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A Simple Method for Controlling the Degree of PEGylation of Dynorphin A (1—13)

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Dynorphin A (1—13) was chemically modified by reaction with succinimidyl propionate-monomethoxy polyethylene glycol (mPEG-SPA). To determine the degree and the optimized condition for PEGylation of dynorphin A, the reactions were monitored in different pH buffers at different molar ratios by reversed-phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results showed that the degree of PEGylation for dynorphin A could easily be controlled through adjustment of the molar ratios and the pH. The degree of PEGylation of dynorphin A increased as the molar ratio of dynorphin to MPEG-SPA and the pH increased.

Keywords dynorphin A, PEGylation degree, molar ratio, pH

Introduction

Dynorphin A (1—13) is a single polypeptide consisting of 13 amino acids (1604 Da), which has analgesic effect and improves ischemia-induced amnesia.[1] As a polypeptide, dynorphin A is subject to chemical and enzymatic degradation and has a short biological half-life in the body. To overcome the rapid clearance of polypeptide from the blood circulation and its *in vivo* instability, chemical modification of therapeutic polypeptides with polyethylene glycol (PEG) has been utilized as a novel approach.[2] It might be the most widely used polymer for drug conjugation to obtain functional bioconjugates possessing increased resistance to proteolytic degradation, increased solubility, decreased immunogenicity, and low toxicity.[3,4] Difficulty in the physicochemical characterization of PEGylated polypeptides is due to their heterogeneity with respect to the distribution of both the number and position of attached PEG molecules and inherent polydispersity of PEG itself.

The number of PEG molecules attached affects the pharmacological properties of the PEGylated polypeptides.[5,6] Despite the obvious importance of controlling the PEGylation, there have been relatively few published studies on controlling the degree of PEGylation of dynorphin A. MALDI-TOF MS has recently been demonstrated as a convenient and powerful method to characterize PEGylated polypeptides and proteins.[7,8] In this paper, the utility of RP-HPLC and MALDI-TOF MS for monitoring the formation of PEGylated dynorphin A was investigated by varying the molar ratio of PEG molecules to peptide and the pH of the reaction medium.

Experimental

Materials

Succinimidyl propionate-monomethoxy PEG (mPEG-SPA, 4880 Da) and dynorphin A (1—13) (1604 Da) were purchased from Beijing Kaizheng and Sinoasis Pharmaceuticals, respectively. α-Cyano-4-hydroxycinnamic acid (α-CHCA) and trifluoroacetic acid (TFA, HPLC grade) were obtained from Sigma. Acetonitrile (HPLC grade) was supplied from Fisher Scientific. All other chemicals were of analytical grade and obtained commercially.

Preparation and separation of PEGylated dynorphin A

mPEG-SPA-4.9k, corresponding to different molar excesses, was added to a dynorphin A solution (3 mg/mL) in 50 mmol•L^{-1} phosphate buffers of different pH values (pH 6—8). The reaction mixtures were shaken gently at 30 ℃ for 60 min. The PEGylation reactions were quenched by addition of 0.2 mL of 1 mol•L^{-1} glycine solution. The PEG-dynorphin A mixture was applied to a Diamosil C18(2) column (250 mm ×4.6 mm, 5 μm, Dikma) for RP-HPLC analysis with a Waters 2695—2996 instrument by UV detection at 210 nm at 30 ℃. Gradient elution was carried out at a flow-rate of 1.0 mL/min with solvent A (0.1% TFA in

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water) and solvent B (0.1% TFA in acetonitrile). The following gradient profile was chosen: 5%—50% B over 35 min. After an additional elution for 10 min with 100% B followed by 10 min with 5% B, the system was ready for the next injection. The HPLC fractions corresponding to respective peaks were collected by a Waters 600E-2487 HPLC instrument, separately, purged off with nitrogen, and stored at 4 ℃.

Identification of PEGylation number

The respective peaks were directly applied to an MALDI-TOF-MS instrument and the MALDI-TOF-MS was carried out using an autoflex III smartbean (Bruker, German). Samples were prepared by mixing each peak fraction with the matrix solution, which was a saturated solution of α-CHCA in 50% of water in acetonitrile. The sample mixture was spotted into a well of the sample plate and dried by vacuum evaporation prior to mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen lasers were averaged for each spectrum in a linear mode, and positive ion TOF detection was performed using an accelerating voltage of 25 kV. The external calibration was performed using a mixture of peptide mass standards, the mixture of angiotensin I, ACTH (clip 1—17), ACTH (clip 18—39), ACTH (clip 7—38), and bovine insulin.

Results and discussion

Dynorphin A (1—13) contains three primary amino groups (one α-amino group at the *N*-terminus and the two ε-amino groups of Lys^{11} and Lys^{13}), which are highly active to PEGylation reaction (Figure 1).

Tyr^{1}-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-**Lys**^{11}-Leu-**Lys**^{13}-OH

Figure 1 Primary structure of dynorphin A (1—13).

The peptide dynorphin A was PEGylated using mPEG-SPA-4.9k at a molar ratio of 1∶2 and pH 7.5. The representative PEG-dynorphin was separated by RP-HPLC into four peaks (a—d) (Figure 2). The first peak (a) was identified to be unmodified dynorphin A as shown in Figure 2. The other peaks were isolated, and

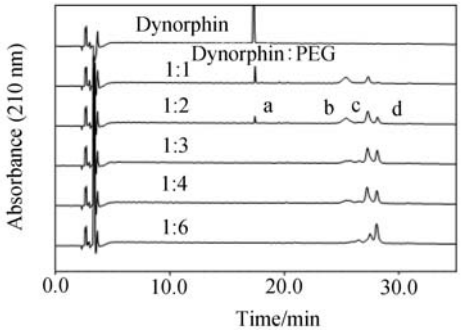

Figure 2 RP-HPLC diagrams for the reactions at different molar ratios of the reactants at pH 7.5.

their molecular masses were determined by MALDI-TOF MS. The MS peaks had a normal distribution, as expected for a polymer, with peak separation of 44 mass units, corresponding to the monomer mass of PEG.[9] The molecular weight of the second peak (b) (retention time＝25.4 min) showed a main distribution approximately at 6500 indicating mono-PEGylation (Figure 5). The third peak (c) (retention time＝27.3 min) was found to be approximately at 11500, which corresponded to the di-PEG-dynorphin. The fourth peak (d) (retention time＝28.2 min) was identified as tri-PEG-dynorphin with a molecular mass of 16500.

The PEGylation reaction of peptides was controlled by varying the stoichiometry of the components and the reaction pH. The effect of molar ratios of the reactants and the reaction pH on the PEGylation reaction of dynorphin was monitored by RP-HPLC (Figures 2 and 3). As expected, the yield of PEGylated dynorphin increased as the molar ratio of dynorphin to MPEG-SPA increased from 1∶1 to 1∶6 (Figure 2).

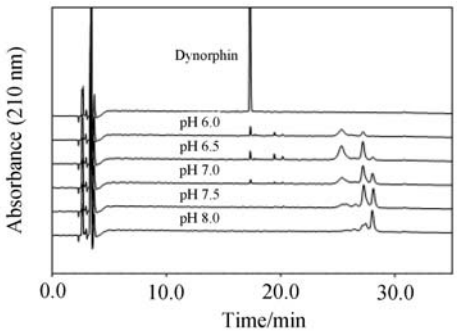

Figure 3 RP-HPLC diagrams for the reactions at different pH at molar ratio of 1∶3 (dynorphin A∶PEG).

Figures 4a and 4b showed MALDI-TOF MS of PEG and dynorphin at 1 mol•L^{-1} glycine solution, respectively. The molecular weight of PEG and dynorphin were 4880.8 and 1604.3, respectively.

With increasing PEG content, a significant increase in the amount of tri-PEG-dynorphin was observed (Figure 2). The yield of tri-PEG-dynorphin was highest at a molar ratio of 1∶6. At a molar ratio of 1∶1, mono-PEG-dynorphin and di-PEG-dynorphin were more predominant than tri-PEG-dynorphin, and the amount of tri-PEG-dynorphin was few. At a molar ratio of 1∶3, dynorphin was completely reacted.

The dependence of PEGylation of dynorphin on the pH values of the mixtures was also monitored at a molar ratio of 1∶3, varying the pH values from 6.0 to 8.0 (Figure 3). The production of PEGylated dynorphin increased as the pH increased. However, the production of PEGylation at pH 8.0 was lower than that at pH 7.5, which may be attributable to the rapid hydrolysis of *N*-hydroxysuccinimidyl ester of MPEG-SPA in a basic condition.[5] At pH 6.0, the amount of tri-PEG-dynorphin was hardly any, and increased with increasing pH value. So mono-PEG-dynorphin and di-PEG-dynorphin could be efficiently produced at pH 6.0. At pH 7.5, the amount

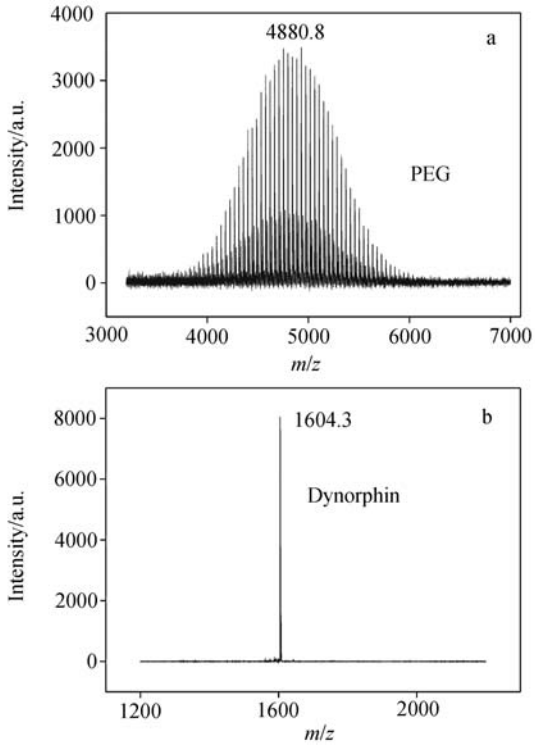

Figure 4 MALDI-TOF MS of PEG (a) and MS of dynorphin (b).

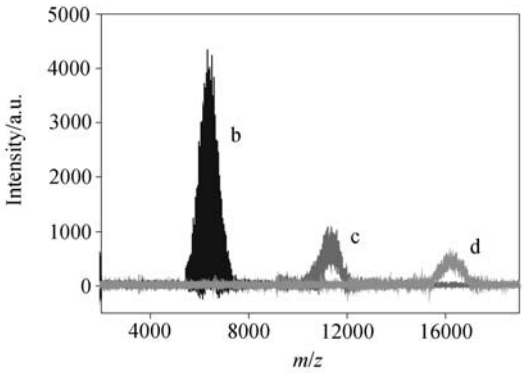

Figure 5 ALDI-TOF MS of the second peak (b), the third peak (c) and the fourth peak (d).

of intact dynorphin significantly decreased. The yield of tri-PEG-dynorphin was highest, while mono-PEG-dynorphin was lowest at pH 8.0. This effect is mainly related to the difference in reactivity between α-(*N*-terminus) and ε-amino (Lys residue) groups at different pH. The primary amine residues in protein have different pK_a values: pK_a 7.8 for *N*-terminal α-amino group and 10.1 for ε-amino group in lysine residues. Moreover, the conjugation of PEG derivatives to primary amine groups of EGF takes place primarily via a nucleophilic substitution reaction: the attack of unprotonated amine group to the carbonyl groups of *N*-hydroxysuccinimide.[10] It suggested that all three primary amines (one α and two ε) were equally reactive to PEGylation at high pH, whereas, at low pH, selective unprotonation of the *N*-terminal α-amine group would be more reactive than the ε-amine groups in lysine residues. So the yield of tri-PEG-dynorphin was highest, while mono-PEG-dynorphin was lowest at high pH, such as pH 8.0, and the amount of mono-PEG-dynorphin was higher at low pH. The effect of molar ratios and pH on the amount of di-PEG-dynorphin was relatively small.

Conclusion

In this study, preparation and identification of PEGylated dynorphin A were investigated. The degree of PEGylation for dynorphin A could easily be controlled through adjustment of the molar ratios and pH. The amount of mono-PEG-dynorphin was higher at low pH and molar excess of PEG, while tri-PEG-dynorphin was higher at high pH and molar excess of PEG under conditions investigated. The effect of molar ratios and pH on the amount of di-PEG-dynorphin was relatively small.

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