molar ratio at room temperature for 3 h in 20 mM sodium phosphate, 0.5% SDS, pH 7.0. The reaction was terminated by adding 100 μL of ethylene glycol. Then, the reaction mixture was loaded onto a Sephadex G-25 column with a running buffer of 20 mM acetate acid and 0.1% SDS, pH 4.0, at a flow rate of 1 mL/min. The peak containing N-terminal-oxidized protein was collected for the next PEGylation step. Protein concentration was determined by UV absorbance at 280 nm with an extinction coefficient of 1.5 mL/(mg cm).[24]

N-Terminal PEGylation of IFN-β-1b by mPEG-HZ. The N-terminal-periodated protein (0.35 mg/mL) was reacted with 5 and 20 kDa mPEG-HZ polymer separately at a 1:5 molar ratio in 20 mM sodium acetate and 0.1% SDS, pH 4.0. The PEGylation reaction was conducted at 25 °C for 20 h. A 1 M sodium cyanoborohydride solution was added to the reaction mixture to a final concentration of 10 mM. To investigate the effect of the reduction of the coupling bond on the activity of the protein, a parallel bioconjugation was conducted without the participation of sodium cyanoborohydride.

N-Terminal PEGylation of IFN-β-1b by mPEG-ALD. IFN-β-1b (1 mg/mL) was reacted separately with 5 and 20 kDa mPEG-ALD polymers at a 1:5 molar ratio in 20 mM sodium acetate and 0.5% SDS, pH 4.0. The PEGylation reaction was conducted at 25 °C for 20 h. A 1 M sodium cyanoborohydride solution was added to the reaction mixture to a final concentration of 10 mM. Yields of all PEGylations were determined by SDS-PAGE with Quality One software.

Purification of Mono-PEGylated IFN-β-1b. The reaction mixture (0.5 mL) was loaded onto a Prepacked HiLoad 16/60 Superdex 200 prep grade column that was equilibrated in a

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running buffer of 20 mM sodium phosphate, 100 mM Na_2SO_4, and 0.1% SDS, pH 7.0, using an ÄKTA purifier protein purification system (GE Healthcare, Sweden). Protein peaks were collected and analyzed by SDS-PAGE. Then, the mono-PEGylated IFN-β-1b was loaded onto a Sephadex G-25 column with 10 mM sodium phosphate and 5% mannitol, pH 7.4. The desalted peak was subsequently dialyzed using a 10k MW cutoff membrane against 10 mM sodium phosphate and 5% mannitol, pH 7.4, at 4 °C for 24 h. The PEGylated proteins were diafiltered to a final concentration of 0.2−0.5 mg/mL.

SDS-PAGE. The PEGylation and purification results were analyzed by SDS-PAGE according to the methods of Laemmli.[26] The reaction mixture and the purified mono-PEGylated IFN-β-1b were combined with a reducing 5× sample-loading buffer (Tris-Glycine 5× SDS sample buffer plus 10% β-mercaptoethanol), heated at 95−100 °C for 5 min, and applied to 15% Tris-Glycine gels. Gels were stained and analyzed for protein using Coomassie brilliant blue dye.

MALDI-TOF MS. The molecular weight of protein was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on an Autoflex III (Bruker, USA) with α-cyano-4-hydroxycinnamic acid as the matrix. The operation mode was reflective mode with positive ion detection. The accelerating voltage was 19 000 V, and the delayed extraction time was 200 ns. Samples (0.2 mg/mL) were dissolved in water and then mixed with saturated matrix solution (CH_3CN/H_2O 1:1, v/v) at a volume ratio of 1:1.

Circular Dichroism (CD). CD Spectra of IFN-β-1b and PEGylated IFN-β-1b were collected using a J-810 spectrometer (Jasco, Japan) in the wavelength range of 200−250 nm at 25 °C. Protein solutions were diluted with their corresponding background buffer to 0.2 mg/mL for immediate analysis. The cuvette path length was 1 mm for far-UV-region measurements. Each spectrum was scanned three times, and the average spectrum was plotted.

Intrinsic Emission Fluorescence. Intrinsic emission fluorescence spectra of IFN-β-1b and its PEGylated forms were analyzed using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Protein solutions were diluted with their corresponding buffer to 0.2 mg/mL for immediate analysis. Spectra were recorded on a 1 cm path-length cuvette with an excitation slit width of 5 nm and an emission slit width of 2.5 nm. The excitation wavelength was set at 280 nm for the specific excitation of tryptophan residues, and emission spectra were recorded from 310 to 400 nm at a constant slit of 1 nm.

In Vitro Antiviral Activity Assay. The in vitro antiviral activity was assayed using WISH cells and VSV. WISH cells were seeded in 96-well (2.5×10^4 cells/well) microtiter plates in 0.1 mL of culture medium and cultivated for 6 h. Serial dilutions of 0.1 mL of IFN-β-1b standard and PEGylated IFN-β-1b samples were added and incubated at 37 °C and 5% CO_2 for 24 h. Supernatants were discarded, and 0.1 mL of VSV was added to each well followed by incubation at 37 °C and 5% CO_2 for another 24 h. Ten microliters of CCK-8 assay reagent was added to each well, and the plates were further incubated for 1.5 h at 37 °C to measure the antiviral activity.[27] The plate was read at 450 nm in a 96-well plate reader (Bio-Rad model 550, USA). IFN-β-1b standard and PEGylated IFN-β-1b were analyzed in triplicate on each plate.

Antiproliferation Assay. Lewis lung cells were distributed on 96-well microtiter plates (4000 cells/well) in 0.1 mL of DMEM culture medium containing 10% fetal bovine serum and 1% penicillin and streptomycin. After cultivating the cells for 12 h, the media was discarded, 0.1 mL of serial dilutions of IFN-β-1b standard and PEGylated IFN-β-1b was added (the initial concentration was 100 ng/mL). Cells continued to grow for 4 days, and the cell antiproliferation activity was measured by CCK-8 assay.

Pharmacokinetic Analyses of IFN-β-1b and PEG-IFN-β-1b. Sprague−Dawley rats (200−220 g, four/group) were injected subcutaneously with IFN-β-1b or its PEGylated conjugates at a dose of 0.1 mg/kg body weight. Sampling of blood (300 μL) was taken via the retro-orbital plexus into vials containing EDTA at times prior to protein administration and at 2 min, 60 min, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, and 96 h after protein administration. The blood was centrifuged at 5000g at 4 °C for 5 min, and the plasma was collected and immediately stored at −70 °C until analysis. Concentrations of compounds were determined by ELISA. Data were calculated by GraphPad Prism software.

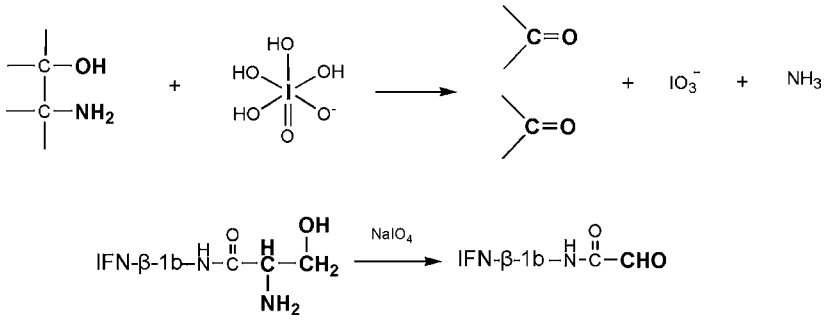
Immunogenicity Analyses of IFN-β-1b and PEG-IFN-β-1b. Sprague−Dawley rats (200−220 g, four/group) were injected subcutaneously with IFN-β-1b or its PEGylated conjugates at a dose of 0.1 mg/kg body weight. Each rat received an injection once per week, which was continued for 4 weeks. Plasma was collected before the next injection, and at the end of the fifth and sixth weeks. Rat plasma antibodies were analyzed by indirect ELISA.[24]

Indirect ELISA. ELISA was performed sequentially in a 96-well polystyrene microtiter plate precoated with IFN-β-1b. IFN-β-1b (2.5 ng/mL) was added in wells and incubated 12 h at 4 °C. After washing, rat plasma samples were added in 1:4 serial dilutions, beginning at 1:100 dilutions. After a 1 h incubation at 37 °C and plate washing with PBS, 0.05% Tween-20, pH 7.4, 100 μL of HRP-conjugated goat anti-rat IgG (1:5000) was added, and after a 1 h incubation at 37 °C, TMB substrate was incubated for 10 min and terminated by adding 50 μL of 2 M H_2SO_4. The plates were read at 450 nm. The ELISA antibody titer was expressed as the highest serum dilution giving a positive reaction.[28]

RESULTS AND DISCUSSION

N-Terminal Oxidation of IFN-β-1b. The site-directed PEGylation of this study was based on the highly specific periodation of the N-terminal serine of IFN-β-1b. In previous research, many amino acids could be oxidized by periodate, such as histidine, tyrosine, tryptophan, cysteine, methionine, and N-terminal serine and threonine, at different rates.[29] In general, tryptophan, tyrosine, and histidine are extensively oxidized to unknown colored products, and thioethers are oxidized to sulphoxides or sulphones.[30] Periodation of α-amino alcohols like serine and threonine leads to aldehyde compounds. Because histidine, tyrosine, and tryptophan are much less reactive to attack by periodate than either an N-terminal serine or threonine or the thioether group of methionine in a protein sequence and could be restrained under the reaction conditions that we utilized, they are generally out of consideration in the periodation of protein. Cysteine is also excluded as a possible site of periodination because of its presumed sensitivity to oxidation and because it is not present in IFN-β-1b. Furthermore, it was reported that the conversion of methionine to its sulfoxide was favored by acidification, whereas the N-terminal periodation of serine or threonine was strongly favored at pH 7 to 8.[11] Therefore, the desired periodation reaction of the 2-amino alcohol of the N-terminal serine is fast and of high selectivity, and the potential

Scheme 1. Specific Periodation of N-Terminal Serine of IFN-β-1b by $NaIO_4$



for side reactions can be dismissed by using very low periodate/protein molar ratios (2:1 in our operation) in addition to controlling the pH. The introduction of aldehyde at the N-terminus of IFN-β-1b is shown in Scheme 1. The molecular weight of IFN-β-1b is 19 876 Da, confirmed by MALDI-TOF MS (Figure 1a), and a single aldehyde group is generated after oxidation, resulting in a molecular-weight reduction of 31 (Figure 1b).

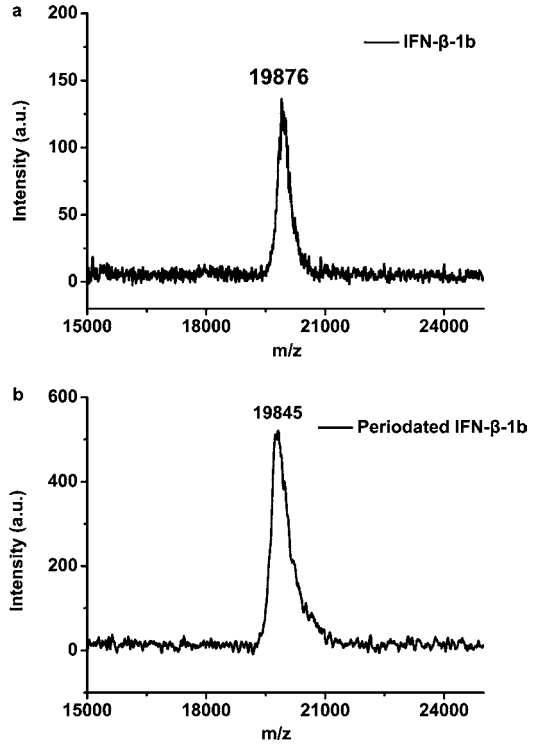


Figure 1. MALDI-TOF mass spectra of proteins: (a) IFN-β-1b and (b) periodated IFN-β-1b.

N-Terminal PEGylation of IFN-β-1b by mPEG-HZ and mPEG-ALD. In this study, two site-specific N-terminal PEGylation strategies were employed to modify IFN-β-1b. The conventional N-terminal modification by aldehyde was conducted by controlling the reaction conditions, such as pH, to adjust the reaction rates between different amino sites. This method can increase the proportion of the mono-PEGylated protein in the reaction mixture but cannot eliminate the possibility of the production of multimodified products and positional isomers. Therefore, a PEGylation strategy through N-terminal periodation followed by site-directed modification using mPEG-HZ was developed to solve these problems and was found to be a highly specific coupling reaction that generated only N-terminal mono-PEGylated conjugates. To confirm the effectiveness of hydrazide modification, a comparison was conducted between the well-known aldehyde-based method and the novel hydrazide-based strategy. The selectivity, velocity, and yield of the two methods were experimentally investigated and plotted against different reaction conditions such as pH, reaction time, and molar ratio of PEG/protein.

Influence of pH on the Selectivity and Homogeneity of N-Terminal PEGylation. PEGylations were performed on IFN-β-1b by mPEG-ALD and mPEG-HZ separately at different pH. Figure 2 shows that some multimodified products were

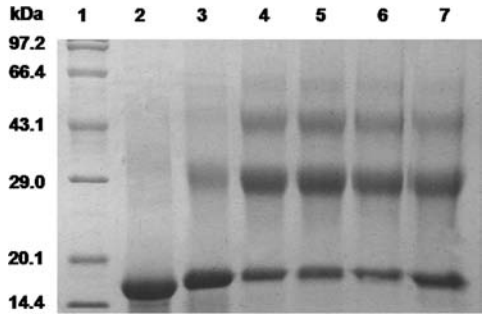


Figure 2. SDS-PAGE analyses of PEGylation with 5 kDa mPEG-ALD at different pH: molecular weight marker (lane 1), original IFN-β-1b (lane 2), and IFN-β-1b PEGylated by 5 kDa mPEG-ALD at pH 4 (lane 3), 5 (lane 4), 6 (lane 5), 7 (lane 6), and 8 (lane 7).

inevitably generated at the pH values from 4 to 8 when using mPEG-ALD as the modifier. At pH 4.0, the yield of mono-PEGylated IFN-β-1b was 33.9% with a selectivity of 91.8%, which then rose to 41.6% when the pH was increased to 8 (Table 1). The results also showed that the yield of mono-PEGylated IFN-β-1b increased at the expense of selectivity because the selectivity decreased from 91.8 to 47.0%. The

Table 1. Yields and Selectivity of PEGylation Products in the Aldehyde-Based Process at Different pH

pH	mono-PEGylated IFN-β-1b (%)	multi-PEGylated IFN-β-1b (%)	selectivity (%)
4	33.9	3	91.8
5	35.8	36	51.8
6	37.8	42.4	47.1
7	37.9	43.8	46.6
8	41.6	46.8	47.0

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reason for this is that the selectivity of the aldehyde modification is built on the difference in the pK_a between free amino groups. The lower the pH of the reaction solution, the higher the selectivity of mono-PEGylated protein that can be obtained. On the contrary, pH did not have any influence on the selectivity of PEG-hydrazide modification of IFN-β-1b, which was maintained at 100% in all reactions because only one binding site was available (Figure 3). Meanwhile, a pH

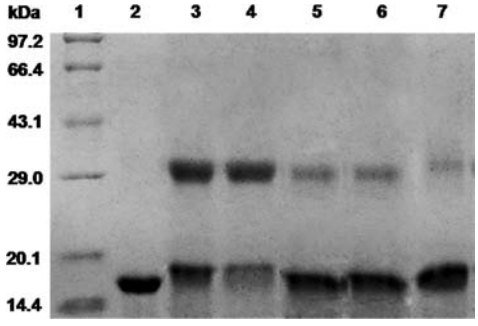

Figure 3. SDS-PAGE analyses of PEGylation with 5 kDa mPEG-HZ at different pH: molecular weight marker (lane 1), original IFN-β-1b (lane 2), and IFN-β-1b PEGylated by 5 kDa mPEG-HZ at pH 4 (lane 3), 5 (lane 4), 6 (lane 5), 7 (lane 6), and 8 (lane 7).

dependence was observed because the yield decreased rapidly with the increasing pH (5 to 8), and a relatively high yield of 51.5% could be achieved at pH 5.0. These results demonstrate that the modification of IFN-β-1b by mPEG-HZ is a robust, site-directed reaction feasible for large-scale preparation.

Influence of PEG-to-Protein Ratio on the Yields of N-Terminal PEGylation. Different PEG-to-protein ratios were applied under the optimized pH conditions in two PEGyltions. In the aldehyde-based process, the mono-PEGylated yield rose continuously with the increasing of PEG-to-protein ratio (Figure 4a) and reached a maximum of 45.4% at the ratio of 20:1. When the PEG-to-protein ratio was higher than 10:1, multiple modified products were generated, and their proportion of the total products rose rapidly. Therefore, the yield of mono-PEGylated product could only reach as high as 38.7% based on the requirement of high selectivity. Whereas, in the hydrazide-based process, the yield of mono-modified protein reached a plateau of 50.8% at the ratio of a 6−8-fold excess of PEG (Figure 4b), which was remarkably increased by a striking 30% compared with that of aldehyde strategy at the same ratio. Furthermore, the selectivity of PEGylation by mPEG-HZ was definitely maintained at 100% at the different ratios.

Influence of Reaction Time on the Velocities and Yields of N-Terminal PEGylation. All reactions were carried out under the optimized pH and PEG-to-protein ratio using the same initial concentration of protein. The mean generation velocity of mono-PEGylated IFN-β-1b in the two methods both kept falling during the reaction time (Table 2). In the first 3 h, the velocity maintained a relatively high value (>2.5 × 10^{-3} mmol/L/h) and declined rapidly as the reaction proceeded. Both reactions reached equilibrium at 8 h, giving a velocity value under 0.05 × 10^{-3} mmol/L/h. The results demonstrate that the rate of hydrazide-based PEGylation was faster than that of the aldehyde process throughout the entire reaction, indicating a higher PEGylation efficiency. As a result, the yield of hydrazide-based PEGylation reached 50.5% in 8 h,

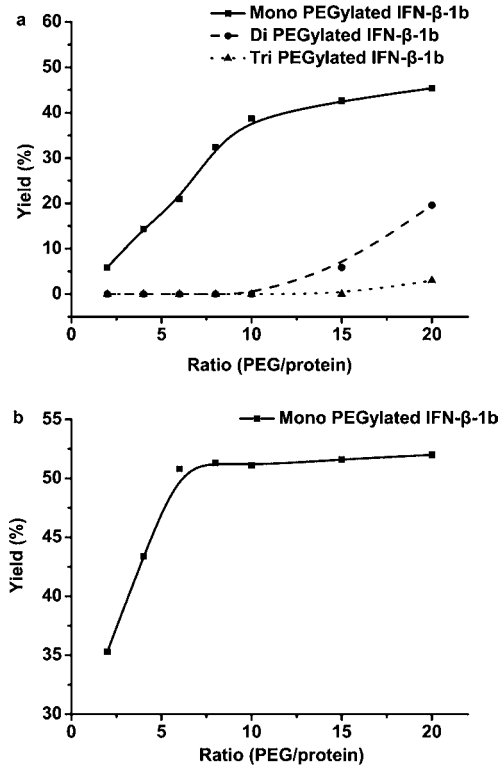

Figure 4. Yields of PEGylation at different PEG-to-protein ratios: (a) PEGylated by 5 kDa mPEG-ALD and (b) PEGylated by 5 kDa mPEG-HZ.

Table 2. Mean Generation Velocity of Mono-PEGylated IFN-β-1b at Different Times

t (h)	K-aldehyde-based PEGylation (10^{-3} mmol/L/h)[a]	K-hydrazide-based PEGylation (10^{-3} mmol/L/h)
0−1	10.1	15.6
1−3	2.73	3.55
3−5	0.75	0.90
5−8	0.40	0.73
8−24	0.01	0.04

[a]The mean production velocity of mono-PEGylated IFN-β-1b.

which is 10% higher than that of the aldehyde-based modification (40.4%).

In conclusion, on the basis of the comparisons on the selectivity, efficiency, and velocity of the two site-directed PEGylation strategies, the hydrazide-based conjugation is significantly superior to the aldehyde-based modification on the N-terminal amino group of the protein in many aspects, including product homogeneity, feasibility for process control, and easier operability.

A hydrazone bond is formed by the condensation of hydrazides with periodated IFN-β-1b after elimination of the carbonyl oxygen as water, resulting in a metastable bond (Scheme 2). A hydrazone linkage is known to be stable at pH 6−8 and susceptible to degradation at pH 2−4.[31,32] This property forms the basis for reversible long-term drugs preparation of PEGylated IFN-β-1b. Meanwhile, a more stable product covalently linked by a carbon−nitrogen bond could be stoichiometrically achieved by adding sodium cyanoborohydride as a reducing agent to obtain a long-acting protein in vivo.

To obtain better pharmacokinetic properties (especially prolonged half-life) in clinical applications, the conjugation of

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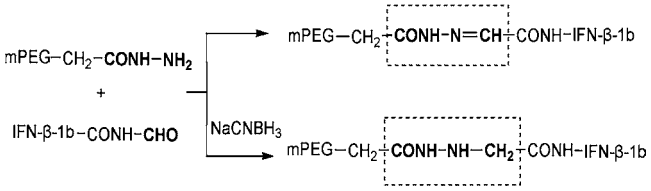
Scheme 2. Bioconjugation of mPEG-HZ and Periodated IFN-β-1b



proteins with higher molecular-weight polyethylene glycol is favored.[33] Therefore, 20 kDa PEG was used in our study for further research.

To separate mono-PEGylated IFN-β-1b from unreacted protein and excessive PEG in the reaction mixture, a Hiload 16/60 Superdex 200 prep grade column with a running buffer containing 0.1% SDS to control aggregation was utilized. Residual SDS in the running buffer was removed by desalting and dialysis, and the final concentration of SDS was undetectable by acridine-orange assay. The results shown in Figure 5 demonstrate that mono-PEGylated proteins with a high purity (>95%) are obtained.

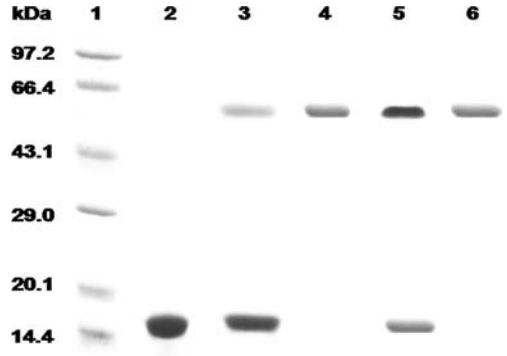


Figure 5. SDS-PAGE analyses of PEGylation mixtures and purified proteins: molecular weight marker (lane 1), original IFN-β-1b (lane 2), mPEG-ALD modification mixture (lane 3), mono-ALD-20k-PEG-IFN-β-1b (lane 4), mPEG-HZ modification mixture (lane 5), and mono-HZ-20k-PEG-IFN-β-1b (lane 6).

Characterization of PEG-IFN-β-1b. *MALDI-TOF MS.* The molecular weight of mono-HZ-20k-PEG-IFN-β-1b was determined by MALDI-TOF MS (Figure 6). The spectrum confirmed the mono conjugate of PEG-IFN-β-1b with a m/z centered at 44 695 Da, consistent with the sum of the molecular weights of IFN-β-1b and 20 kDa PEG-HZ, revealing a great homogeneity.

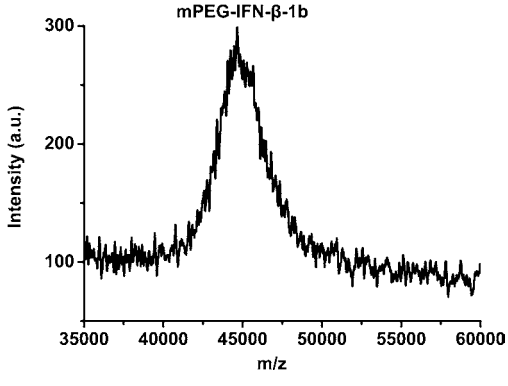


Figure 6. MALDI-TOF MS spectrum of mono-HZ-20k-PEG-IFN-β-1b.

Circular Dichroism (CD). To detect the conformational changes of IFN-β-1b in the PEGylation, circular dichroism spectra were analyzed, and the results are presented in Figure 7.

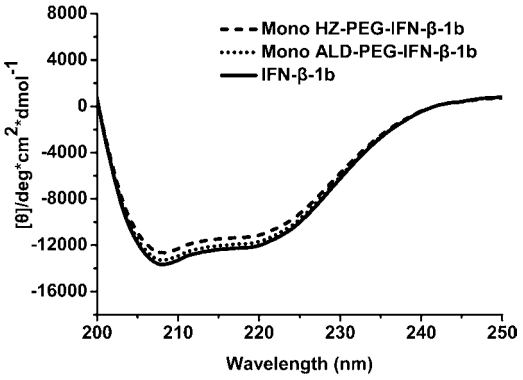


Figure 7. Circular dichroism analyses of IFN-β-1b (solid line), purified mono-ALD-20K-PEG-IFN-β-1b (dotted line), and purified mono-HZ-20K-PEG-IFN-β-1b (dashed line). For each sample, protein concentrations was 0.2 mg/mL in 20 mM sodium phosphate buffer, pH 7.0.

The CD profile of mono-PEGylated IFN-β-1b was comparable with that of the native form, with characteristic minima observed at 208 and 222 nm, indicating helix structure. It was found that both of the PEGylation approaches of IFN-β-1b, mPEG-HZ and mPEG-ALD, preserved the protein's secondary structure. These results are consistent with previous studies that indicated few secondary-structure losses occurred when IFN-β-1b was PEGylated by different PEG polymers.[8,34,35]

Intrinsic Emission Fluorescence. Fluorescence spectra were measured to analyze the differences between the tertiary structure of IFN-β-1b and that of mono-PEGylated IFN-β-1b. The results are shown in Figure 8. The maximum emission wavelength of IFN-β-1b was 334 nm, and a blue shift was detected for mono-PEGylated IFN-β-1b (330 nm), indicating compact packing of the protein structure, which could be attributed to the interaction between PEG and protein. IFN-β-1b was wrapped in the PEG chain, contributing to the compact

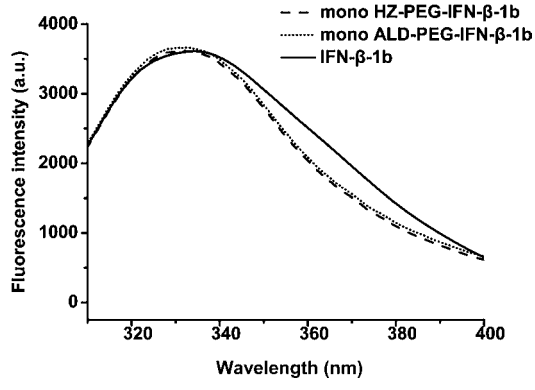


Figure 8. Intrinsic fluorescence spectra of IFN-β-1b and mono-PEGylated IFN-β-1b. The spectra were recorded at protein concentrations of 0.2 mg/mL, as outlined in the experimental procedures.

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structure, and tyrosine residues were embedded after PEGylation.

In Vitro Antiviral Activity Assay. The in vitro antiviral activities of IFN-β-1b and the conjugates with different PEGs were compared for the ability of IFN-β-1b to inhibit the VSV amplification. The in vitro potency of PEGylated IFN-β-1b modified by mPEG-HZ and mPEG-ALD remained at 62.1 to 67.6% of the native IFN-β-1b, respectively (Table 3). The

Table 3. Antiviral Activities of IFN-β-1b and Mono-PEGylated IFN-β-1b[a]

compound	activity (IU/mg)	activity retention (%)
IFN-β-1b	3×10^7	100
mono-HZ-20k-PEG-IFN-β1b[b]	2.03×10^7	67.6
mono-HZ-20k-PEG-IFN-β1b[c]	2.01×10^7	67.0
mono-ALD-20k-PEG-IFN-β1b	1.86×10^7	62.1

[a]The antiviral activities of mono-PEGylated IFN-β-1b were calculated using a standard IFN-β-1b (3×10^7 IU/mg) as reference. [b]The reducing mono-PEGylated IFN-β-1b. [c]The nonreducing mono-PEGylated IFN-β-1b.

retention of activity was much higher than that of the marketed PEGylated IFN-α, PEG-INTRON and Pegasys, whose in vitro antiviral activities were about 28 and 7% of the unmodified protein separately.[3,36] Furthermore, it was also as high as the maximum potency of the reported amino PEGylated IFN-β-1b, whose in vitro retention was about 65%.[24] The antiviral activity of mono-PEGylation IFN-β-1b modified by mPEG-HZ (2.03×10^7 IU/mg) did not show apparent distinction between that of the nonreducing form (2.01×10^7 IU/mg), whereas it was 8.2% higher than those derived from mPEG-ALD modification (1.86×10^7 IU/mg).

Antiproliferation Assay. The half-maximal inhibitory concentration (IC_{50}) was measured to evaluate the effectiveness of IFN-β-1b conjugates in inhibiting antiproliferation of carcinoma cells (Lewis lung cells).[37] IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro, where a higher IC_{50} value indicates a lower potency. The reducing mono-HZ-PEG-IFN-β-1b showed the maximum cytotoxic activity on LLC (IC_{50} = 178 pg/mL), which was slightly stronger than that of mono-ALD-PEG-IFN-β-1b (IC_{50} = 194 pg/mL). However, the hydrazone-linked mono-HZ-PEG-IFN-β-1b showed weaker potency (IC_{50} = 441 pg/mL), which may be due to the readily degradable nature of the double bond between the protein and PEG chain (Table 4). Overall, IFN-β-1b displayed an increased antiproliferative capacity after being PEGylated.

Pharmacokinetic Analyses of IFN-β-1b and PEG-IFN-β-1b. The in vivo pharmacokinetic behaviors of the conjugates were assayed by ELISA following subcutaneous injection. The mean serum concentration−time curves are shown in Figure 9, and

Table 4. Antiproliferation Activity of IFN-β-1b and Mono-PEGylated IFN-β-1b

compound	IC_{50} (pg/mL)	activity increase
IFN-β-1b	788	1
mono-ALD-PEG-IFN-β-1b	194	4.06
mono-HZ-PEG-IFN-β-1b[a]	178	4.42
mono-HZ-PEG-IFN-β-1b[b]	441	1.78

[a]The reducing mono-PEGylated IFN-β-1b. [b]The nonreducing mono-PEGylated IFN-β-1b.

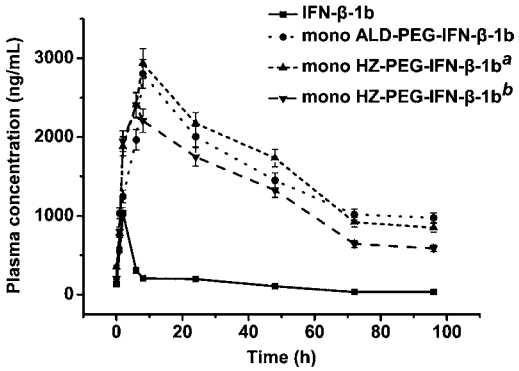

Figure 9. Pharmacokinetics profiles of IFN-β-1b and PEGylated conjugates. Rats were subjected to subcutaneous administration at 0.1 mg protein/kg body weight. Plasma concentrations were measured at the indicated time points post administration by ELISA. [a]Reducing mono-PEGylated IFN-β-1b. [b]Nonreducing mono-PEGylated IFN-β-1b.

the pharmacokinetic parameters are shown in Table 5. The half-life of native IFN-β-1b was about 2 h, whereas those of mono-PEGylated IFN-β-1b were significantly prolonged to 8 h. The concentration of PEGylated IFN-β-1b did not decline rapidly but maintained a relatively high level for a long time, indicating the improvement imparted by PEGylation for enhancing a protein drug's therapeutic efficacy. All three species of conjugates displayed no apparent half-life time difference, probably as a result of having the same PEG molecular weight. For AUC values, PEGylation increased the total exposure of IFN-β-1b when administered in vivo. The reducing form of mono-HZ-PEG-IFN-β-1b exhibited greater plasma exposure than the other three. The enhanced exposure of mono-HZ-PEG-IFN-β-1b compound was expected to provide a much more potent drug than unmodified IFN-β-1b. These results demonstrated that the reducing form of mono-HZ-PEG-IFN-β-1b would have the best pharmacokinetic properties in clinical application.

Immunogenicity Analyses of IFN-β-1b and PEG-IFN-β-1b. Immunogenicity is a crucial concern in the development and drug approval of protein therapeutics, including PEGylated proteins.[38,39] Binding antibodies were commonly encountered, and neutralizing antibodies against commercial IFN-β-1b (Betaseron) and other IFN-β compounds have been reported.[40] In this study, we measured the immunoassays titers to assay the immunogenicity of IFN-β-1b after PEGylation. Rats received 0.1 mg/kg subcutaneous injections of the compounds once weekly for 4 weeks. Rat plasma samples were collected each week (continued for 6 weeks) and analyzed by indirect ELISA. As shown in Figure 10, the rat IgG response to the three PEGylated IFN-β-1b compounds was markedly diminished in week 2 through week 6 compared with the IgG response to unmodified IFN-β-1b, showing that PEGylation can significantly weaken the original immunogenicity of protein. In this immunoassay, native IFN-β-1b coated the plates, and the bound rat IgG was specifically detected by anti-rat IgG reagents.

Researchers have reported that the immunogenicity of a PEGylated protein changes with different leaving groups of PEG derivatives because some PEG derivatives are immunogenic and antigenic.[41] The application of Pegloticase, a PEGylated uric acid-specific enzyme approved in 2010 by the U.S. FDA, has been hampered by the occurrence of PEG

Table 5. Pharmacokinetic Parameters in Rats[a]

compound	dose (mg/kg)	AUC (ng × h/mL)[b]	half-life (h)
IFN-β-1b	0.1	13 632 ± 2863	2.0 ± 0.4
mono-ALD-PEG-IFN-β-1b	0.1	146 300 ± 16 239	7.8 ± 0.6
mono-HZ-PEG-IFN-β-1b[c]	0.1	156 507 ± 17 372	8.4 ± 1.0
mono-HZ-PEG-IFN-β-1b[d]	0.1	122 002 ± 16 104	7.6 ± 0.6

[a]Data for each molecule represent the mean ± standard deviation calculated from four animals. [b]Area under the curve. [c]The reducing mono-PEGylated IFN-β-1b. [d]The nonreducing mono-PEGylated IFN-β-1b.

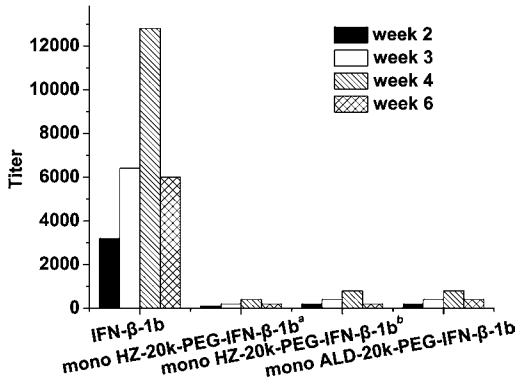

Figure 10. Immunogenicity of IFN-β-1b and PEGylated IFN-β-1b in rats. Indirect ELISA of IgG response in rats to IFN-β-1b at different weeks. Plasma from the rats injected with either of these test articles were incubated independently on microtiter wells along with the captured native IFN-β-1b followed by detection with anti-rat IgG-HRP reagent. [a]Reducing mono-PEGylated IFN-β-1b. [b]Nonreducing mono-PEGylated IFN-β-1b.

antibodies, resulting in increased drug clearance and loss of efficacy.[42] Because the hydrazide-based PEGylation can reduce the immunogenicity of proteins with N-terminal Ser and Thr, it could be expected to provide a promising approach to achieve a less immunogenic PEG-uricase for use in clinical applications.

CONCLUSIONS

IFN-β-1b was PEGylated successfully via two site-directed PEGylation strategies, including aldehyde- and hydrazide-based chemistry. By analyzing the reaction parameters such as pH, PEG-to-protein ratio, and reaction time, comparisons of the two methods for their selectivity, velocity, and yield were investigated. For the modification by mPEG-ALD, multi-modified conjugates and positional isomers are generally produced during the process, leading to difficulties in purification and medical evaluation procedures. Our results confirmed that the PEGylation selectivity was pH dependent, but complete selectivity was hard to achieve even under the optimum conditions. On the contrary, excellent selectivity was observed in the pH range of 4−8 by the hydrazide-based PEGylation. Moreover, the hydrazone-conjugation product demonstrated remarkably higher yields. The homogeneity of mono-HZ-PEG-IFN-β-1b contributed to better performance in many aspects, including binding-site specificity, in vitro and in vivo activity, antiproliration potency, and immunogenicity, which is a significant advancement in the field of biomedicine and for clinical applications. Although this site-specific strategy was presently utilized for modification of proteins with N-terminal serine or threonine, this application could be realized for other proteins by the introduction of serine or threonine to the N-terminus through gene engineering. The hydrazide-based strategy for IFN-β-1b offers a promising prospect for the development of engineered biomolecules such as polymer−protein conjugates.

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Notes

The authors declare no competing financial interest.

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